RITA LEVI MONTALCINI: THREE CRUCIAL STEPS TOWARD STOCKOLM

P. CALISSANO

European Brain Research Institute, Rome, Italy

The Nobel prize award often represents not only a breakthrough discovery in a given field of investigation, but places that discovery in a sort of definitive position within that field. Sometimes, however, that breakthrough only constitutes the beginning of a still largely uncharted route. This is, in my opinion, the case of the discovery of NGF.

This protein was identified by Rita Levi Montalcini, under the supervision of Viktor Hamburger, for its *nerve growth promoting activity* - hence the acronym of NGF - exerted on two peripheral nerve cell populations, forming the sensory and sympathetic ganglia. The subsequent development of a rapid and semiquantitative test, based on the so called *halo effect*, and the unequivocal demonstration of the physiological role of NGF - with the ingenious immunological knock down procedure known as *immunosympathectomy* - established the revolutionary nature of NGF discovery. It is worth noting that this experiment anticipated by some decades the knock out genetic manipulations widely used to assess the function of a gene and of the corresponding, coded protein.

Interestingly, most of Rita's experiments did not contain statistical data. The demonstration of the diffusible nature of the growth promoting activity by mouse sarcoma tumors, *the halo effect,* and the *immunosympathectomy* were so pronounced that they did not require any statistics.

But more had to come. Thanks also to the astonishing improvements of genetics and molecular biology, the following decades continued to open new routes for NGF and a scientific dream of Rita gained access to what she often referred to as the *organismic role* of NGF. Today, such a dream is witnessed by an incredible number of scientific reports.

This is not the place to review such achievements, but to remember the unique person who worked as an indomitable miner and believed, more than anybody else, in the golden nature of the precious metal she had found. Indeed, Rita herself considered her scientific achievements a detective story or a saga, rather than the fruit of a scientific inductive route. In this connection, two posters, the first hanging in the tissue culture room of the small Center of Neurobiology of CNR which I noticed during my interview as a postdoc in 1965, the second, - in subsequent years - in her office as Director of the Laboratory of Cell Biology of CNR, underline her personality as a scientist and as a socially involved person.

The poster in the tissue culture room of the Center of Neurobiology, which Rita directed after her return to Italy in 1963, signed by Albert Einstein, stated that "Imagination is more important than knowledge". Such an artistic-like attitude was Rita's general approach toward experimental science. In several informal conversations that I had the honour and pleasure of having during my long collaboration and friendship with her, Rita asserted that often an excessive knowledge in a given field of investigation could exert a sort of inhibitory action on its experimental planning: how could one conceive an entirely new approach with a mind excessively filled with previous data on that topic? Sometimes it is more fruitful to ignore some data in order to have the courage to try to get entirely new ones. This was a scientific approach that every young investigator

Mailing address:
Pietro Calissano
European Brain Research Institute
Via del Fosso di Fiorano, 64 - Rome, Italy
Phone: +3906501703004
Email: calissano@inmm.cnr.it

2279-5855 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

would obviously prefer, and often provided entirely novel experimental approaches.

The second poster was hanging in her office when, in the decade 1969-1979, Rita directed the Laboratory of Cell Biology of the CNR. It was a picture of Martin Luther King with the words "a man who is not ready to die for his ideas is not fit to live". This was, in my opinion, Rita's attitude toward social issues. The sacrifice of men like the black leader probably hit her heart when, together with several colleagues of the Rebstok laboratory of the Washington University, we joined thousands of people in a peaceful march in support of the ideals of the black leader who had been hit to death.

Rita has provided several examples of her interest and involvement in social issues long before she was nominated life-Senator of the Italian Republic. In later years, she became a staunch supporter of women and of the underprivileged. Great admirer in her youth of the work of Albert Schweitzer in Africa, Rita has established a foundation to assist young African girls with scholarships to further their education in their own country.

The last, but certainly not the least, important achievement in her long career was the foundation of the European Brain Research Institute (EBRI), established in 2005 with the aim of creating a research center entirely devoted to study brain functioning and neuropathologies. The goal was to encourage young Italian investigators to return to Italy or young foreigners to spend a period in our country. EBRI soon became the point of attraction for two other research institutes also involved in neuroscience: the Santa Lucia Foundation and the Institute of Neurobiology and Molecular Medicine of CNR. Within a few years, the three institutes had established the most important Italian center of research in neuroscience.

Today, EBRI has established its internationally recognized position in neuroscience and in her last years Rita could finally and fully enjoy her double dream: the realization of her idea of NGF as a diffusible factor playing a crucial role in several organ functions and the creation of the physical place where such a role can be further investigated and its clinical use as a therapeutic agent put into operation.

I like to remember Rita with the words of Primo Levi - the eminent Italian writer who experienced persecution during the war: "A tiny lady with an indomitable will and the countenance of a princess".

DISCOVERY OF VEGF, A KEY REGULATOR OF ANGIOGENESIS

N. FERRARA

University of California San Diego Medical Center, La Jolla, CA, USA

The existence of factors capable of inducing proliferation of cells and tissues has been hypothesized since the beginning of the last century. As early as 1913, Carrel described the ability of tissues extracts to stimulate cell growth in cultured tissue explants (Carrel, 1913). In the 1920s, these observations were extended to isolated fibroblasts (Carrel & Ebeling, 1923a; Carrel & Ebeling, 1923). In the same period, Spemann's seminal discovery of the "organizer" in blastula stage amphibian embryos (Hamburger, 1969) was followed by the demonstration that "chemical transmission" directs the establishment of the vertebrate body axis (Hamburger, 1997). Early pioneers in the angiogenesis field also speculated that biochemical mediators are responsible for the growth of blood vessels during tumorigenesis and other pathological conditions (reviewed in Ferrara, 2002). In 1939, the observation that experimental tumors strongly induce vascular growth led Ide and colleagues to hypothesize the action of a tumor-derived "blood vessel growth stimulating factor" (Ide et al, 1939). A decade later, Michaelson postulated the existence of a diffusible angiogenic factor ("Factor X"), responsible for the development of the normal retinal vasculature and for pathological neovascularization associated with diabetic retinopathy and other retinal disorders (Michaelson, 1948).

However, very limited progress in furthering these seminal observations was possible at that time, primarily due to the enormous technological challenges in isolating and characterizing such growth or differentiation factors, which typically are active at extremely low concentrations.

In the 1950s, the extraordinary collaboration between a great developmental biologist, Rita Levi-Montalcini, and a great biochemist, Stanley Cohen, in the laboratory of a major pioneer, Viktor Hamburger, resulted in the Nobel Prize-winning discovery, purification and biological characterization of the first defined growth factor, nerve growth factor (NGF) (Levi-Montalcini et al, 1954; Cohen & Levi-Montalcini, 1956; Cohen & Levi-Montalcini, 1957). This remarkable personal and scientific journey has been chronicled elsewhere (Levi-Montalcini, 1975; Hamburger, 1993). Suffices to say here that Levi-Montalcini's early work not only provided insights into the development of the sympathetic nervous system that were entirely confirmed using gene targeting technologies decades later (Cattaneo, 2013), but it also represents a key milestone in the quest to elucidate the mechanisms of cell growth and differentiation. It encouraged efforts that ultimately resulted in discovery and isolation of many unrelated factors, which regulate a variety of fundamental biological processes.

This brief review illustrates the discovery and therapeutic applications of VEGF, a growth factor implicated in angiogenesis.

DISCOVERY OF ANGIOGENIC FACTORS

As above noted, early investigators implicated hypothetical angiogenic factors in a number of proliferative processes. In the 1970s, efforts to isolate such factor(s) began (Folkman et al, 1971). The greatest challenge was purifying these proteins to homogeneity, in order to obtain a partial amino acid sequence that could be used to design probes suitable

Corresponding Author: Napoleone Ferrara
University of California San Diego Medical Center
3855 Health Sciences Drive, Mail Code # 0819,
La Jolla CA, 92093, USA
Phone: (650) 882-6822
Fax: (858) 534-2157
E-mail: nferrara@ucsd.edu

2279-5855 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

for cDNA cloning (Ferrara, 2002). Several molecules were identified and characterized as angiogenesis inducers in the subsequent decade, including EGF, TGF-α, aFGF, bFGF, angiogenin, etc. (reviewed in (Ferrara, 2002; Folkman & Klagsbrun, 1987). However, while these factors were able to promote angiogenesis in various bioassays, initial attempts to directly link them to angiogenesis were largely unsuccessful (reviewed in (Ferrara, 2002; Klagsbrun & D'Amore, 1991). For example, antibodies to bFGF, the molecule that at that time was perhaps the most promising candidate as a regulator of angiogenesis (Gospodarowicz et al, 1987), had no effect on tumor growth in vivo (Dennis & Rifkin, 1990). Subsequently, it become known that bFGF is mainly an intracellular protein, due to the fact that the bFGF gene does not encode a conventional secretory signal peptide (Abraham et al, 1986), while earlier studies had implicated diffusible molecules in tumor angiogenesis (Greenblatt & Shubick, 1968). Therefore, many investigators in the field felt that some key angiogenic factors had yet to be discovered.

Independent efforts contributed to the discovery of VEGF. In 1983, Senger and Dvorak described the identification in the conditioned medium of a tumor cell line of a protein that induced vascular leakage when injected in the guinea pig skin, which they named vascular permeability factor (VPF) (Senger et al, 1983). These authors hypothesized that VPF may be a mediator of the high permeability properties of tumor blood vessels (Senger et al, 1983). However, these efforts did not yield the full purification of the VPF protein. The lack of amino acid sequence data precluded cDNA cloning and establishing the identity of VPF. Therefore, very limited progress in elucidating the significance and function of VPF took place during the following several years (Ferrara, 2009).

In 1989, we reported the isolation and cloning of a novel heparin-binding endothelial cell mitogen secreted by bovine pituitary follicular cells (Ferrara & Henzel, 1989; Leung et al, 1989). This effort began while I was a postdoctoral fellow at the Reproductive Endocrinology Center at the University of California, San Francisco in the mid 1980s. While culturing various cell types from bovine pituitary, I isolated a population of non-hormone secreting cells that we subsequently identified as follicular or folliculo-stellate cells (Ferrara et al, 1986; Ferrara et al, 1987). The function of follicular cells was unclear, but it had been observed that their foot-like projections establish intimate contacts with the pituitary perivascular spaces, a finding that led early investigators to suggest, among several possibilities, that follicular cells might play some role in the maintenance of the pituitary vasculature (Forbes, 1972; Vila-Porcile, 1972). In the course of these studies, I discovered that follicular cell conditioned medium was strongly mitogenic to cultured endothelial cells. Because this mitogenic activity was detectable at high level in the medium, I speculated that the molecule responsible for such activity was a soluble protein and therefore likely to be different from bFGF. In 1988 I joined Genentech, where I had the opportunity to pursue the isolation of this growth factor. In 1989 we were able to determine the NH_2-terminal amino acid sequence of the purified protein. This analysis proved that the molecule was distinct from bFGF or any known protein in available databases (Ferrara & Henzel, 1989; Leung et al, 1989). Because this molecule appeared to have growth-promoting activity selectively for vascular endothelial cells, I proposed the term "vascular endothelial growth factor" (VEGF). The NH_2-terminal amino acid sequence of bovine VEGF enabled the design of oligonucleotide probes to screen cDNA libraries and we were able to identify bovine and human clones encoding multiple molecular species (isoforms) of VEGF, likely due to alternative mRNA splicing (Leung et al, 1989). A typical signal sequence preceded the NH_2-terminus determined by protein sequencing (Leung et al, 1989), confirming our initial hypothesis that VEGF was a secreted protein.

After our cDNA cloning paper was accepted for publication (Leung et al, 1989), we learned that a group at the Monsanto Company had submitted at about the same time a manuscript reporting on the cloning of VPF (Keck et al, 1989). These investigators described a human clone which encoded a protein identical to one of the VEGF isoforms that we discovered, $VEGF_{189}$ (Keck et al, 1989). This group had followed up on the aforementioned early work by Senger et al (1983) and was able to purify and sequence VPF. Therefore, it appeared that a single molecule possesses both mitogenic and permeabity-enhancing activities. This was entirely unexpected

because other endothelial cell mitogens did not induce vascular permeability.

EARLY STUDIES IMPLICATING VEGF IN THE REGULATION OF ENDOTHELIAL CELL GROWTH

The cloning of VEGF, termed also VEGF-A, was followed by the discovery of several related molecules, VEGF-B (Olofsson et al, 1996), VEGF-C (Joukov et al, 1996), VEGF-D (Orlandini et al, 1996; Achen et al, 1998) and placenta growth factor (Maglione et al, 1991). This family of factors generated at that time significant interest in the field (Ferrara, 2002; Alitalo et al, 2005; Ferrara et al, 2003). However, much more work was needed before one could conclude that VEGF (or any of these related molecules) plays a physiologically significant role. On the other hand, our early findings were quite encouraging. For example, the VEGF isoforms appeared well suited to generate biochemical gradients, a requirement for angiogenesis *in vivo* (Ferrara, 2010a), due to their differential affinity for heparan-sulfate proteoglycans in the cell surface and extracellular matrix, which in turn markedly affected their diffusibility characteristics (Houck et al, 1992; Park et al, 1993).

A key question was whether VEGF plays a role as an angiogenic factor *in vivo*. The earliest evidence that VEGF expression is correlated to blood vessel growth was from a study testing the hypothesis that VEGF may be implicated in a key physiological process. It is well established that follicular growth and the development of the ovarian *corpus luteum* (CL) are dependent on the growth of new capillary vessels (Bassett, 1943). We tested whether VEGF mRNA expression is related the blood vessel growth in the rat ovary. We found that the VEGF mRNA is expressed at low levels in the avascular granulosa cells, but is strongly up-regulated in the highly vascularized CL (Phillips et al, 1990). These early findings represented purely correlative evidence, but subsequent studies using potent VEGF inhibitors provide compelling evidence that VEGF is required for CL angiogenesis (Ferrara et al, 1998). Another key evidence was the finding that the VEGF high affinity binding sites are largely restricted to the vascular endothelium *in situ* as assessed by ligand autoradiography in tissue sections from both developing (Jakeman et al, 1993) and adult (Jakeman et al, 1992) rats, suggesting that the selectivity of VEGF for cultured endothelial cells could be extended to the *in vivo* context. Taken together, these initial findings suggested that VEGF was an interesting candidate as a potential regulator of angiogenesis.

Major steps toward a greater understanding of the function of VEGF were the discoveries of two tyrosine kinase VEGF receptors. The first VEGF receptor to be identified was the fms-like tyrosine kinase (Flt-1), known subsequently as VEGFR-1 (Shibuya et al, 1990; De Vries et al, 1992). Subsequently, a highly homologous tyrosine kinase, known as KDR, Flk-1, or VEGFR-2, was also shown to bind VEGF (Terman et al, 1992). There is now agreement that VEGFR-2 is the major signaling receptor that mediates most of the biological activities of VEGF (Ferrara et al, 2003; Chung et al, 2010). Interestingly, other studies implicated VEGF-C and VEGF-D in the regulation of lymphatic angiogenesis through binding to VEGFR-3, a tyrosine kinase receptor highly homologous to VEGFR-1 and VEGF-2 (reviewed in Alitalo et al, 2005).

Later on, the discovery that neuropilin-1, a molecule implicated in axon guidance (Neufeld et al, 2002), functions also as a co-receptor for heparin-binding VEGF isoforms and modulates the activation of VEGFR-2 (Soker et al, 1998), added a new level of complexity to our understanding of VEGF signaling. It also emphasized that existence of common molecular mechanisms guiding nerve fibers and blood vessels, which tend to course alongside each other throughout the body (Carmeliet & Tessier-Lavigne, 2005).

VEGF AS AN ANGIOGENIC FACTOR *IN VIVO* AND A POTENTIAL THERAPEUTIC TARGET

To advance our understanding of the role of VEGF *in vivo*, we developed genetic (Ferrara et al, 1996; Gerber et al, 1999a) and pharmacological tools such as neutralizing anti-VEGF monoclonal antibodies (Kim et al, 1992) and high affinity soluble receptors (Davis-Smith et al, 1996). Inactivation of the *VEGF* gene in mice provided compelling evidence for the crucial role of this molecule in developmental angiogenesis. In 1996 our group (Ferrara et al, 1996) and

Carmeliet's group (Carmeliet et al, 1996) reported that VEGF is essential for normal embryonic vasculogenesis and angiogenesis. Strikingly, inactivation of even a single *VEGF* allele in mice resulted in defective vascular development and early embryonic lethality. Further studies documented the crucial role of VEGF in postnatal organ (Gerber et al, 1999a) and skeletal (Gerber et al, 1999b) growth, using chimeric soluble VEGF receptors as function blockers.

In 1993 we reported that anti-VEGF antibodies inhibit growth of several human tumor cell lines when implanted in immunodeficient mice, while the antibodies had no direct inhibitory effects on tumor cell proliferation (Kim et al, 1993). These findings provided the earliest direct evidence that tumor growth is angiogenesis-dependent. These observations were then extended to many tumor models, using a variety of VEGF inhibitors (Ferrara, 2002) and paved the way for subsequent clinical development of VEGF inhibitors as cancer therapeutics.

In 1994, in collaboration with Aiello and King at the Joslin Diabetes Center in Boston, we reported that VEGF is highly expressed in the ocular fluids of patients with ischemic retinal disorders such as proliferative diabetic retinopathy (Aiello et al, 1994). Proof of concept studies in animal models documented the essential role of VEGF in retinal ischemia-induced neovascularization (Aiello et al, 1995; Adamis et al, 1996). There is now agreement that VEGF is indeed is "Factor X" hypothesized by Michaelson (1948).

To test the hypothesis that blocking VEGF may have impact on human diseases, we developed reagents suitable for clinical trials: a humanized version of an anti-VEGF monoclonal antibody (Presta et al, 1997), which is presently known as bevacizumab or Avastin (Ferrara et al, 2004), and ranibizumab, or Lucentis, an affinity-matured Fab variant of bevacizumab (Chen et al, 1999). Clinical trials testing these inhibitors in cancer and age-related macular degeneration (AMD) began in 1997 and 2000, respectively.

IMPACT OF VEGF INHIBITORS ON HUMAN DISEASE

Work over the last two decades has demonstrated that VEGF is the key regulator of physiological angiogenesis. As noted, even a partial VEGF loss is incompatible with embryonic development. Therefore, the discovery of VEGF has had a major impact on basic vascular biology. Importantly, VEGF blockers inhibited physiological and pathological angiogenesis in a variety of models. Clinical studies have also established that inhibiting VEGF confers benefits to human patients, leading to multiple drug approvals. The first VEGF inhibitor to be approved by the FDA is bevacizumab (February 2004), following a randomized phase III study showing that adding bevacizumab to cytotoxic chemotherapy resulted in increased median survival and progression-free survival in patients with previously untreated metastatic colorectal cancer (Hurwitz et al, 2004). Subsequent clinical trials established the benefits of bevacizumab therapy in multiple malignancies (Sandler et al, 2006; Escudier et al, 2008; Burger et al, 2011), leading to additional FDA approvals. Furthermore, several small molecule VEGF receptor tyrosine kinase inhibitors, including sunitinib, sorafenib and pazopanib, have been approved by the FDA for cancer therapy (reviewed in Ellis & Hicklin, 2008).

Blocking VEGF with ranibizumab resulted in a substantial benefit, including gaining visual acuity, in patients with neovascular AMD (Rosenfeld et al, 2006; Brown et al, 2006). Other VEGF inhibitors including bevacizumab and the VEGF-trap, a chimeric soluble receptor, also resulted in improved vision (Ferrara, 2010b). Interestingly, recent studies indicate that the availability of VEGF inhibitors has had a dramatic impact on the clinical course of AMD, to the extent that AMD may no longer be the leading cause of blindness in the elderly population (Campbell et al, 2012). To date, ten different VEGF pathway inhibitors have been approved for treatment of cancer or intraocular neovascular disorders (Singh & Ferrara, 2012).

REFERENCES

Abraham JA, *et al.* (1986) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**(4763):545-548.

Achen MG, *et al.* (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proceedings of the National Academy of Sciences of the United States of America* **95**(2):548-553.

Adamis AP, *et al.* (1996) Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. *Arch-Ophthalmol* **114**(1):66-71.

Aiello LP, *et al.* (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. [see comments]. *New England Journal of Medicine* **331**(22):1480-1487.

Aiello LP, *et al.* (1995) Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc-Natl-Acad-Sci-U-S-A* **92**(23):10457-10461 issn: 10027-18424.

Alitalo K, Tammela T, & Petrova TV (2005) Lymphangiogenesis in development and human disease. *Nature* **438**:946-953.

Bassett DL (1943) The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle. *Am. J. Anat.* **73**:251-278.

Brown DM, *et al.* (2006) Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *The New England journal of medicine* **355**(14):1432-1444.

Burger RA, *et al.* (2011) Incorporation of bevacizumab in the primary treatment of ovarian cancer. *The New England journal of medicine* **365**(26):2473-2483.

Campbell JP, Bressler SB, & Bressler NM (2012) Impact of availability of anti-vascular endothelial growth factor therapy on visual impairment and blindness due to neovascular age-related macular degeneration. *Archives of ophthalmology* **130**(6):794-795.

Carmeliet P, *et al.* (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**(6573):435-439 issn: 0028-0836.

Carmeliet P & Tessier-Lavigne M (2005) Common mechanisms of nerve and blood vessel wiring. *Nature* **436**(7048):193-200.

Carrel A (1913) Artificial Activation of the Growth in Vitro of Connective Tissue. *The Journal of experimental medicine* **17**(1):14-19.

Carrel A & Ebeling AH (1923) Action of Serum on Fibroblasts in Vitro. *The Journal of experimental medicine* **37**(6):759-765.

Carrel A & Ebeling AH (1923) Survival and Growth of Fibroblasts in Vitro. *The Journal of experimental medicine* **38**(5):487-497.

Cattaneo A (2013) Immunosympathectomy as the first phenotypic knockout with antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **110**(13):4877-4885.

Chen Y, *et al.* (1999) Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *Journal of molecular biology* **293**(4):865-881.

Chung AS, Lee J, & Ferrara N (2010) Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer* **10**(7):505-514.

Cohen S & Levi-Montalcini R (1956) A Nerve Growth-Stimulating Factor Isolated from Snake Venom. *Proceedings of the National Academy of Sciences of the United States of America* **42**(9):571-574.

Cohen S & Levi-Montalcini R (1957) Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180. *Cancer research* **17**(1):15-20.

Davis-Smyth T, Chen H, Park J, Presta LG, & Ferrara N (1996) The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. *EMBO Journal* **15**(18):4919-4927.

Dennis PA & Rifkin DB (1990) Studies on the role of basic fibroblast growth factor in vivo: inability of neutralizing antibodies to block tumor growth. *Journal of Cellular Physiology* **144**(1):84-98.

de Vries C, *et al.* (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* **255**(5047):989-991.

Ellis LM & Hicklin DJ (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* **8**(8):579-591.

Escudier B, *et al.* (2008) Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. *Lancet* **370**(9605):2103-2111.

Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* **2**(10):795-803.

Ferrara N (2009) Vascular Endothelial Growth Factor. *Arterioscler Thromb Vasc Biol* **29**:789-791.

Ferrara N (2010a) Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. *Mol. Biol. Cell* **21**(5):687-690.

Ferrara N (2010b) Vascular endothelial growth factor and age-related macular degeneration: from basic science to therapy. *Nature medicine* **16**(10):1107-1111.

Ferrara N, *et al.* (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF

gene. *Nature* **380**(6573):439-442.

Ferrara N, *et al.* (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nature medicine* **4**:336-340.

Ferrara N, Fujii DK, Goldsmith PC, Widdicombe JH, & Weiner RI (1987) Transport epithelial characteristics of cultured bovine pituitary follicular cells. *American Journal of Physiology* **252**(3 Pt 1):E304-312.

Ferrara N, Gerber HP, & LeCouter J (2003) The biology of VEGF and its receptors. *Nature Med.* **9**:669-676.

Ferrara N, Goldsmith P, Fujii D, & Weiner R (1986) Culture and characterization of follicular cells of the bovine anterior pituitary and pars tuberalis. *Methods in Enzymology;* **124**:245-53.

Ferrara N & Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochemical & Biophysical Research Communications* **161**(2):851-858.

Ferrara N, Hillan KJ, Gerber HP, & Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature reviews. Drug discovery* **3**(5):391-400.

Folkman J, Merler E, Abernathy C, & Williams G (1971) Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.* **133**:275-288.

Folkman J & Klagsbrun M (1987) Angiogenic factors. *Science* **235**:442-447.

Forbes MS (1972) Fine structure of the stellate cell in the pars distalis of the lizard, Anolis carolinensis. *Journal of morphology* **136**(2):227-245.

Gerber HP, *et al.* (1999a) VEGF is required for growth and survival in neonatal mice. *Development* **126**:1149-1159.

Gerber HP, *et al.* (1999b) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Med.* **5**:623-628.

Gospodarowicz D, Ferrara N, Schweigerer L, & Neufeld G (1987) Structural characterization and biological functions of fibroblast growth factor. *Endocrine Reviews* **8**(2):95-114.

Greenblatt M & Shubick P (1968) Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. *Journal of the National Cancer Institute* **41**:111-124.

Hamburger V (1969) Hans Spemann and the organizer concept. *Experientia* **25**(11):1121-1125.

Hamburger V (1993) The history of the discovery of the nerve growth factor. *Journal of neurobiology* **24**(7):893-897.

Hamburger V (1997) A historic moment: the discovery of the chemical transmission of embryonic inductions. *Developmental neuroscience* **19**(4):293-296.

Houck KA, Leung DW, Rowland AM, Winer J, & Ferrara N (1992) Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J-Biol-Chem* **267**(36):26031-26037.

Hurwitz H, *et al.* (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* **350**:2335-2342.

Ide AG, Baker NH, & Warren SL (1939) Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. *Am. J. Roentgenol.* **42**:891-899.

Jakeman LB, Armanini M, Philips HS, & Ferrara N (1993) Developmental expression of binding sites and mRNA for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrinology* **133**:848-859.

Jakeman LB, Winer J, Bennett GL, Altar CA, & Ferrara N (1992) Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *Journal of Clinical Investigation* **89**(1):244-253.

Joukov V, *et al.* (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO-J* **15**(7):1751 issn: 0261-4189.

Keck PJ, *et al.* (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246**(4935):1309-1312.

Kim KJ, Li B, Houck K, Winer J, & Ferrara N (1992) The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors* **7**(1):53-64.

Kim KJ, *et al.* (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. *Nature* **362**:841-844.

Klagsbrun M & D'Amore PA (1991) Regulators of angiogenesis. *Annu Rev Physiol* **53**:217-239.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, & Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**(4935):1306-1309.

Levi-Montalcini R, Meyer H, & Hamburger V (1954) In vitro experiments on the effects of mouse sarcomas 180

and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer research* **14**(1):49-57.

Levi-Montalcini R (1975) Nerve growth factor. *Science* **187**(4172):113.

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, & Persico MG (1991) Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *ProcNatlAcadSci USA* **88**(20):9267-9271.

Michaelson IC (1948) The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. *Trans. Ophthalmol. Soc. U.K.* **68**:137-180.

Neufeld G, *et al.* (2002) The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends Cardiovas. Med.* **12**:13-19.

Olofsson B, *et al.* (1996) Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc-Natl-Acad-Sci-U-S-A* **93**(6):2576-2581 issn: 0027-8424.

Orlandini M, Marconcini L, Ferruzzi R, & Oliviero S (1996) Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family [corrected; erratum to be published]. *Proc-Natl-Acad-Sci-U-S-A* **93**(21):11675-11680.

Park JE, Keller G-A, & Ferrara N (1993) The vascular endothelial growth factor isoforms (VEGF): Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Molecular Biology of the Cell* **4**:1317-1326.

Phillips HS, Hains J, Leung DW, & Ferrara N (1990) Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* **127**(2):965-967.

Presta LG, *et al.* (1997) Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res.* **57**:4593-4599.

Rosenfeld PJ, *et al.* (2006) Ranibizumab for neovascular age-related macular degeneration. *N. Engl. J. Med.* **355**:1419-1431.

Sandler A, *et al.* (2006) Paclitaxel-Carboplatin Alone or with Bevacizumab for Non-Small-Cell-Lung Cancer. *N. Engl. J. Med.* **355**:2542-2550.

Senger DR, *et al.* (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**:983-985.

Shibuya M, *et al.* (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase (flt) closely related to the fms family. *Oncogene* **8**:519-527.

Singh M & Ferrara N (2012) Modeling and predicting clinical efficacy for drugs targeting the tumor milieu. *Nat Biotechnol* **30**(7):648-657.

Soker S, Takashima S, Miao HQ, Neufeld G, & Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**(6):735-745.

Terman BI, *et al.* (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochemical & Biophysical Research Communications* **187**(3):1579-1586.

Vila-Porcile E (1972) [The network of the folliculo-stellate cells and the follicles of the adenohypophysis in the rat (pars distalis)]. *Zeitschrift fur Zellforschung und mikroskopische Anatomie* **129**(3):328-369.

NOVEL EXPERIMENTAL MODELS TO STUDY NEUROTROPHIN FUNCTION

F. CESCA[1], G. SCHIAVO[2], F. BENFENATI[1]

[1]Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Genova, Italy; [2]Sobell Department of Motor Neuroscience and Movement Disorders, Institute of Neurology, University College London, London, UK

THE CLASSICAL "NEUROTROPHIC HYPOTHESIS" AND NOVEL NEUROTROPHIN FUNCTIONS

In the history of science, few research papers stand out amongst uncountable publications, acting as foundations of the vast building of modern research. Amongst such papers, a prominent position is undisputedly held by the publication in which Stanley Cohen, Rita Levi-Montalcini and Viktor Hamburger first described a novel "nerve growth factor" (NGF), a target-derived pro-survival factor for sensory and sympathetic neurons derived from ganglia isolated from chick embryos (Cohen et al., 1954). This seminal work paved the path for the subsequent discovery of the other members of the mammalian neurotrophin (NT) family, namely brain-derived neurotrophic factor (BDNF), neurotrophin-3 and -4 (NT-3 and NT-4), together with their specific tropomyosin-related kinase (Trk) receptors TrkA, TrkB and TrkC, as well as the pan-neurotrophin receptor $p75^{NTR}$ (Bibel and Barde, 2000). In the nineties, the improvements and widespread application of mouse genetics allowed the generation of several lines of genetically-modified mice lacking single neurotrophins or their receptors. This work, mostly performed by the research groups led by Mariano Barbacid, Rudolf Jaenisch, Luis Parada, Heidi Phillips, and Louis Reichardt (Klein et al., 1993; Crowley et al., 1994; Ernfors et al., 1994a; Ernfors et al., 1994b; Klein et al., 1994), provided a wealth of information on the physiological roles of the neurotrophin/receptor complexes. A general picture therefore emerged, whereby neurons of the peripheral nervous system rely on one or just few neurotrophins, while a higher level of complexity and redundancy applies to neurons of the central nervous system (CNS) (Bibel and Barde, 2000). However, these initial findings also made clear that the function of NTs extended far beyond the original and somehow simplistic role of pro-survival factors for neurons, aimed at matching neuronal populations to the size of their targets. In this respect, for example, the complete deletion of all TrkC isoforms caused a range of cardiovascular defects that for the first time implied a role for neurotrophins in tissues other than the nervous system (Tessarollo et al., 1997). At the molecular level, several groups have contributed over the years to describe in detail the signalling pathways initiated by the binding of neurotrophins to their receptors, and the molecular mechanisms underlying the transport of specific signalling endosomes from the axon terminal to the cell body (for a recent review on the subject, see (Harrington and Ginty, 2013)). More recently, a novel hypothesis has been put forward, whereby Trk receptors (TrkA and TrkC) are not any more considered "just" pro-survival factors, but rather as dependence receptor. In other words, Trks could act as "double edged sword" molecules, actively promoting neuronal survival and differentiation when bound to their cognate ligands, and similarly, actively promoting cell death in absence of ligands, contrasting (or rather revising) the generally accepted tenet by

Corresponding Author:
Fabrizia Cesca, PhD
Department of Neuroscience and Brain Technologies,
Istituto Italiano di Tecnologia
Via Morego, 30 – 16163 Genova, Italy
Phone: +39 010 71781788; Fax: +39 010 71781230
Email: fabrizia.cesca@iit.it

2279-5855 (2013)

which neurotrophin withdrawal would just unmask default death pathways (Tauszig-Delamasure et al., 2007; Nikoletopoulou et al., 2010; Ichim et al., 2012; Dekkers and Barde, 2013). An additional level of complexity in the NT system is provided by the presence of $p75^{NTR}$, and by the ever growing set of functions attributed to pro-neurotrophins, which are known to trigger a wide range of cellular responses, independently of the classical "mature NT-Trk" axis (Kotlyanskaya et al., 2013). It is now clear that the balance between the precursor and mature forms of NTs, as well as the repertoire of Trk / $p75^{NTR}$ receptors expressed by the single neuron and their relative localisation in discrete sub-domains, will ultimately determine the outcome of every neurotrophin-based input. These outcomes span an array of biological effects, ranging between pro-survival and pro-death pathways (Lu et al., 2005). Besides such basic "life or death" decisions, NTs were soon implicated into the more subtle regulation of synapse development and function, playing important roles into the refinement of connectivity in complex neural circuits (Poo, 2001). Such a fine action mostly relies on the activity-dependent secretion of NTs, in particular of BDNF, which in mature neuronal circuits is regulated in a synapse-specific fashion. Dendritic localization of BDNF mRNA transcripts allows the fast and specific translation of BDNF at synaptic sites, where it can act in both an autocrine and paracrine manner to modulate dendritic development and synaptic plasticity (Kuczewski et al., 2010). Another very interesting field of investigation, which has progressively gained importance, concerns the ability of the various components of the NT pathways to cross-talk with different signalling routes. In one of the earliest reports on this subject (Lee and Chao, 2001), Trk receptors were shown to be activated in hippocampal neurons also in absence of neurotrophins. This effect was mediated by adenosine binding to A_{2A} receptors, which belong to the G-protein coupled receptor superfamily. A_{2A} receptors were able to transactivate Trks by phosphorylation via Src family kinases (Lee and Chao, 2001). Successively, ephrinA5 was shown to interact with TrkB on retinal ganglion cell axons. Depending on the presence of the cognate Eph ligand, ephrinA5 was able to either promote or inhibit TrkB signalling, axonal branching and synaptogenesis (Marler et al., 2008). An increasing number of pathways have been recently associated to the NT-Trk system. Pühringer and colleagues have showed how transactivation of TrkB and TrkC by the epithelial growth factor receptor (EGFR) is required to drive migration of newborn cortical neurons, independently of their "classical" ligands BDNF and NT-3 (Puehringer et al., 2013). Wnt signalling is also coming to light as an important modulator of NT stimuli. In fact, Wnt-dependent pathways have been shown to mediate axonal growth in developing sympathetic neurons by modulating NGF-TrkA signalling (Bodmer et al., 2009), and to cooperate with BDNF-TrkB in the molecular events driving dendrite growth and dendritic spine formation in primary cortical neurons (Hiester et al., 2013). Last but not least, the NGF-TrkB pathway has been implicated in the modulation of endocannabinoid signalling in developing cholinergic neurons (Keimpema et al., 2013).

The integration between previously unrelated pathways is emerging as a widespread modality of signalling, and more links and connections are likely to emerge in the future. The maintenance of such an integrated signalling network is apt to achieve the coordination of the myriad of cellular and molecular processes required for the correct development and functioning of the nervous system.

NOVEL EXPERIMENTAL MODELS TO STUDY NT FUNCTION

Chick, mouse and rat embryos have been used over the years to dissect the biological functions of NTs. The main reason for the choice of these vertebrate models is that simpler organisms such as *D. melanogaster* or *C. elegans* cannot be used as they lack a canonical NT system (Benito-Gutierrez et al., 2006). While this manuscript was in preparation, McIlroy and colleagues reported that Toll receptors 6 and 7 act as receptors for *D. melanogaster* neurotrophins DNT1 and DNT2, thus opening a potential new area of investigation in the neurotrophin field, using *Drosophila* as model organism (McIlroy et al., 2013). The full complement of NTs and Trks have been identified in fish, together with two additional isoforms (NT-6 and NT-7) with no orthologs yet described in vertebrates. Despite the similarities between zebrafish and mammalian NTs,

and the many advantages of the former compared to rodents, zebrafish was never fully accepted as model organism in the NT field (Heinrich and Lum, 2000).

The analysis of the NT- and Trk mouse knockout strains has been fundamental to demonstrate the importance of NT signalling during both nervous system development and in adulthood. These mouse models have also helped to appreciate the differences and commonalities between the physiological roles of NTs in the central vs the peripheral nervous system. With few exceptions, full or conditional deletion of one or more NTs or Trk receptors causes a wide range of deficits, making it difficult to dissect the subtle mechanisms underlying different aspects of NT physiology. With the goal of dissecting in time and space the mechanisms controlling the NT-Trk system, researchers in the field turned to the analysis of mouse lines lacking single components of NT-dependent pathways, which are expected to give a more restricted, and henceforth specific, range of phenotypes. Unexpected contributions to a better understanding of NT physiology have been recently provided by the analysis of mouse models lacking proteins that had not been previously associated with these growth factors. In this review, we aim to give an overview of the most significant recent developments on three key topics: (i) molecular mechanisms of NT signalling; (ii) synaptic functions of NT; (iii) NTs in neuropsychiatric diseases.

(i) Molecular mechanisms of NT signalling.

Upon NT binding on the plasma membrane, Trk receptors are internalised. They can then either signal locally, or be retrogradely transported to the neuronal cell body, where they initiate a different set of biochemical cascades whose final outcome is the long-term modulation of gene transcription (Heerssen and Segal, 2002). These cellular events have been extensively studied, however, some recent work has further clarified the molecular events regulating receptor trafficking and signalling. The generation of mutant mouse lines bearing the neuron-specific deletion of the serine/threonine phosphatase calcineurin shed light onto the molecular mechanisms underlying NGF-triggered endocytosis of TrkA in sympathetic neurons, demonstrating how the dephosphorylation of dynamin by calcineurin itself is a necessary step for TrkA endocytosis (Bodmer et al., 2011). Taking advantage of the genetic deletion of sortilin, it was possible to demonstrate a new role for this membrane receptor in mediating the anterograde transport of Trk receptors, in addition to its established function as co-receptor for $p75^{NTR}$ (Vaegter et al., 2011). New important insights on the mechanisms regulating the retrograde transport of TrkB were provided in a recent work, in which snapin was identified as an adaptor for the microtubule motor protein dynein. The analysis of primary neurons isolated from snapin knockout animals was instrumental to demonstrate that the interaction between snapin and dynein is required for the retrograde transport of activated TrkB receptor, and is essential for dendritic development in cortical neurons (Zhou et al., 2012).

Our understanding of the complex signalling network initiated by NTs has also being revolutionized by several recent studies. In support of the view that Wnt is an important NT effector, the analyses of neuronal cultures lacking Wnt5a and of a Wnt5a knockout mouse line unveiled a previously uncharacterized pathway, in which NGF modulates sympathetic neuron development by promoting the activation of Wnt5a-mediated signalling (Bodmer et al., 2009). Similarly, MAP kinase phosphatase 1 (MKP-1) activity was described to shape axon branches in response to BDNF by inactivating c-jun N-terminal kinase (JNK) and destabilizing microtubules (Jeanneteau et al., 2010). Moreover, studies on a knockout mouse for kinase D interacting protein / ankyrin repeat membrane spanning (Kidins220/ARMS) have been instrumental to reveal new mechanisms of neurotrophin signalling through the Kidins220/ARMS adaptor protein. Interestingly, Kidins220/ARMS might integrate NT and vascular endothelial growth factor (VEGF) signalling during cardiovascular development, and possibly in neuronal differentiation (Cesca et al., 2011; Cesca et al., 2012). On the same line, the analysis of mice lacking glucocorticoid-induced tumor necrosis factor receptor-related (GITR) showed how this protein and its ligand (GITRL) promote NGF signalling in sympathetic neurons, by phosphorylating extracellular signal-regulated kinases (Erk) 1 and 2 (O'Keeffe et al., 2008).

The nature of signalling endosomes containing NT is still controversial, since several types of

organelles have been linked to the axonal transport of Trk receptors, including early endosomes, multivesicular bodies (MBV) and late endosomes (Bhattacharyya et al., 2002; Salehi et al., 2006). Clearly, both early and late endosomal proteins have been associated with signalling endosomes. The small GTPase Rab5 is an early endosomal marker and plays a key role in the formation and fusion of early endosomes, whilst Rab7 is localized on late endosomes and lysosomes (Mizuno-Yamasaki et al., 2012). Interestingly, both these markers have been associated with NT signalling endosomes in different cell types. For example, NGF and TrkA were found in Rab5-positive early endosomes in sensory neurons (Howe et al., 2001; Delcroix et al., 2003), whereas Rab7 has been shown to be a key player in the axonal retrograde transport of TrkB and $p75^{NTR}$ in motor neurons (Deinhardt et al., 2006) and to participate in TrkA signalling via ERK1/2 in PC12 cells (Saxena et al., 2005a; Saxena et al., 2005b). Rab7 and Rab7-interacting proteins were also identified via a proteomic analysis in TrkA-positive endosomes isolated from PC12 cells stimulated with NGF (Harrington et al., 2011). Trk-positive signalling endosomes may therefore undergo a transition from Rab5 to Rab7 correspondent to endosome maturation (Rink et al., 2005) to engage in fast and long-range axonal retrograde transport.

Activated Trks are able to recruit several adaptors to their cytoplasmic domain, each of which is able to trigger specific intracellular responses. Understanding the contribution of each signal transduction pathway has been hampered so far by the fact that full and conditional knockout strategies impair the full set of intracellular NT adaptors and pathways. Recently, a mouse knockin strain for a phospho-mutant form of TrkB ($TrkB^{S478A/S478A}$) has been generated, allowing a better understanding of the role of Cdk5 phosphorylation of TrkB receptors in the process of synaptic plasticity and memory formation (Lai et al., 2012).

(ii) Synaptic function of NTs.

In addition to promoting neuronal survival and differentiation, NTs are now recognized as essential modulators of synaptic plasticity. In this context, a functional loop has been described whereby synaptic activity drives synthesis and secretion of NTs, which in turn modulate synaptic efficacy and morphology (Poo, 2001). Accordingly, mouse lines displaying defects in synaptic physiology have provided valuable information about the role played by NTs in this specialized neuronal compartment. Few examples are discussed in the following paragraph.

Synapsins (Syns) are synaptic vesicle proteins known to play an essential role in the mobilization of SV pools at pre-synaptic terminals (Cesca et al., 2010). Recently, work from our group has demonstrated that beyond their well-established role in the regulation of the SV cycle, Syns represent an essential part of the mechanism controlling BDNF-mediated enhancement of post-tetanic potentiation (PTP) (Valente et al., 2012). Thus the presynaptic protein synapsin I (syn I), which plays a well-established role in the regulation of synaptic vesicle trafficking, acts as a coincidence detector for high frequency stimulation and BDNF-mediated TrkB activation. Tetanic stimulation increases syn I phosphorylation at Ca^{2+} calmodulin kinase / protein kinase A sites through the intraterminal Ca^{2+} build-up, thus increasing the availability of vesicles for release and the readily releasable pool (RRP) size. Concomitantly, BDNF further increases the RRP size by triggering convergent phosphorylation of syn I at the Erk sites. As an activity-dependent signal, BDNF has also been proposed to have a role in homeostatic plasticity, particularly in the synaptic upscaling that follows prolonged activity deprivation (Rutherford et al., 1998). Further contributions to our understanding of the role of NTs in presynaptic physiology were provided by other mouse models. For example, Kidins220/ARMS has been recently suggested to participate in the modulation of the BDNF-induced potentiation of synaptic plasticity in excitatory neurons (Cesca et al., 2012). Synaptotagmin IV (SytIV), which localises to BDNF-containing vesicles, also controls the rate of SV exocytosis by modulating the release of BDNF from the post-synaptic site (Dean et al., 2009). The interaction of TrkB with the actin-based motor myosin VI and the adaptor protein GIPC1 provides another mechanism by which BDNF modulates synaptic vesicle (SV) release and the short-term synaptic plasticity properties of neurons (Yano et al., 2006). **Figure 1** summarizes the principal sites of action of BDNF at pre- and post-synaptic compartments.

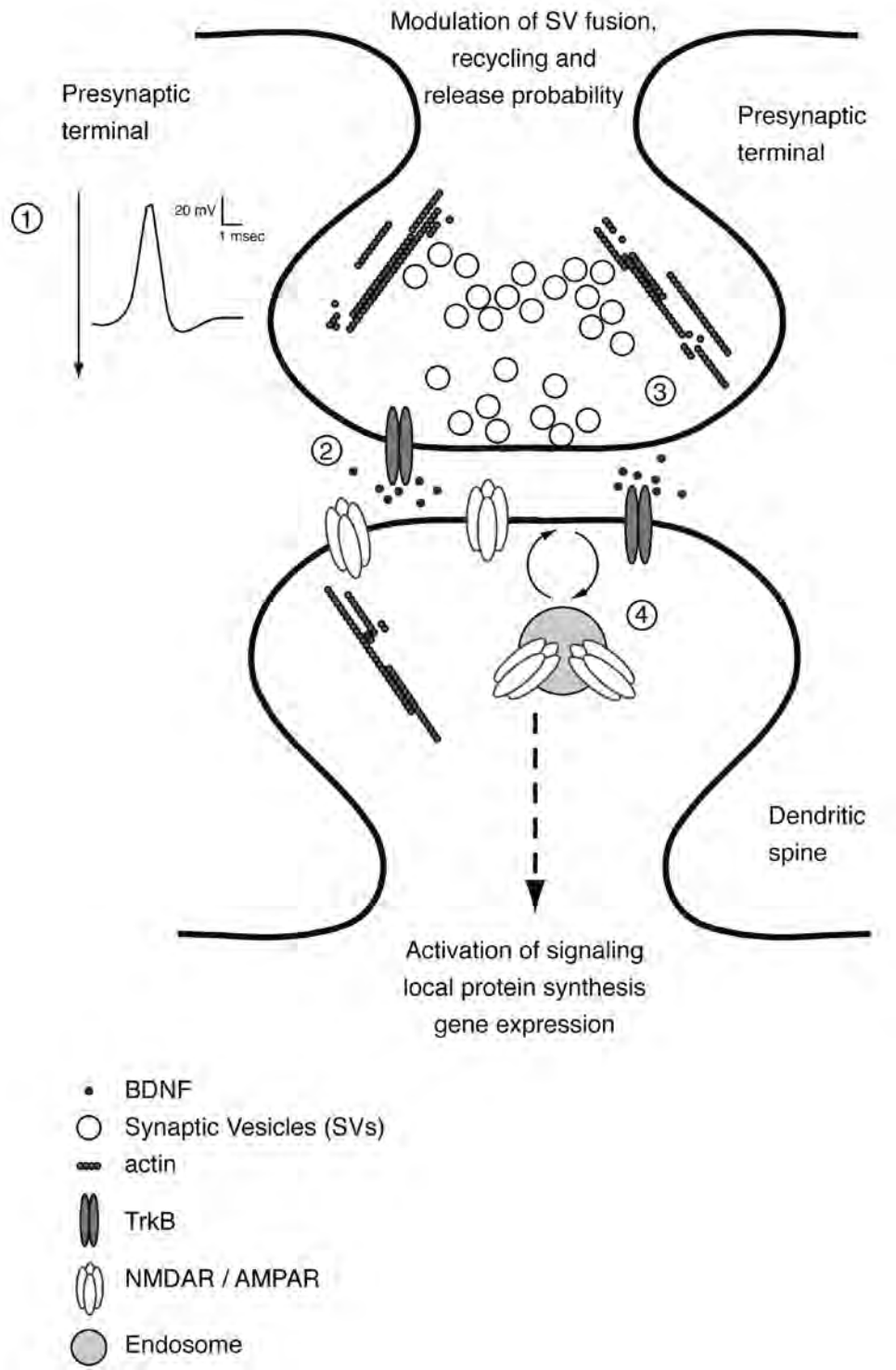


Fig. 1. *BDNF at the pre- and post-synaptic compartment. Neuronal activity (1) triggers BDNF release. At presynaptic level, BDNF-induced TrkB activation (2) plays a role in the control of SV fusion, recycling and release probability (3), mostly via Ca^{2+}-mediated signalling pathways. Post-synaptically, BDNF triggers the activation of several signalling pathways, which lead to both local and fast protein synthesis, cytoskeletal remodelling and long-term changes in gene expression. In addition, BDNF participates in the complex events modulating Hebbian and homeostatic plasticity, by controlling the insertion / removal of AMPAR and NMDAR to and from the post-synaptic membrane (4).*

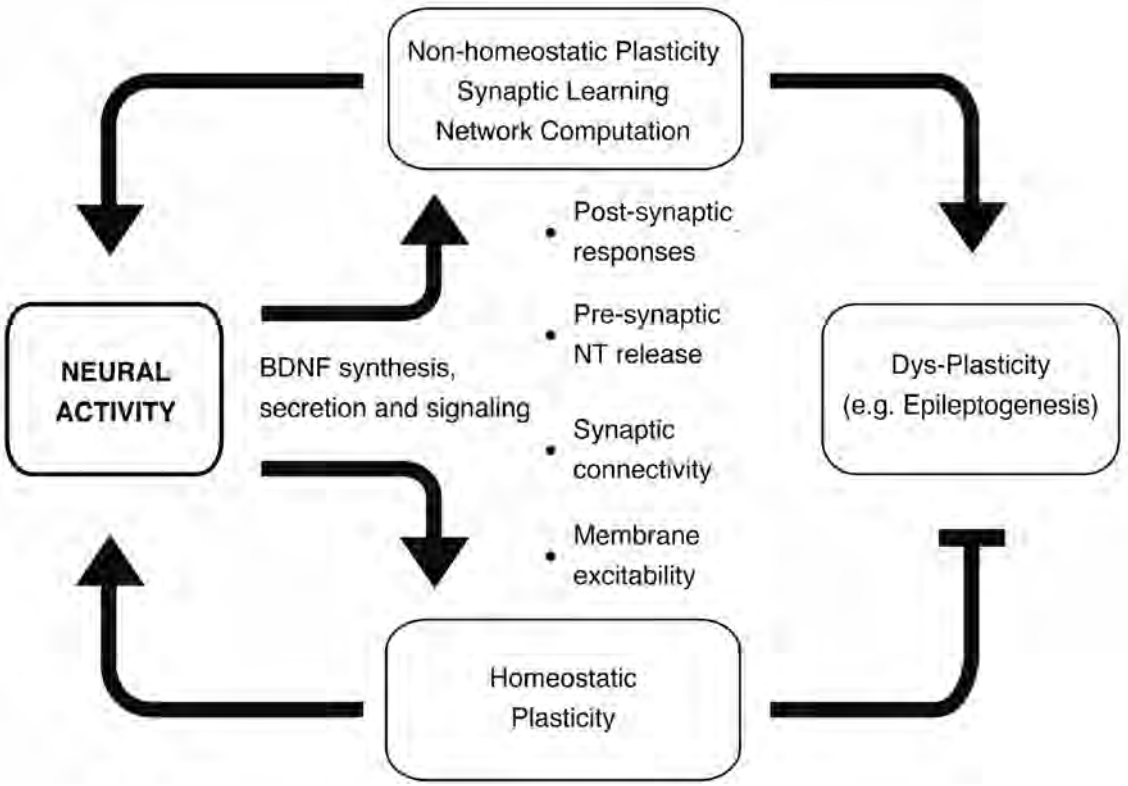


Fig. 2. *BDNF-mediated plasticity occurring under physiological and pathological conditions.*

(iii) NTs and neuropsychiatric diseases.

Given their physiological importance, NT pathways have often been considered primary targets for therapeutic intervention. Several clinical trials are presently underway to test the efficacy of compounds enhancing or inhibiting NT signalling. While a comprehensive survey of such trials is beyond the scope of this review, we discuss few examples that best represent the pathogenic role of NTs and/or the possibility of using NTs as therapeutic tools for neuropsychiatric diseases.

NTs, and in particular BDNF, have been proposed to act as critical modulators of depressive behaviour on the basis of human genetic studies and analyses of knockout and transgenic mouse models (Jiang and Salton, 2013). A large body of evidence suggests that BDNF is involved in depression, leading to the so-called "neurotrophin hypothesis of depression" (Dwivedi, 2013). It has been shown that the single Val-Met nucleotide polymorphism in the human proBDNF gene is associated with a dysregulation of BDNF secretion that impacts behaviour and cognition and has pro-depressant effects. Indeed, lower levels of BDNF were found in depressed patients, an effect that could be rescued by antidepressant drugs.

As BDNF is part of a general mechanism for activity-dependent synaptic modification, diseases characterised by altered excitability (such as epilepsy or neuropathic pain) in some cases are associated with abnormal BDNF signalling. In the presence of unbalanced excitatory/inhibitory synaptic transmission, the initial hyperactivity may induce release of BDNF that further potentiates excitatory transmission and triggers a positive feedback that leads to the overt disease state. Indeed, several studies have found that BDNF is upregulated in epileptogenic areas, and consequently that inhibition of BDNF signalling prevents epileptogenesis (Binder, 2004). A general scheme of the homeostatic and non-homeostatic plasticity phenomena driven

by BDNF, occurring under physiological and pathological (*dys-plasticity*) conditions, is presented in Figure 2.

A reduction of BDNF expression in cortical neurons has been associated with the presence of mutant huntingtin (htt), the culprit of Huntington's disease (HD). By employing knockin animals expressing the pathogenic form of htt, it was possible to demonstrate that reduced supply of BDNF to the striatum is one of the main causes of striatal neuron death in HD (Liot et al., 2013). Reduced BDNF transcription has been described in HD, probably caused by an increase activity of the transcriptional repressor RE1-silencing transcription factor (REST) (Zuccato et al., 2003), and also in mouse models of Rett syndrome, in association with mutations in the transcription factor methyl-CpG binding protein 2 (MeCP2) (Li and Pozzo-Miller, 2013). Along the same line, knockin animals for BDNF Val66Met polymorphism (Chen et al., 2006) have allowed to gather a wealth of information on the molecular mechanisms underlying some forms of genetic predispositions to anxiety and depressive disorders.

NGF has been long considered a promising therapeutic target to treat Alzheimer's disease (AD) due to the high vulnerability of basal forebrain cholinergic neurons, which are strongly dependent on NGF for their survival, in AD. Several transgenic mouse models of AD have been created, based on the proteins that are most commonly mutated in AD patients, such as α-synuclein, amyloid precursor protein (APP), presenilin, Tau and many others (for a recent review, see (LaFerla and Green, 2012). Amongst these, a mouse line expressing an anti-NGF antibody (AD11 mouse model) is particularly relevant to the topic of this review. Such mouse strain was recently used to demonstrate that an imbalance in the relative amount of proNGF/NGF could be one of the main factors leading to neurodegeneration in AD brains, by favouring $p75^{NTR}$ to TrkA signalling (Capsoni et al., 2010). Further support to this hypothesis is provided by the finding that the amyloid precursor protein (APP) is a downstream target of activated TrkA receptors, and takes part in the intracellular pathways stimulated by NGF. This study was made possible by the availability of two mouse lines, one knockin for a phosphorylation mutant of APP, and one bearing the deletion of full-length APP (Matrone et al., 2011). Very recently, defects in TrkB signalling have been described in a mouse model of Angelman syndrome. These mice bear a full deletion of the Ube3A ubiquitin protein ligase and provide an additional link between BDNF signalling and cognitive impairments/autism (Cao et al., 2013).

FUTURE PERSPECTIVES

Since the initial characterization of NTs as target-derived pro-survival factors for peripheral neurons, a much broader role has now been recognized for this family of growth factors, both within and outside of the nervous system. The research in this field has greatly benefitted, and will continue to do so, from the analysis of seemingly unrelated experimental models, i.e. mouse lines lacking signalling or synaptic proteins such as Wnt, Kidins220/ARMS, calcineurin, sortilin, snapin, synapsin, syt IV (Bodmer et al., 2009; Dean et al., 2009; Bodmer et al., 2011; Vaegter et al., 2011; Cesca et al., 2012; Valente et al., 2012; Zhou et al., 2012) and many others, as discussed in previous sections. Possible applications are expected in the medical field, especially for the treatment of neurodegenerative pathologies (for a comprehensive review on the subject, see (Allen et al., 2013)). NTs might also be used as therapeutic molecules also to treat diseases outside of the nervous system, such as some types of cancer (Roesler et al., 2011).

After decades of biochemical research, many (although not all) intracellular pathways activated by NT have been described at molecular level. What would then improve our understanding of the dynamics of NT signalling activation? This process must be tightly modulated, both at a broader scale in a tissue- and cell-specific manner, but also at single cell level. Indeed, signalling pathways undergo a very strict spatial and temporal modulation. To delve into the precise regulation of signalling dynamics, traditional biochemical techniques are not any more up to the task, as they lack the spatial and temporal resolution required, for example, to monitor the rapid and sequential activation of signalling proteins in different compartments of the same cell. With the advent of optogenetics, tools are now available that allow to interrogate cellular processes with unprecedented precision in time and space. As the

number of applications of optogenetics continues to increase, the repertoire of opto-probes designed to target specific signalling pathways is constantly growing. Advanced tracking techniques have become available, which allow to follow the intracellular trafficking of receptors and scaffold proteins, such as the recombinant *F*ibronectin *in*trabodies *g*enerated with m*R*NA display (FingRs). These recently published tools bind endogenous neuronal proteins and incorporate a transcriptional regulation system that ties FingR expression to the level of the target (Gross et al., 2013). Besides molecule tracking, of particular interest are optogenetic techniques for controlling protein-protein interaction that rely on plant-derived photosensitive modules such the PhytochromeB/phytochrome interacting factor 3 (PhyB/PIF3) system (Levskaya et al., 2009), or the Light-Oxygen-Voltage (LOV) domains (Wu et al., 2009). It is highly likely that more of such probes will be engineered in the near future, creating an "*in vitro* / *in vivo* opto-signalling toolbox" comprising probes and sensors able to span multiple signalling events, from the fast and local activation of signalling proteins, to the long-term modulation of gene expression.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Italian Ministry of University and Research (PRIN to F.B.), the Compagnia di San Paolo-Torino (to F.B.), the EC FP7-HEALTH-2013-INNOVATION integrating project "Desire" (to F.B. and F.C.), and Cancer Research UK and Weizmann UK Making Connections (G.S). The support of Telethon-Italy (Grants GGP09134 and GGP13033 to F.B.) is also acknowledged.

REFERENCES:

Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK (2013) GDNF, NGF and BDNF as therapeutic options for neurodegeneration. *Pharmacology & therapeutics* **138**:155-175.

Benito-Gutierrez E, Garcia-Fernandez J, Comella JX (2006) Origin and evolution of the Trk family of neurotrophic receptors. *Molecular and cellular neurosciences* **31**:179-192.

Bhattacharyya A, Watson FL, Pomeroy SL, Zhang YZ, Stiles CD, Segal RA (2002) High-resolution imaging demonstrates dynein-based vesicular transport of activated Trk receptors. *Journal of neurobiology* **51**:302-312.

Bibel M, Barde YA (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev* **14**:2919-2937.

Binder DK (2004) The role of BDNF in epilepsy and other diseases of the mature nervous system. *Advances in experimental medicine and biology* **548**:34-56.

Bodmer D, Ascano M, Kuruvilla R (2011) Isoform-specific dephosphorylation of dynamin1 by calcineurin couples neurotrophin receptor endocytosis to axonal growth. *Neuron* **70**:1085-1099.

Bodmer D, Levine-Wilkinson S, Richmond A, Hirsh S, Kuruvilla R (2009) Wnt5a mediates nerve growth factor-dependent axonal branching and growth in developing sympathetic neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**:7569-7581.

Cao C, Rioult-Pedotti MS, Migani P, Yu CJ, Tiwari R, Parang K, Spaller MR, Goebel DJ, Marshall J (2013) Impairment of TrkB-PSD-95 signaling in Angelman syndrome. *PLoS biology* **11**:e1001478.

Capsoni S, Tiveron C, Vignone D, Amato G, Cattaneo A (2010) Dissecting the involvement of tropomyosin-related kinase A and p75 neurotrophin receptor signaling in NGF deficit-induced neurodegeneration. *Proc Natl Acad Sci U S A* **107**:12299-12304.

Cesca F, Baldelli P, Valtorta F, Benfenati F (2010) The synapsins: key actors of synapse function and plasticity. *Prog Neurobiol* **91**:313-348.

Cesca F, Yabe A, Spencer-Dene B, Arrigoni A, Al-Qatari M, Henderson D, Phillips H, Koltzenburg M, Benfenati F, Schiavo G (2011) Kidins220/ARMS is an essential modulator of cardiovascular and nervous system development. *Cell Death Dis* **2**:e226.

Cesca F, Yabe A, Spencer-Dene B, Scholz-Starke J, Medrihan L, Maden CH, Gerhardt H, Orriss IR, Baldelli P, Al-Qatari M, Koltzenburg M, Adams RH, Benfenati F, Schiavo G (2012) Kidins220/ARMS mediates the integration of the neurotrophin and VEGF pathways in the vascular and nervous systems. *Cell Death Differ* **19**:194-208.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* **314**:140-143.

Cohen S, Levi-Montalcini R, Hamburger V (1954) A

Nerve Growth-Stimulating Factor Isolated from Sarcom as 37 and 180. *Proc Natl Acad Sci U S A* **40**:1014-1018.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD, et al. (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**:1001-1011.

Dean C, Liu H, Dunning FM, Chang PY, Jackson MB, Chapman ER (2009) Synaptotagmin-IV modulates synaptic function and long-term potentiation by regulating BDNF release. *Nature neuroscience* **12**:767-776.

Deinhardt K, Salinas S, Verastegui C, Watson R, Worth D, Hanrahan S, Bucci C, Schiavo G (2006) Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. *Neuron* **52**:293-305.

Dekkers MP, Barde YA (2013) Developmental biology. Programmed cell death in neuronal development. *Science* **340**:39-41.

Delcroix JD, Valletta JS, Wu C, Hunt SJ, Kowal AS, Mobley WC (2003) NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. *Neuron* **39**:69-84.

Dwivedi Y (2013) Involvement of brain-derived neurotrophic factor in late-life depression. *The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry* **21**:433-449.

Ernfors P, Lee KF, Jaenisch R (1994a) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**:147-150.

Ernfors P, Lee KF, Kucera J, Jaenisch R (1994b) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**:503-512.

Gross GG, Junge JA, Mora RJ, Kwon HB, Olson CA, Takahashi TT, Liman ER, Ellis-Davies GC, McGee AW, Sabatini BL, Roberts RW, Arnold DB (2013) Recombinant probes for visualizing endogenous synaptic proteins in living neurons. *Neuron* **78**:971-985.

Harrington AW, Ginty DD (2013) Long-distance retrograde neurotrophic factor signalling in neurons. *Nature reviews Neuroscience* **14**:177-187.

Harrington AW, St Hillaire C, Zweifel LS, Glebova NO, Philippidou P, Halegoua S, Ginty DD (2011) Recruitment of actin modifiers to TrkA endosomes governs retrograde NGF signaling and survival. *Cell* **146**:421-434.

Heerssen HM, Segal RA (2002) Location, location, location: a spatial view of neurotrophin signal transduction. T*rends in neurosciences* **25**:160-165.

Heinrich G, Lum T (2000) Fish neurotrophins and Trk receptors. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience* **18**:1-27.

Hiester BG, Galati DF, Salinas PC, Jones KR (2013) Neurotrophin and Wnt signaling cooperatively regulate dendritic spine formation. *Molecular and cellular neurosciences* **56C**:115-127.

Howe CL, Valletta JS, Rusnak AS, Mobley WC (2001) NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. *Neuron* **32**:801-814.

Ichim G, Tauszig-Delamasure S, Mehlen P (2012) Neurotrophins and cell death. *Experimental cell research* **318**:1221-1228.

Jeanneteau F, Deinhardt K, Miyoshi G, Bennett AM, Chao MV (2010) The MAP kinase phosphatase MKP-1 regulates BDNF-induced axon branching. *Nature neuroscience* **13**:1373-1379.

Jiang C, Salton SR (2013) The Role of Neurotrophins in Major Depressive Disorder. *Translational neuroscience* **4**:46-58.

Keimpema E, Tortoriello G, Alpar A, Capsoni S, Arisi I, Calvigioni D, Hu SS, Cattaneo A, Doherty P, Mackie K, Harkany T (2013) Nerve growth factor scales endocannabinoid signaling by regulating monoacylglycerol lipase turnover in developing cholinergic neurons. *Proc Natl Acad Sci U S A* **110**:1935-1940.

Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M (1993) Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell* **75**:113-122.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M (1994) Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. *Nature* **368**:249-251.

Kotlyanskaya L, McLinden KA, Giniger E (2013) Of proneurotrophins and their antineurotrophic effects. *Science signaling* **6**:pe6.

Kuczewski N, Porcher C, Gaiarsa JL (2010) Activity-dependent dendritic secretion of brain-derived neurotrophic factor modulates synaptic plasticity. *The European journal of neuroscience* **32**:1239-1244.

LaFerla FM, Green KN (2012) Animal models of Alzheimer disease. Cold Spring Harbor perspectives in medicine 2.

Lai KO, Wong AS, Cheung MC, Xu P, Liang Z, Lok KC, Xie H, Palko ME, Yung WH, Tessarollo L, Cheung ZH, Ip NY (2012) TrkB phosphorylation by Cdk5 is required for activity-dependent structural plasticity and spatial memory. *Nature neuroscience* **15:**1506-1515.

Lee FS, Chao MV (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci U S A* **98**:3555-3560.

Levskaya A, Weiner OD, Lim WA, Voigt CA (2009) Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature* **461**:997-1001.

Li W, Pozzo-Miller L (2013) BDNF deregulation in Rett syndrome. *Neuropharmacology*.

Liot G, Zala D, Pla P, Mottet G, Piel M, Saudou F (2013) Mutant Huntingtin alters retrograde transport of TrkB receptors in striatal dendrites. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**:6298-6309.

Lu B, Pang PT, Woo NH (2005) The yin and yang of neurotrophin action. *Nature reviews Neuroscience* **6**:603-614.

Marler KJ, Becker-Barroso E, Martinez A, Llovera M, Wentzel C, Poopalasundaram S, Hindges R, Soriano E, Comella J, Drescher U (2008) A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**:12700-12712.

Matrone C, Barbagallo AP, La Rosa LR, Florenzano F, Ciotti MT, Mercanti D, Chao MV, Calissano P, D'Adamio L (2011) APP is phosphorylated by TrkA and regulates NGF/TrkA signaling. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**:11756-11761.

McIlroy G, Foldi I, Aurikko J, Wentzell JS, Lim MA, Fenton JC, Gay NJ, Hidalgo A (2013) Toll-6 and Toll-7 function as neurotrophin receptors in the Drosophila melanogaster CNS. *Nature neuroscience*.

Mizuno-Yamasaki E, Rivera-Molina F, Novick P (2012) GTPase networks in membrane traffic. *Annual review of biochemistry* **81**:637-659.

Nikoletopoulou V, Lickert H, Frade JM, Rencurel C, Giallonardo P, Zhang L, Bibel M, Barde YA (2010) Neurotrophin receptors TrkA and TrkC cause neuronal death whereas TrkB does not. *Nature* **467**:59-63.

O'Keeffe GW, Gutierrez H, Pandolfi PP, Riccardi C, Davies AM (2008) NGF-promoted axon growth and target innervation requires GITRL-GITR signaling. *Nature neuroscience* **11**:135-142.

Poo MM (2001) Neurotrophins as synaptic modulators. *Nature reviews Neuroscience* **2**:24-32.

Puehringer D, Orel N, Luningschror P, Subramanian N, Herrmann T, Chao MV, Sendtner M (2013) EGF transactivation of Trk receptors regulates the migration of newborn cortical neurons. *Nature neuroscience* **16**:407-415.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. *Cell* **122**:735-749.

Roesler R, de Farias CB, Abujamra AL, Brunetto AL, Schwartsmann G (2011) BDNF/TrkB signaling as an anti-tumor target. *Expert review of anticancer therapy* **11:**1473-1475.

Rutherford LC, Nelson SB, Turrigiano GG (1998) BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. *Neuron* **21**:521-530.

Salehi A, Delcroix JD, Belichenko PV, Zhan K, Wu C, Valletta JS, Takimoto-Kimura R, Kleschevnikov AM, Sambamurti K, Chung PP, Xia W, Villar A, Campbell WA, Kulnane LS, Nixon RA, Lamb BT, Epstein CJ, Stokin GB, Goldstein LS, Mobley WC (2006) Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. *Neuron* **51**:29-42.

Saxena S, Bucci C, Weis J, Kruttgen A (2005a) The small GTPase Rab7 controls the endosomal trafficking and neuritogenic signaling of the nerve growth factor receptor TrkA. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**:10930-10940.

Saxena S, Howe CL, Cosgaya JM, Steiner P, Hirling H, Chan JR, Weis J, Kruttgen A (2005b) Differential endocytic sorting of p75NTR and TrkA in response to NGF: a role for late endosomes in TrkA trafficking. *Molecular and cellular neurosciences* **28**:571-587.

Tauszig-Delamasure S, Yu LY, Cabrera JR, Bouzas-Rodriguez J, Mermet-Bouvier C, Guix C, Bordeaux MC, Arumae U, Mehlen P (2007) The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. *Proc Natl Acad Sci U S A* **104**:13361-13366.

Tessarollo L, Tsoulfas P, Donovan MJ, Palko ME, Blair-Flynn J, Hempstead BL, Parada LF (1997) Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis. *Proc Natl Acad Sci U S A* **94**:14776-14781.

Vaegter CB, Jansen P, Fjorback AW, Glerup S, Skeldal S, Kjolby M, Richner M, Erdmann B, Nyengaard JR, Tessarollo L, Lewin GR, Willnow TE, Chao MV, Nykjaer

A (2011) Sortilin associates with Trk receptors to enhance anterograde transport and neurotrophin signaling. *Nature neuroscience* **14**:54-61.

Valente P, Casagrande S, Nieus T, Verstegen AM, Valtorta F, Benfenati F, Baldelli P (2012) Site-specific synapsin I phosphorylation participates in the expression of post-tetanic potentiation and its enhancement by BDNF. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**:5868-5879.

Wu YI, Frey D, Lungu OI, Jaehrig A, Schlichting I, Kuhlman B, Hahn KM (2009) A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* **461**:104-108.

Yano H, Ninan I, Zhang H, Milner TA, Arancio O, Chao MV (2006) BDNF-mediated neurotransmission relies upon a myosin VI motor complex. *Nature neuroscience* **9**:1009-1018.

Zhou B, Cai Q, Xie Y, Sheng ZH (2012) Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. *Cell reports* **2**:42-51.

Zuccato C, Tartari M, Crotti A, Goffredo D, Valenza M, Conti L, Cataudella T, Leavitt BR, Hayden MR, Timmusk T, Rigamonti D, Cattaneo E (2003) Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nature genetics* **35**:76-83.

THE NEXT 50 YEARS OF NGF

M.V. CHAO

Departments of Cell Biology, Physiology & Neuroscience and Psychiatry New York, USA

The identification of the first growth factor, NGF, over 50 years ago changed the field of biology in many unexpected ways. Growth factors now have a prominent role in cancer, stem cells, cell differentiation and maladaptive consequences, such as psychiatric and neurodegenerative disorders. What will transpire in the next 50 years? Here we explore unanswered questions related to neurotrophic factors in the future. If solved, the answers would represent breakthroughs with lasting and widespread impact upon many biological problems.

This year we witnessed the passing of Rita Levi-Montalcini, whose characterization of NGF with Stanley Cohen led to its purification and the discovery of many growth factors (Cohen, 1960; Levi-Montalcini and Cohen, 1960). The discovery of NGF was the result of an effort to answer a question that stymied many investigators--what inductive or diffusible substances made at target tissues are responsible for controlling nerve growth? The discovery of NGF resulted from solving the problem of how the periphery affected neuronal survival and nerve fiber growth. The occasion of the 100th birthday celebration for Levi-Montalcini in 2009 bought both her and Cohen together in Rome (Figure 1).

Looking back at these events, we marvel at the confluence of events that gave rise to NGF, and also EGF. The landmark experiments were a result of a combination of expertise in different disciplines—embryology, neuroanatomy and biochemistry that came together at Washington University with Viktor Hamburger, Rita Levi-Montalcini and Stanley Cohen. Levi-Montalcini arrived from Turin in 1947, upon the invitation of Viktor Hamburger, to pursue target-derived signals. Hamburger invited Cohen in 1953 to work with Levi-Montalcini on the isolation of NGF. Cohen was anchored in the Microbiology Department, where a large number of luminary biochemists, such as Arthur Kornberg and Paul Berg, were available for advice. There are many excellent reviews of this historic period (Levi-Montalcini, 1987; Cowan, 2001; Cohen, 2008).

From the serendipitous observation that snake venom contained large quantities of NGF, it became possible to purify the protein from mouse submaxillary glands. EGF was discovered unexpectedly from an assay of crude fractions of NGF that resulted in precocious opening of eyelids and keratinization of the skin (Cohen, 1962). These discoveries resulted from keen powers of observation combined with deductive logical reasoning. Now antibodies against the EGF receptor, Tarceva and Erbitux, are now used widely in a variety of cancers.

Growth factors figure in all areas of biology, including immunology, development, stem cells, tumor biology and neuropsychiatric disorders. It is noteworthy that BDNF is implicated in anxiety and depression and neuregulin, a member of the EGF family, is associated with schizophrenia (Chao et al, 2008). The rich history and far-reaching effects of growth factors makes us pause and consider. What are the major questions that will be resolved in the future? Which problems, if solved, would provide a

Corresponding Author: Moses V. Chao
Departments of Cell Biology, Physiology & Neuroscience and Psychiatry
Skirball Institute of Biomolecular Medicine
New York University Langone Medical Center
540 First Avenue
New York, New York 10016 USA

2279-5855 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

genuine impact in the field of neuroscience? Of many questions, we will consider several existing biological problems. Though the focus is on the NGF family of trophic factors, answering these questions will ultimately help to understand how complex neural circuits are formed and how to treat neurodegenerative diseases, two problems of intense current interest.

HOW ARE LONG DISTANCE SIGNALS TRANSMITTED?

Since the time of Rene Descartes and centuries before, it has been a mystery how signals are sent from the periphery to the spinal cord and brain. In the past 50 years, it has been established that trophic factors generate signals that are conveyed over long distances from the nerve terminal to the cell body. How is this accomplished? Vesicles containing the ligand and receptor are delivered to the cell body, where signals are transduced to ensure neuronal survival and differentiation.

It is widely accepted that NGF and its receptors, TrkA and p75, become internalized and are retrogradely transported to the cell body (Ye et al, 2003) in "signaling endosomes" (Grimes et al, 1997). There is also a counter proposal that internalization of NGF is not required for a retrograde response (MacInnis and Campenot, 2002). In this case, survival signals occur without uptake and transport of NGF in distal axons. Indeed, Trk receptors can be activated in the absence of neurotrophin binding in a process called transactivation (Lee and Chao, 2001; Jeanneteau et al, 2008). Unraveling the exact mechanisms that account for retrograde transport of neurotrophins and their receptors will reveal the cellular requirements for propagating trophic signals over long distances.

Why is this issue important? Transport of trophic receptors is not limited to the NGF family. The GDNF family, retrograde transport in sensory neurons with its binding receptor, GDNF family α-receptors (Leitner et al, 1999). Many other proteins, such as TGF-β, FGF, IGF, LIF and CNTF are similarly taken up and transported in axons. Hence, the mechanisms that account for transport and recognition of different ligands and their receptors will determine a whole host of activities, including cell survival, axon pruning, protein sorting and synapse formation. Since transport vesicles would orient the intracellular domain of the receptors to face the cytoplasm, each receptor would be available for interactions with cytoplasmic proteins and can produce many signaling outcomes.

How neurotrophic signals are transmitted will depend upon how the receptors are transported and localized. The intracellular location of ligand-receptor complexes requires an understanding of the transport machinery and how motors—dynein and actin-based motors, or kinesin-based motors—are regulated. Scaffold proteins associated with neurotrophin signaling provide a link with actin-based and dynein-based motors. In addition, studies of how herpes simplex virus (HSV-1) and rabies virus undergo axonal transport and become activated represent important directions for future research. Infection and latency of HSV-1 in sensory neurons, a common event in the human population, is controlled by NGF signaling (Wilcox et al, 1980). Latency results in suppression of the virus in neurons that is maintained for years by continuous signaling through phosphatidylinositol 3-kinase and TrkA tyrosine kinase activities (Camarena et al, 2010). These findings indicate that growth factors exert an impact in keeping viral latency in check. For instance, it is known that anti-NGF treatment in humans and in rabbits may lead to viral reactivation associated with keratitis and potentially blindness (Mauro et al, 2007; Lambiase et al, 2008). Ablation of NGF signaling therefore can serve as a reactivation viral stimulus.

TREATMENTS FOR NEURODEGENERATIVE DISEASES

Can neurodegenerative diseases, such as Alzheimer's and Huntington's disease be treated with neurotrophic factors? A central hypothesis is the lack of specific neurotrophic factors contributes to age-related neurodegeneration (Appel, 1981). While there were disappointing clinical trials in the 1990s to administer BDNF and NGF for ALS and different neuropathies (Thoenen and Sendtner, 2002), there is still considerable promise in using neurotrophins for neurodegenerative diseases. Plenty of evidence indicates that ablation or lowering of trophic factors as a function of age increases the neurodegenerative process. Reversal of this process represents a viable

therapeutic approach if the mechanisms that lead to age-related neurodegeneration are defined in the future. Several examples strongly support this hypothesis.

Decreased levels of NGF and BDNF have been implicated in Alzheimer's disease (AD) by many studies. In the hippocampus, a deficit in BDNF and TrkB receptors have been identified in CA1 pyramidal cells, analyzed from postmortem tissue of patients with AD (Ginsberg et al, 2010). Reduced levels of BDNF have also been reported in mouse models of AD. Deficits in BDNF-dependent functions have been reported, such as reduced synaptic plasticity (Marchetti and Marie, 2012). The idea that reduced BDNF or TrkB caused the impairments was supported by a study which showed that increasing BDNF levels restored cognitive function (Nagahara et al, 2009).

There are many compelling lines of evidence that BDNF can explain Huntington's disease (HD). BDNF is a major target of mutant huntingtin protein in the CNS. Decreased BDNF levels in the striatum have been detected in human HD subjects and mouse models of HD (Zuccato and Cattaneo, 2007). Significantly, a conditional mouse model in which BDNF has been specifically reduced in the cortex results in dendritic loss resulted in an animal with features closely resembling features of HD. They include dendritic loss and age-dependent depletion of striatal medium spinal neurons (Baquet et al, 2004). Moreover, gene expression profiling indicates that depletion of BDNF in the cortex more closely resembles presymptomatic human HD than other established transgenic models (Strand et al, 2007). These results suggest that striatal-specific atrophy in HD may be a consequence of a decrease of cortical BDNF by mutant huntingtin.

How does a loss of cortical BDNF affect the striatum? Over 90% of striatal BDNF is derived from the cortex through anterograde transport (Zuccato and Cattaneo, 2007). Surprisingly, the striatum does not produce BDNF. Anterograde transport of BDNF therefore plays a key element to explain the age-dependent loss of trophic support from the cortex to the striatum. Moreover, it has been shown that huntingtin acts as a scaffold protein to facilitate transport of BDNF and that accumulation of mutant huntingtin protein results in a decrease in BDNF vesicle transport (Gauthier et al, 2004).

Huntington's disease is caused by a polyglutamine expansion in the huntingtin protein, which results in abnormal motor movements, personality changes, cognitive decline and early death. The selective loss of BDNF (and other transported trophic factors) in the cortex can explain the psychiatric disorders associated with HD and also the late onset of the loss of striatal neurons that leads to deficits in motor function. Indeed, the cortical-striatal circuitry is probably disrupted by the loss of BNDF through mechanisms involving synaptic dysfunction. Indeed, survival of striatal neurons and motor deficits in HD models can be rescued with BDNF (Gharami et al, 2008).

Increasing the levels of BDNF through physical exercise, antidepressants or other novel activities (Cotman et al, 2007) will be beneficial in HD and other neurodegenerative diseases, without having to resort to the administration of BNDF, an approach associated with undesirable side effects and uncertain pharmacokinetics. Hence, certain disorders, such as Huntington's and Alzheimer's disease, could be delayed or diminished if increasing the levels of neurotrophins, such as BDNF, can be accomplished.

THE IMPORTANCE OF CLEAVAGE

The cleavage of proteins by proteolysis is a universal mechanism that is associated not only with protein turnover, but also with the generation of intracellular signals. Neurotrophins are generated from pro-neurotrophins, which are cleaved intracellularly by furin or pro-convertases to release the mature protein that forms stable, non-covalent dimers. The pro-region has been believed to be important in proper folding and intracellular sorting of neurotrophins. Pro-NGF, the precursor of NGF, binds to p75 with a greater affinity than mature NGF and can lead to cell death (Figure 2). In patients with AD, pro-NGF levels are elevated in the cortex (Fahnestock et al, 2001). The precursor form of neurotrophins are more selective ligands for the p75 neurotrophin receptor than mature forms and are more effective in inducing p75-dependent apoptosis (Lee et al, 2001; Beattie et al, 2002). These observations are important, since they indicate the biological actions of the neurotrophins are regulated by proteolytic cleavage,

with pro-forms preferentially activating p75 and mature forms selectively activating Trk receptors (Figure 2).

The functional outcomes of γ-secretase cleavage of p75 are important, since the same events occur with amyloid precursor protein (APP). Also, p75 fragments may play a role in the association with ephrinA (Lim et al. 2008) and Nogo receptor (Domeniconi et al. 2005). Metalloproteases are associated with the Eph A receptor (Janes et al, 2005), suggesting that the actions of p75 may depend upon specific enzymes that are associated with other co-receptors. As the p75 receptor is frequently unregulated after injury or inflammation, its cleavage represents an early event that precedes APP cleavage. Thus, cleavage of p75 may have a predictive value in neurodegeneration. Controlling the cleavage of neurotrophins and the p75 receptor has many consequences in cell survival, growth of axons and dendrites and growth cones.

REGRESSIVE EVENTS

One problem in developmental neurobiology is why one axon branch survives and other axon branches become less competitive. A prediction of the neurotrophic hypothesis is that competition between axon branches leads to the strengthening of one branch and withdrawal or pruning of the others. How this applies to elimination of axons is not well understood. Synapse elimination results in a reduction of axons that innervates each postsynaptic cell maybe due to neurotransmitter release, activity-dependent effects, neuronal firing patterns or epigenetic mechanisms (Colman et al, 1997). Several groups have proposed that elimination of cells and axon retraction may be carried out by a p75-dependent mechanism and that neurotrophins such as BDNF may promote local axon degeneration (Cao et al. 2007; Deppmann et al, 2008; Singh et al, 2008; Yang et al, 2009) and growth cone collapse (Deinhardt et al, 2012).

Trk receptors transduce neurotrophin signaling events using the same downstream components as other growth factors, such as EGF, FGF and PDGF. The generation of specific signals through a similar set of tyrosine kinase substrates is not fully understood (Chao 1992). Explanations include the time course of signaling, the local concentration of ligand, the location and the affinity of specific proteins with Trk and p75 receptors. Adaptor proteins are specialized for protein trafficking and degradation; others for axonal transport; and still others may be unique for axon guidance. An example is the Nedd4-2, an E-3 ligase, which ubiquinates TrkA receptors through a PPXY sequence at the C-terminus of TrkA (Arevalo et al, 2006). Nedd4-2 has a strong E3 ligase activity that stimulates lysosomal degradation of the TrkA receptor. The TrkB receptor contains an APXY sequence at the same location, but is incapable of binding Nedd4-2 and undergoing ubiquitination. Indeed, the phosphorylation of the PPXY tyrosine residue can also result in the recruitment of phospholipase C-γ. This implies that a competition exists for binding to the PPXY sequence by two proteins that give completely different outcomes.

Another mechanism relevant to pruning is the consequence of withdrawal of growth factors. Deprivation of trophic support not only impacts on the ability of neurons to survive, but also sets into motion a series of molecular events. Removal of NGF from neurons leads to increases in Bim and caspase activity, resulting in the cleavage of APP (Matrone et al, 2008; Nikolaev et al, 2009), which are preceded by retrograde axonal signaling (Mok et al. 2009). These observations indicate that the events that immediately follow withdrawal of trophic factors are just as important as the signaling cascades that are triggered with neurotrophin addition. Growing evidence supports a direct link of neurotrophin levels and Trk tyrosine kinase receptor activity and the processing of APP (Matrone et al, 2011). Defining the events that occur after removal of trophic factors will reveal mechanisms of axon retraction and elimination, two early events that have been associated with many neurodegenerative diseases.

NGF AND PAIN

A frequent outcome of clinical trials using neurotrophins for neuropathy and neurodegeneration is pronounced pain (Johagen et al, 1998; Thoenen and Sendtner, 2002). Though an undesirable effect, this result likely reflects a normal function of neurotrophins in the adult nervous system in the development of hyperalgesia after inflammatory in-

jury in the adult nervous system. NGF and BDNF are frequently elevated after inflammation or injury. This response results in greater nociceptor sensitization through rapid modulation of heat and vanilloid receptors. Notably, there are sequence variants in NGF and the TrkA receptor that are associated with congenital insensitivity to pain (Einarsdottir et al, 2004; Indo, 2009). It has become clear that a normal function of NGF-TrkA signaling in the adult nervous system is in the control of pain (Mantyh et al, 2011), which was suggested by the original observations of abundant amounts of NGF in snake venom (Cohen, 1959).

Therefore, blocking NGF with antagonists may provide pain relief. This has now been borne out by a number of studies using anti-NGF antibodies and Fc-receptor fusion proteins in cases of chronic knee pain caused by osteroarthritis (Lane et al, 2010); bone cancer pain (Sevcik et al, 2005); autoimmune arthritis (Shelton et al, 2005); bone fracture (Sabasovich et al, 2008); and neuropathic pain (Ugolini et al, 2007). All these studies support the use of anti-NGF in decreasing pain.

One mechanism for NGF-induced hyperalgesia is through an interaction of the TrkA receptor with the TRPV1 capsaicin receptor, a non-selective cation channel that is activated by heat, noxious vanilloid compounds such as capsaicin, or extracellular protons (Caterina et al, 1997). The ability to antagonize NGF action during pain though new therapeutics to diminish the expression of NGF or signaling of its receptor has the potential of ameliorating neuropathic and inflammatory pain. It is necessary to identify the cells that release NGF and BDNF under these conditions.

The mechanism of action of NGF and BDNF can be further addressed by following a growing number of ion channels that are associated with the Trk tyrosine kinases. These interactions can produce an array of different effects lasting from milliseconds to days that generates changes upon synaptic transmission and plasticity. The Trk receptor has been implicated with many different ion channels, such as TRP channels exemplified by TRPC3 and the TRPV1 capsaisin receptor (Chuang et al, 2001), KCNQ channels, Na^+ and AMPA and NMDA receptors (Chao, 2003). The p75 receptor is associated with an inward rectifying potassium channel (Coulson et al, 2008). Several interactions are striking, such as an association of TrkB with the voltage sensitive $Na_V1.9$ sodium channel. Other associations such as the store operated cation TRPC3 channel give a delayed inward current, which is specific to BDNF and not other growth factors such as FGF and IGF-1 (Li et al, 1999). The specificity of these ion channel interactions is critical. For AMPA receptor trafficking, there are many mechanisms of exocytotic and endocytotic events that determine activity-dependent changes of synaptic efficacy. These could well be influenced by BDNF signaling. The relationship between trophic factors and ion channels will likely answer many questions concerning the ability of neurotrophins to be responsive to activity dependent changes in the nervous system and to change synaptic activity.

OBESITY

One of the most striking and reproducible phenotypes of mice heterozygous for BDNF and its TrkB receptor is increased food intake (hyperphagia) and obesity. Reduced levels of BDNF or TrkB signaling in mice and humans are associated not only with obesity, but also some cases of increased aggressive behavior and hyperactivity (Kernie et al, 2000; Rios et al, 2001). In studies of subjects that exhibited severe obesity, a number of sequence variants were discovered in the TrkB receptor (Yeo et al, 2004; Gray et al, 2007). While the phenotypes are pronounced, the mechanisms that account for weight gain are completely mysterious.

Nevertheless, monoclonal antibody agonists against the TrkB receptor have been shown to be effective in eliciting anti-obesity effects in mouse models, similar to the reduction in obesity from administration of NT-4, another ligand of TrkB (Tsao et al, 2008). These experiments suggest that TrkB signaling can exert an impact upon either appetite, accumulation of body fat or body weight, and suggest that levels of BDNF are directly involved in metabolic energy homeostasis and mammalian feeding behavior. One prediction is that obesity might be controlled by agents that increase BDNF levels or enhance signaling through TrkB receptors at specific hypothalamic sites. Increased bridging of research in neural circuits and endocrinology will provide greater insights into the mechanism by which BDNF

has such a strong influence upon obesity.

NEW DRUGS FOR PSYCHIATRY

Psychiatric disorders such as depression, bipolar disease and schizophrenia are debilitating mental illnesses that are influenced by many genetic and environmental factors. While little is known about the neural circuits that underlie mood disorders, growth factors have emerged as susceptibility genes. BDNF has been implicated in the pathophysiology of depression, anxiety, bipolar disease and other psychiatric disorders. BDNF represents a prominent secreted protein that facilitates learning and memory. In addition, neuregulin-1 has been tightly linked as candidate gene for schizophrenia. Lack of neuregulin-1 function may be involved in dysregulation of synaptogenesis and synaptic plasticity in the adult.

There is considerable genetic and physiological evidence that now support NGF and BDNF as central factors in behavior and memory (Connor et al, 2009; Nagahara et al, 2009). A key BNDF polymorphism, Val66Met, has now been firmly associated with many behavioral disorders in human populations (Egan et al, 2003). This amino acid change is not localized in mature BDNF itself, but rather in the precursor polypeptide, proBDNF. The Val66Met polymorphism is significant, since the Met-BDNF form is impaired in activity-dependent secretion at synapses (Chen et al, 2006), resulting in miss-sorting and lower activity-dependent secretion of BDNF. Individuals carrying the Met amino acid at the position 66 performed weaker in verbal episodic memory tests, and functional MRI tests of hippocampal function also showed an abnormal pattern of activation during cognitive tests (Egan et al, 2003). This polymorphism also affects patterns of memory extinction (Yu et al, 2009). These observations suggest that neurotrophins can play a role in hippocampal function and memory, due to the efficacy of activity-dependent secretion of BDNF and its subsequent effects upon LTP and synaptic plasticity in the human population.

Increases in neuronal activity or exercise result in elevated BDNF hippocampal levels that affect cognitive abilities, whereas exposure to stress dramatically decreases BDNF levels in the adult brain. Clinical studies have indicated that serum levels of BDNF are decreased in patients with major depressive disorders. Heterozygous BDNF knockout mice display enhanced aggressiveness and obesity that can be counteracted by administration of Prozac (fluoxetine) indicating a role for serotonin uptake (Lyons et al, 1999). This raises several questions. Can growth factor signaling be used to design new drugs for psychiatric illnesses? Why do antidepressants take so long to become efficacious?

Unlike neurological disorders that have few treatments, there are many drugs available for psychiatric disorders. However, there have been few novel drugs that have been discovered in the last few decades. The underlying mechanisms for many drugs are still elusive, but further studies of the action of growth factors will contribute to our understanding of the pharmacology and the neural circuits that underlie psychiatric illnesses. Many common downstream enzymatic activities, PTEN, AKT, PI-3K, MAPK, PLC-γ, mTOR, have been identified for neuregulins and neurotrophins. One treatment with rapid anti-depressant effects is through the NMDA receptor antagonist, ketamine, which is involved in mediating mTOR-mediated protein synthesis (Li et al, 2010), as well as BDNF action. Because the actions of these growth factors have effects upon LTP, LTD and other forms of synaptic plasticity, it is likely that small molecule agonists that promote these enzymatic activities may prove to be clinically relevant. It is also likely that other growth factors also influence these complex disorders.

DRUGS FOR LEARNING AND MEMORY

There is considerable evidence that NGF and BDNF signaling is involved in learning and memory. While BDNF signaling is established in hippocampus-dependent learning and plasticity, NGF also has effects in acquisition and retention of memory tasks in the hippocampus (Chen et al, 1997). Increases in activity-dependent events, antidepressants and exercise (Cotman et al, 2007) have raised the possibility that small molecules that could increase neurotrophin levels or Trk receptor signaling could be protect against memory impairment or increase learning. The identification of small molecule agonists (Pehar et al. 2006) such as ampakines (Lynch et al, 2008), and zinc (Huang et al, 2008) and the ability of glucocorticoids, adenosine and other G protein coupled

Fig. 1. *Recent photos of Rita Levi-Montalcini (born April 22, 1909 in Turin, Italy) and Stanley Cohen (born November 17, 1922 in Brooklyn, New York). They worked together from 1953-1959 with Viktor Hamburger at Washington University in St. Louis.*

ligands to transactivate Trk receptors (Jeanneteau et al, 2008) offer new opportunities to find targets for drug discovery.

As opposed to G protein-coupled receptors, the search for agonists and antagonists for Trk and p75 receptors has lagged behind. While there has been progress in the X-ray crystal structures of the extracellular domains of p75 and Trk receptors, many questions remain regarding the stoichiometry and mechanism of how neurotrophins interact with their receptors. Therefore, structures of NGF bound to p75 and Trk offer the opportunity to begin to pursue therapeutic drug design, a goal that has eluded the trophic factor field.

Several issues have arisen from structural studies of the neurotrophins and their receptors, Trk and p75. These receptors can form complexes that contribute to high affinity binding (Hempstead et al, 1991), however, the exact configuration of this association is not clear from the structures of TrkA and p75 with NGF (Wiesmann et al, 1999; Wehrman et al, 2007). Both symmetrical and asymmetrical complex have been proposed for p75 binding to NGF dimers (He and Garcia, 2004; Aurikko et al, 2005). The p75 receptor is unusual in that it also interacts with leucine-repeat rich proteins, such as Nogo receptor and LINGO, which are involved in regeneration (Mi et al, 2004) and sortilin, a trafficking receptor that brings the precursor form of NGF, proNGF (Nykjaer et al, 2004) together with p75 (Figure 2).

Because allosteric bending has been seen with NGF binding to p75 (He and Garcia, 2004), conformational changes in the ligand and receptor may account for the structural discrepancies that have been reported. They may also account for older observations that the on- and off- binding rates to p75 differ with each neurotrophin (Bothwell, 1995). Additionally, since proNGF has a higher affinity for p75 than mature NGF, changes of conformation or stoichiometry may accompany the binding of proNGF to p75. Previous studies of Trk receptors have suggested that conformation changes in the TrkA receptor can ac-

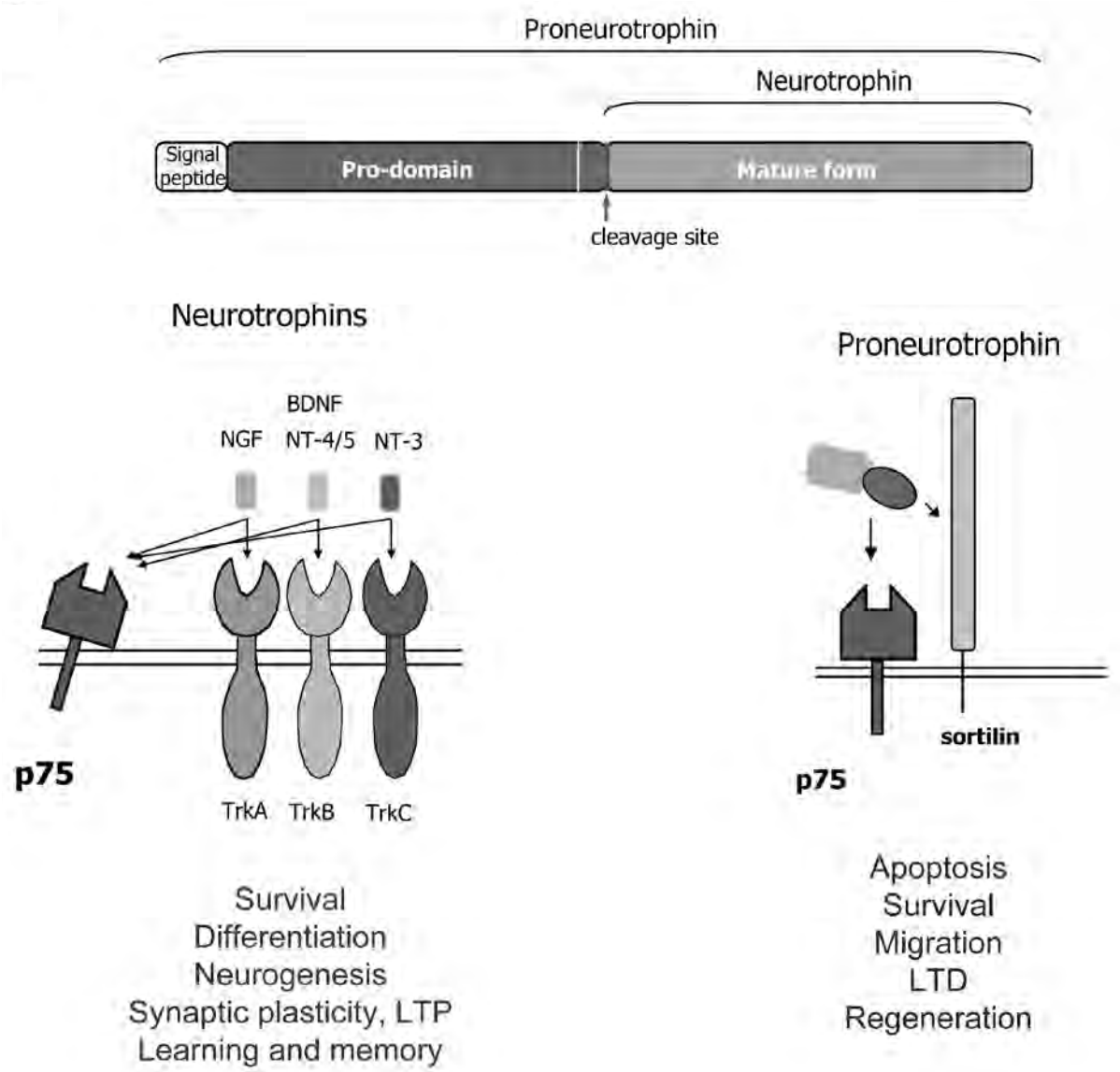


Fig. 2. *Neurotrophins and their receptors. All neurotrophins are initially produced as 30-35 kDa precursor proteins containing a signal peptide, sites for glycosylation and pairs of basic amino acids that are recognized by processing enzymes, such as furin and metalloproteinases. These enzymes release a biologically active 12-14 kDa mature form of neurotrophin. Pro-neurotrophins bind to the p75 receptor with a higher affinity than mature NGF. The preferential binding of mature neurotrophins to Trk can lead to differential outcomes. Trk receptors mediate differentiation and survival signaling through MAP kinase, PI-3 kinase and PLC-γ pathways. Trk family members recruit and increase the tyrosine phosphorylation of phospholipase C-γ, and the ARMS/Kidins220 and SHC adaptor proteins. The p75 receptor predominantly signals to activate NF-κB and JNK.*

count for changes in its NGF binding properties Trk receptor dimerization and activation has been observed in the absence of ligand. A single point mutation in the extracellular domain of TrkA (P203A) distant from the NGF binding site, resulted in increased binding (Arevalo et al, 2001), suggesting that a conformation change was created by the P203 extracellular TrkA mutation. These observations emphasize the need for more structural studies of neurotrophins with their receptors and co-receptors.

There are only a handful of conserved receptor systems—G protein coupled receptors, Sonic Hedgehog, WNT, steroid receptors and receptor tyrosine kinases. Therefore, future computational analysis should be able to predict signaling cascades determined by different protein-protein interactions, phosphorylation sites and other post-translational events, such as ubiquitination. This information, along with spatial intracellular localization of signaling complexes, will explain how specificity is achieved by different classes of signaling molecules.

PERSPECTIVE

The neurotrophic hypothesis explained how neurons are eliminated during development as a result of cell death from deprivation of trophic factors. The elaboration of this concept by Rita Levi-Montalcini and Viktor Hamburger was an important advance that addressed an urgent question posed in the 1930s. The solution came from testing a competition mechanism involving limiting amounts of trophic factors. The hypothesis does not exclude other competitive roles for neurotrophic factors, most notably, the relevance to higher order functions, such as the circuits involved in pain, fear, depression, anxiety and neurological disorders such as Huntington's and Alzheimer's diseases. Neurotrophins do not exist in *Drosophila melanogaster* or *Caenorhabditis elegans*, even though other polypeptide growth factors, such as EGF, FGF, TGF-β and insulin, are highly conserved in these species and play important developmental roles. The evolution of neurotrophins as a family implies their actual functions are more directly involved in mediating specialized activities, such as pain, obesity, learning, retention of memory and behavior. Future insights to these areas of neuroscience research may well come from using trophic factors as probes for the nervous system, analogous to the Indian parable of "blind men and an elephant". That is, to resolve complicated questions and intractable problems, it pays to take different perspectives and integrate across different fields of scientific inquiry.

REFERENCES

Appel SH (1981) A unifying hypothesis for the cause of amyotrophic lateral sclerosis, parkinsonism and Alzheimer's disease. *Ann Neurol* **10**: 499-505.

Arevalo J, Conde B, Hempstead BL, Chao MV, Martin-Zanca D (2001) A novel mutation within the extracellular domain of TrkA causes constitutive receptor activation. *Oncogene* **20**: 1229-1234.

Arevalo JC, Waite J, Rajagopal R, Beyna M, Chen ZY, Lee FS, Chao MV (2006) Cell survival through Trk neurotrophin receptors is differentially regulated by ubiquitination. *Neuron* **50**: 549-559.

Aurikko J, Ruotolo B, Grossman JG, Moncrieffe MC, Stephens E, Leppanen VM, Robinson CV, Saarma M, Bradshaw RA, Blundell TL (2005) Characterization of symmetric complexes of nerve growth factor and the ectodomain of the pan-neurotrophin receptor, p75NTR. *J Biol Chem* **280**: 33453-33460.

Baquet ZC, Gorski JA, Jones KR (2004) Early striatal dendrite deficits followed by neuron loss with advanced age in the absence of anterograde cortical brain-derived neurotrophic factor. *J Neurosci* **24**: 4250-4258.

Beattie M, Harrington AW, Lee R, Kim JY, Boyce SL, Longo, FM, Bresnahan JC, Hempstead BL, Yoon, SO (2002) ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. *Neuron* **36**: 375-386.

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. *Ann. Rev. Neurosci.* **18**: 223-253.

Cao L, Dhilla A, Mukai J, Blazeski R, Lodovichi C, Mason CA, Gogos JA (2007) Genetic modulation of BDNF signaling affects the outcome of axonal competition in vivo. *Current Biology* **17**: 911-921.

Camarena V, Kobayashi M, Kim JY, Roehm P, Perez R, Garner J, Wilson AC, Mohr I, Chao MV (2010) Nature and duration of growth factor signaling through receptor tyrosine kinases regulates HSV-1 latency in neurons. *Cell Host Microbe* **8**: 320-330.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**: 816-824.

Chao MV (1992) Growth factor signaling: where is the specificity? *Cell* **68**: 995-997.

Chao MV (2003) Neurotrophins and their receptors: A convergence point for many signaling pathways. *Nature Rev Neuroscience* **4**: 299-309.

Chao MV (2008) Growth factors and psychiatric disorders. Novartis Foundation, Chichester UK, John Wiley & Sons.

Chen K, Nishimura M, Armanini MP, Crowley C, Spencer SD, Phillips HS (1997) Disruption of a single al-

lele of the nerve growth factor gene results in atrophy of basal forebrain cholinergic neurons and memory deficits. *J Neuroscience* **17**: 7288-7296.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* **314**: 140-143.

Chuang H, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PIP2-mediated inhibition. *Nature* **411**: 957-962.

Cohen S (1959) Purification and metabolic effects of a nerve growth-promoting protein from snake venom. *J Biol Chem* **234**: 1129-1137.

Cohen S (1960) Purification of a nerve growth-promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. *Proc Natl Acad Sci* **46**: 302-311.

Cohen S (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. *J Biol Chem* **237**: 1555-1562.

Cohen S (2008) Origins of growth factors: NGF and EGF. *J Biol Chem* **283**: 33793-33797.

Colman H, Nabekura J, Lichtman JW (1997) Alterations in synaptic strength preceding axon withdrawal. *Science* **275**: 324-325.

Conner JM, Franks KM, Titterness AK, Russell, K, Merrill DA, Christie BR, Sejnowski TJ, Tuszynski MH (2009) NGF is essential for hippocampal plasticity and learning. *J Neuroscience* **29**: 10883-10889.

Cotman C, Berchtold N, Christie LA (2007) Exercise builds brain health: key roles of growth factor cascades and inflammation. *Trends Neurosci* **30**: 464-472.

Coulson EJ, May LM, Osborne SL, Reid K, Underwood CK, Meunier FA, Bartlett PF, Sah P (2008) p75 neurotrophin receptor mediates neuronal cell death by activating GIRK channels through phosphatidylinositol 4,5-bisphosphate. *J Neuroscience* **28**: 315-324.

Cowan W (2001) Viktor Hamburger and Rita Levi-Montalicini: the path to the discovery of nerve growth factor. *Ann Rev Neuroscience* **24**: 551-600.

Deinhardt K, Kim T, Spellman DS, Mains RE, Eipper BA, Neubert TA, Chao MV, Hempstead BL (2011) Neuronal growth cone retraction relies on proneurotrophin receptor signaling through Rac. *Science Signaling* **4**: ra82.

Deppmann CD, Mihalas S, Sharma N, Lonze BE, Niebur E, Ginty DD (2008) A model for neuronal competition during development. *Science* **320**: 369-373.

Domeniconi M, Zampieri N, Spencer T, Hilaire M, Mellado W, Chao MV, Filbin MT (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron* **46**: 849-55.

Egan M, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* **112**: 257-269.

Einarsdottir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, Holmgren G, Holmberg D, Holmberg M (2004) A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Human Mol Genet* **13**: 799-805.

Fahnestock M, Michalski B, Xu B, Coughlin MD (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factgor in brain and is increased in Alzheimer's brain. *Mol Cell Neurosci* **18**: 210-220.

Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, De Mey J, MacDonald ME, Lessmann V, Humbert S, Saudou F (2004) Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell* **118**: 127-38.

Gharami K, Xie Y, An JJ, Tonegawa S, Xu B (2008) Brain-derived neurotrophic factor over-expression in the forebrain ameliorates Huntington's disease phenotypes in mice. *J Neurochem* **105**: 369-379.

Ginsberg SD, Allred, MJ, Counts SE, Cataldo AM, Neve RL, Jiang Y, Wuu J, Chao MV, Mufson EJ, Nixon RA Che S (2010) Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer's disease progression. *Biol Psychiatry* **68**: 885-893.

Gray J, Yeo G, Hung C, Keogh J, Clayton P, Banerjee K, McAulay A, O'Rahilly S, Farooqi IS (2007) Functional characterization of human NTRK2 mutations identified in patients with severe early-onset obesity. *Int J Obesity* **31**: 359-364.

Grimes M, Beattie E, Mobley WC (1997) A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. *Proc Natl Acad Sci USA* **94**: 9909-9914.

He XL, Garcia K (2004) Structure of nerve growth factor complexed with the shared neurotrophin recpetor

p75. *Science* **304**: 870-875.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires co-expression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* **350**: 678-683.

Huang YZ, Pan E, Xiong ZQ, McNamara JO (2008) Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse. *Neuron* **57**: 546-558.

Indo Y (2009) Nerve growth factor, interoception, and sympathetic neuron: lesson from congenital insensitivity to pain with anhidrosis. *Auton Neurosci* **147**: 3-8.

Janes PW, Saha N, Barton WA, Kolev MV, Wimmer-Kleikamp SH, Nievergall E, Blobel CP, Himanen JP, Lackmann M, Nikolov DB (2005) Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. *Cell* **123**: 291-304.

Jeanneteau F, Garabedian MJ, Chao MV (2008) Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *Proc Natl Acad Sci USA* **105**(12): 4862-7.

Johagen M, Nordberg A et al. (1998) Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimers disease. *Dement Geriat Cogn* **9**: 246-257.

Kernie S, Liebl D, Parada LF (2000) BDNF regulates eating behavior and locomotor activity in mice. *EMBO J* **19**: 1290-1300.

Lambiase A, Coassin M, Costa N, Lauretti P, Micera A, Ghinelli E, Aloe L, Rama P, Bonini S (2008). Topical treatment with nerve growth factor in an animal model of herpetic keratitis. *Graefes Arch. Clin Exp Ophthalmol* **246**: 121–127.

Lane NE, Schnitzer TJ, Birbara CA, Mokhtarani M, Shelton DL, Smith MD, Brown MT (2010) Tanezumab for the treatment of pain from osteoarthritis of the knee. *New Engl J Med* **363**: 1521-1531.

Lee FS, Chao MV (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci USA* **92**: 3555-3560.

Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. *Science* **294**: 1945-8.

Leitner ML, Molliver DC, Osborne PA, Vejsada R, Golden JP, Lampe PA, Kato AC, Milbrandt J, Johnson EM (1999) Analysis of the retrograde transport of glial cell line-derived neurotrophic factor (GDNF), neuturin, and persephin suggests that in vivo signaling for the GDNF family is GFRα coreceptor-specific. *J Neuroscience* **19**: 9322-9331.

Levi-Montalcini R (1987) The nerve growth factor: thirty-five years later. *Science* **237**: 1154-1164.

Levi-Montalcini R, Cohen S (1960) Effects of an extract of the mouse submaxillary salivary glands on the sympathetic system of mammals. *Ann N.Y. Acad. Sci* **84**: 324-341.

Li HS, Xu XZ, Montell C (1999) Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. *Neuron* **24**: 261-273.

Li N, Lee B, Liu R-J, Banasr M, Dwyer JM, Iwata M, Li X-Y, Aghajanian G, Duman RS (2010) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science 329: 959-964.

Lim YS, McLaughlin T, Sung TC, Santiago A, Lee KF, O'Leary DD (2008) p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping. *Neuron* **59**: 746-58.

Lynch G, Rex CS, Chen LY, Gall CM (2008) The substrates of memory: defects, treatments, and enhancement. *Eur J Pharmacol* **585**: 2-13.

Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, Tessarollo L (1999) Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc Natl Acad Sci USA* **96**: 15239-15244.

MacInnis B, Campenot R (2002) Retrograde support of neuronal survival without retrograde transport of nerve growth factor. *Science* **295**: 1536-1539.

Mantyh PW, Koltzenburg M, Mendell LM, Tive L, Shelton, DL (2011) Antagonism of nerve growth factor-TrkA signaling and the relief of pain. *Anesthesiology* **115**: 189-204.

Marchetti C and Marie H (2012) Hippocampal synaptic plasticity in Alzheimer's disease: what have we learned so far from transgenic models. *Rev Neuroscience* **190**: 21-26.

Matrone C, Ciotti MT, Mercanti D, Marolda R, Calissano P (2008) NGF and BDNF signaling control amyloidogenic route and Abeta production in hippocampal neurons. *Proc Natl Acad Sci USA* **105**: 13139-13144.

Matrone C, Barbagallo AP, LaRosa LR, Florenzano F, Ciotti MT, Mercanti D, Chao MV, Calissano P, D'Adamio L (2011) APP is phosphorylated by TrkA and regualtes NGF/TrkA signaling. *J Neuroscience* **31**: 11756-11761.

Mauro C, Pietro L, and Emilio CC (2007) The use of nerve growth factor in herpetic keratitis: a case report. *J Med Case Reports* **1**: 124.

Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nature Neurosci* **7**: 221-228.

Mok SA, Lund K, Campenot RB (2009) A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures. *Cell Res* **19**: 546-560.

Nagahara AH, Merrill DA, Coppola G, Tskada S, Schroeder BE, Shaked GM, Wang L, Blesch A, Kim A, Conner JM, Rockenstein E, Chao MV, Koo EH, Geschwind D, Masliah E, Chiba, AA, Tuszynski MH (2009) Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nature Med.* 15, 331-337.

Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M (2009) APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature* **19**: 981-989.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* **427**: 843-848.

Pehar M, Cassina P, Vargas MR, Xie Y, Beckman JS, Massa SM, Longo FM, Barbeito L (2006) Modulation of p75-dependent motor neuron death by a small non-peptidyl mimetic of the neurotrophin loop 1 domain. *Eur J Neurosci* **24**: 1575-80.

Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R (2001) Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinology* **15**: 1748-1757.

Sabasovich I, Wei T, Guo Tz, Zhao R, Shi X, Li X, Yeomans DC, Klyukinov M, Kingery WS, Clark JD (2008) Effect of anti-NGF antibodies in a rat tibia fracture model of complex regional pain syndrome type I. *Pain* **138**: 47-60.

Sevcik MA, Ghilardi JR, Peters CM, Lindsay TH, Halvorson KG, Jonas BM, Kubota K, Kuskowski MA, Boustany L, Shelton DL Mantyh PW (2005) Anti-NGF therapy profoundly reduces bone cancer pain and the accompanying increase in markers of peripheral and central sensitization. *Pain* **115**: 128-141.

Shelton DL, Zeller J, Ho WH, Pons J, Rosenthal A (2005) Nerve growth factor mediates hyperalgesia and cachexia in auto-immune arthritis. *Pain* **116**: 8-16.

Singh KK, Park HK, Hong EJ, Kramer BM, Greenberg ME, Kaplan DR, Miller FD (2008) Developmental axon pruning mediated by BDNF-p75-dependent axon degeneration. *Nature Neuroscience* **11**: 649-658.

Strand AD, Baquet ZC, Aragaki AK, Holmans P Yang L, Cleren C, Beal MF, Jones L, Kooperberg C, Olson JM, Jones KR (2007) Expression profiling of Huntington's disease models suggests that brain-derived neurotrophic factor depletion plays a major role in striatal degeneration. *J Neurosci* **27**: 11758-68.

Thoenen H, Sendtner M (2002) Neurotrophins: from enthusiastic expectations through sobering experiences to rational therapeutic approaches. *Nature Neurosci* **5**(Suppl): 1046.

Tsao D, Thomsen HK, Chou J, Stratton J, Hagen M, Loo C, Garcia C, Sloane DL, rosenthal A, Lin JC (2008) TrkB agonists ameliorate obesity and associated metabolic conditions in mice. *Endocrinology* **149**: 1038-1048.

Ugolini G, Marinelli S, Covaceuszach S, Cattaneo A, Pavone F (2007) the function neutralizing anti-TrkA antibody MNAC13 reduces inflammatory and neuropathic pain. *Proc Natl Acad Sci USA* **104**: 2985-2990.

Wehrman T, He X, Raab B, Dukipatti A, Blau H, Garcia KC (2007) Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron* **53**: 25-38.

Wiesmann C, Ultsch MH, Bass SH, de Vos AM (1999) Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. *Nature* **401**: 184-188.

Wilcox CL, Smith RL, Freed CR, and Johnson EM (1990) Nerve growth factor dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. *J Neuroscience* **10**: 1268–1275.

Yang F, Je HS, Ji Y, Nagappan G, Hempstead B, Lu B (2009) Pro-BDNF-induced synaptic depression and retraction at developing neuromuscular synapses. *J Cell Biol* **185**: 727-741.

Ye H, Kuruvilla R, Zweifel LS, Ginty DD (2003) Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. *Neuron* **39**: 57-68.

Yeo GS, Hung CC, Rochford J, Keogh J, Gray J, Sivaramakrishnan S, O'Rahilly S, Farooqi IS (2004) A de novo mutation affecting TrkB associated with severe obesity and developmental delay. *Nature Neurosci* **7**: 1187-1189.

Zuccato C, Cattaneo E (2007) Role of brain-derived neurotrophic factor in Huntington's disease. *Prog Neurobiol* **81**: 294-330.

INTRACELLULAR PROTEIN DEGRADATION: FROM A VAGUE IDEA THROUGH THE LYSOSOME AND THE UBIQUITIN-PROTEASOME SYSTEM AND ON TO HUMAN DISEASES AND DRUG TARGETING

A. CIECHANOVER

The David and Janet Polak Cancer and Vascular Biology Research Center, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel

Between the 1950s and 1980s, scientists were focusing mostly on how the genetic code was transcribed to RNA and translated to proteins, but how proteins were degraded had remained a neglected research area. With the discovery of the lysosome by Christian de Duve it was assumed that cellular proteins are degraded within this organelle. Yet, several independent lines of experimental evidence strongly suggested that intracellular proteolysis was largely non-lysosomal, but the mechanisms involved have remained obscure. The discovery of the ubiquitin-proteasome system resolved the enigma. We now recognize that degradation of intracellular proteins is involved in regulation of a broad array of cellular processes, such as cell cycle and division, regulation of transcription factors, and assurance of the cellular quality control. Not surprisingly, aberrations in the system have been implicated in the pathogenesis of human disease, such as malignancies and neurodegenerative disorders, which led subsequently to an increasing effort to develop mechanism-based drugs.

The concept of protein turnover is hardly 70 years old. Beforehand, body proteins were viewed as essentially stable constituents that were subject to only minor 'wear and tear': dietary proteins were believed to function primarily as energy-providing fuel, which were independent from the structural and functional proteins of the body. The problem was hard to approach experimentally, as research tools were not available. Important research tools that were lacking at that time were stable isotopes. While radioactive isotopes were developed earlier by George de Hevesy (de Hevsey G., Chemistry 1943. In: *Nobel Lectures in Chemistry 1942-1962*. World Scientific 1999. pp. 5-41), they were mostly unstable and could not be used to follow metabolic pathways). The concept that body structural proteins are static and the dietary proteins are used only as a fuel was challenged by Rudolf Schoenheimer in Columbia University in New York City. Schoenheimer escaped from Germany and joined the Department of Biochemistry in Columbia University founded by Hans T. Clarke (Clarke, 1958; Kennedy, 2001; Simoni et al, 2002). There he met Harold Urey who was working in the Department of Chemistry and who discovered deuterium, the heavy isotope of hydrogen, a discovery that enabled him to prepare heavy water, D_2O. David Rittenberg who had recently received his Ph.D. in Urey's laboratory, joined Schoenheimer, and together they entertained the idea of '*employing a stable isotope as a label in organic compounds, destined for experiments in intermediary metabolism, which should be biochemically indistinguishable from their natural analog'* (Clarke, 1958). Urey later succeeded in enriching nitrogen with ^{15}N, which provided Schoenheimer and Rittenberg with a 'tag'

Keywords: ubiquitin, proteasome, protein degradation, lysosome, diseases

Correspondence address: Dr. Aaron Ciechanover
The Polak Cancer and Vascular Biology Research Center
Faculty of Medicine Technion-Israel Institute of Technology
Efron Street, Bat Galim P.O.Box 9649 Haifa 31096. ISRAEL
Tel: +972-4-829-5379, +972-4-829-5356
Fax: +972-4-852-1193
e-mail: c_tzachy@netvision.net.il

2279-5855 (2013)

for amino acids and as a result for the study of protein dynamics. They discovered that following administration of ^{15}N-labled tyrosine to rat, only ~50% can be recovered in the urine, '*while most of the remainder is deposited in tissue proteins. An equivalent of protein nitrogen is excreted*"' (Schoenheimer et al, 1939). They further discovered that from the half that was incorporated into body proteins '*only a fraction was attached to the original carbon chain, namely to tyrosine, while the bulk was distributed over other nitrogenous groups of the proteins'* (Schoenheimer et al, 1939), mostly as an αNH_2 group in other amino acids. These experiments demonstrated unequivocally that the body structural proteins are in a dynamic state of synthesis and degradation, and that even individual amino acids are in a state of dynamic interconversion. Similar results were obtained using ^{15}N-labled leucine (Ratner et al, 1940). This series of findings shattered the paradigm in the field at that time that: (1) ingested proteins are completely metabolized and the products are excreted, and (2) that body structural proteins are stable and static. Schoenheimer was invited to deliver the prestigious Edward K. Dunham lecture at Harvard University where he presented his revolutionary findings. After his untimely tragic death in 1941, his lecture notes were edited Hans Clarke, David Rittenberg and Sarah Ratner, and were published in a small book by Harvard University Press. The editors called the book '*The Dynamic State of Body Constituents'* (1942), adopting the title of Schoenheimer's presentation. In the book, the new hypothesis was clearly presented: '*The simile of the combustion engine pictured the steady state flow of fuel into a fixed system, and the conversion of this fuel into waste products. The new results imply that not only the fuel, but the structural materials are in a steady state of flux. The classical picture must thus be replaced by one which takes account of the dynamic state of body structure'*. However, the idea that proteins are turning over had not been accepted easily, and was challenged as late as the mid-1950s. For example, Hogness and colleagues studied the kinetics of β-galactosidase in *Escherichia coli* and summarized their findings (Hogness et al, 1955): '*To sum up: there seems to be no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover, our experiments have shown that the proteins of growing E. coli are static. Therefore it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a 'dynamic state'*. While the experimental study involved the bacterial β-galactosidase, the conclusions were broader, including also the authors' hypothesis on mammalian proteins. The use of the term '*dynamic state*' was not incidental, as they challenged directly Schoenheimer's studies.

Now, after more than seven decades of research in the field of intracellular proteolysis, and with the discovery of the lysosome and later the ubiquitin-proteasome system, it is clear that the field has been revolutionized. We now recognize that intracellular proteins are turning over extensively, that the process is specific, and that the stability of many proteins is regulated individually and can vary under different conditions. From a scavenger, unregulated and non-specific end process, it has become clear that proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process that plays major roles in a broad array of basic pathways. Among these processes are cell cycle, development, differentiation, regulation of transcription, antigen presentation, signal transduction, receptor-mediated endocytosis, quality control, and modulation of diverse metabolic pathways. Subsequently, it has changed the paradigm that regulation of cellular processes occurs mostly at the transcriptional and translational levels, and has set regulated protein degradation in an equally important position. With the multitude of substrates targeted and processes involved, it has not been surprising to find that aberrations in the pathway have been implicated in the pathogenesis of many diseases, among them certain malignancies, neurodegeneration, and disorders of the immune and inflammatory system. As a result, the system has become a platform for drug targeting, and mechanism-based drugs are currently developed, one of them is already on the market.

THE LYSOSOME AND INTRACELLULAR PROTEIN DEGRADATION

In the mid-1950s, Christian de Duve discovered

the lysosome (see, for example, De Duve et al, 1953; Gianetto & De Duve, 1955 and Figure 1). The lysosome was first recognized biochemically in rat liver as a vacuolar structure that contains various hydrolytic enzymes which function optimally at an acidic pH. It is surrounded by a membrane that endows the contained enzymes latency that is required to protect the cellular contents from their action (see below). The definition of the lysosome was broadened over the years because it had been recognized that the digestive process is dynamic and involves numerous stages of lysosomal maturation together with the digestion of both exogenous proteins (which are targeted to the lysosome through receptor-mediated endocytosis and pinocytosis) and exogenous particles (which are targeted via phagocytosis; the two processes are known as heterophagy), as well as digestion of endogenous proteins and cellular organelles (which are targeted by micro- and macro-autophagy; see Figure 2). The lysosomal/vacuolar system as we currently recognize it is a discontinuous and heterogeneous digestive system that also includes structures that are devoid of hydrolases – for example, early endosomes which contain endocytosed receptor-ligand complexes and pinocytosed/phagocytosed extracellular contents. On the other extreme it includes the residual bodies - the end products of the completed digestive processes of heterophagy and autophagy. In between these extremes one can observe: primary/nascent lysosomes that have not been engaged yet in any proteolytic process; early autophagic vacuoles that might contain intracellular organelles; intermediate/ late endosomes and phagocytic vacuoles (heterophagic vacuoles) that contain extracellular contents/particles; and multivesicular bodies (MVBs) which are the transition vacuoles between endosomes/phagocytic vacuoles and the digestive lysosomes.

The discovery of the lysosome along with independent experiments that were carried out at the same time and that have further strengthened the notion that cellular proteins are indeed in a constant state of synthesis and degradation (see, for example, Simpson, 1953), led scientists to feel, for the first time, that they have at hand an organelle that can potentially mediate degradation of intracellular proteins. The fact that the proteases were separated from their substrates by a membrane provided an explanation for controlled degradation, and the only problem left to be explained was how the substrates are translocated into the lysosomal lumen, exposed to the activity of the lysosomal proteases and degraded. An important discovery in this respect was the unraveling of the basic mechanism of action of the lysosome in autophagy (reviewed in Mortimore & Poso, 1987). Under basal metabolic conditions, portions of the cytoplasm, which contain the entire cohort of cellular proteins, are segregated within a membrane-bound compartment, and are then fused to a primary nascent lysosome and their contents digested. This process was called microautophagy. Under more extreme conditions, starvation for example, mitochondria, endoplasmic reticulum membranes, glycogen bodies and other cytoplasmic entities, can also be engulfed by a process called macroautophagy (see, for example, Ashford & Porter, 1962; the different modes of action of the lysosome in digesting extra- and intracellular proteins are shown in Fig. 2).

However, over a period of more than two decades, between the mid-1950s and the late 1970s, it has become gradually more and more difficult to explain several aspects of intracellular protein degradation based on the known mechanisms of lysosomal activity: accumulating lines of independent experimental evidence indicated that the degradation of at least certain classes of cellular proteins must be non-lysosomal. Yet, in the absence of any 'alternative', researchers came with different explanations, some more substantiated and others less, to defend the 'lysosomal' hypothesis.

First was the gradual discovery that came from different laboratories, that different proteins vary in their stabilities, and their half-life times can span three orders of magnitude, from a few minutes to many days. Thus, the $t_{1/2}$ of ornitihine decarboxylase (ODC) is ~10 min, while that of glucose-6-phosphate dehydrogenase (G6PD) is 15 hours (for review articles, see, for example, Schimke & Doyle, 1970; Goldberg & St. John, 1976). Also, rates of degradation of many proteins were shown to change with changing physiological conditions, such as availability of nutrients or hormones. It was conceptually difficult to reconcile the findings of distinct and changing half lives of different proteins

with the mechanism of action of the lysosome, where the microautophagic vesicle contains the entire cohort of cellular (cytosolic) proteins that are therefore expected to degrade at the same rate. Similarly, changing pathophysiological conditions, such as starvation or re-supplementation of nutrients, were expected to affect the stability of all cellular proteins to the same extent. Clearly, this was not the case.

Another source of concern about the lysosome as the organelle in which intracellular proteins are degraded were the findings that specific and general inhibitors of lysosomal proteases have different effects on different populations of proteins, making it clear that distinct classes of proteins are targeted by different proteolytic machineries. Thus, the degradation of endocytosed/pinocytosed extracellular proteins was significantly inhibited, a partial effect was observed on the degradation of long-lived cellular proteins, and almost no was detected on the degradation of short-lived and abnormal/mutated proteins.

Finally, the thermodynamically paradoxical observation that the degradation of cellular proteins requires metabolic energy, and more importantly, the emerging evidence that the proteolytic machinery uses the energy directly, were in contrast with the known mode of action of lysosomal proteases that under the appropriate acidic conditions, and similar to all known proteases, degrade proteins in an exergonic manner.

The assumption that the degradation of intracellular proteins is mediated by the lysosome was nevertheless logical. Proteolysis results from direct interaction between the target substrates and proteases, and therefore it was clear that active proteases cannot be free in the cytosol which would have resulted in destruction of the cell. Thus, it was recognized that any suggested proteolytic machinery that mediates degradation of intracellular protein degradation must also be equipped with a mechanism that separates - physically or virtually - between the proteases and their substrates, and enables them to associate only when needed. The lysosomal membrane provided this fencing mechanism. Obviously, nobody could have predicted that a new mode of post-translational modification - ubiquitination - could function as a proteolysis signal, and that untagged proteins will remain protected. Thus, while the structure of the lysosome could explain the separation necessary between the proteases and their substrates, and autophagy could explain the mechanism of entry of cytosolic proteins into the lysosomal lumen, major problems have remained unsolved. Important among them were: (i) the varying half lives, (ii) the energy requirement, and (iii) the distinct response of different populations of proteins to lysosomal inhibitors. Thus, according to one model, it was proposed that different proteins have different sensitivities to lysosomal proteases, and their half lives *in vivo* correlate with their sensitivity to the action of lysosomal proteases *in vitro* (Segal et al, 1974). To explain an extremely long half-life of a protein that was nevertheless sensitive to lysosomal proteases, or alterations in the stability of a single protein under various physiological states, it was suggested

Table I. *Resolution of the ATP-dependent proteolytic activity from crude reticulocyte extract into two essentially required complementing activities (adapted from Ref. 38; with permission from Elsevier/Biochem. Biophys. Res. Commun.).*

Fraction	Degradation of [^{3}H]globin (%)	
	-ATP	+ATP
Lysate	**1.5**	**10**
Fraction I	**0.0**	**0.0**
Fraction II	**1.5**	**2.7**
Fraction I and Fraction II	**1.6**	**10.6**

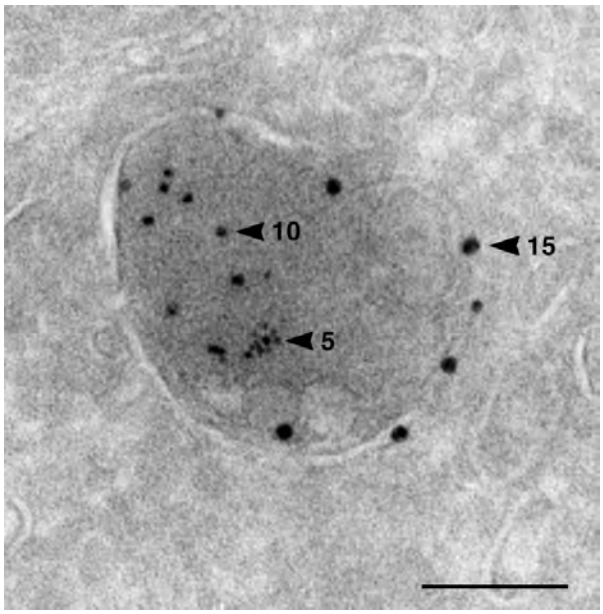


Fig. 1. *The lysosome: Ultrathin cryosection of a rat PC12 cell that had been loaded for 1 hour with bovine serum albumin (BSA)-gold (5 nm particles) and immunolabeled for the lysosomal enzyme cathepsin B (10-nm particles) and the lysosomal membrane protein LAMP1 (15 nm particles). Lysosomes are recognized also by their typical dense content and multiple internal membranes. Bar, 100 nm. Courtesy of Viola Oorschot and Judith Klumperman, Department of Cell Biology, University Medical Centre Utrecht, The Netherlands.*

that although all cellular proteins are engulfed into the lysosome, only the short-lived proteins are degraded, whereas the long-lived proteins exit back into the cytosol: '*To account for differences in half-life among cell components or of a single component in various physiological states, it was necessary to include in the model the possibility of an exit of native components back to the extralysosomal compartment'* (Haider & Segel, 1972). According to a different model, selectivity was determined by the binding affinity of the different proteins to the lysosomal membrane which controls their entry rates into the lysosome, and subsequently their degradation rates (Dean, 1977). For a selected group of proteins, such as the gluconeogenetic enzymes phosphoenol-pyruvate carboxykinase (PEPCK) and fructose-1,6-biphosphatase, it was suggested, though not firmly substantiated, that their degradation in the yeast vacuole was regulated by glucose via a mechanism called 'catabolite inactivation' that possibly involves their phosphorylation. However this regulated mechanism for vacuolar degradation was limited only to a small and specific group of proteins (see for example Muller et al, 1981; reviewed in Holzer, 1989). More recent studies have shown that at least for stress-induced macroautophagy, a general sequence of amino

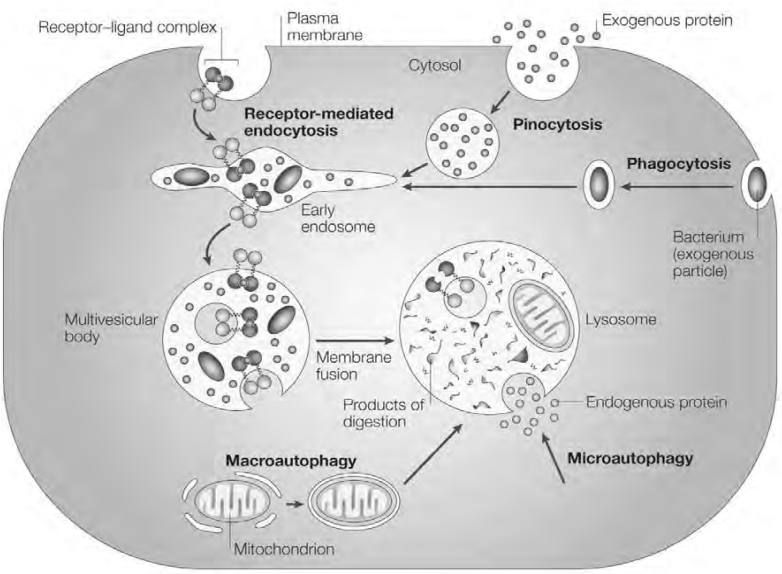


Fig. 2. *The four digestive processes mediated by the lysosome: (i) specific receptor-mediated endocytosis, (ii) pinocytosis (non-specific engulfment of cytosolic droplets containing extracellular fluid), (iii) phagocytosis (of extracellular particles), and (iv) autophagy (micro- and macro-; of intracellular proteins and organelles)(with permission from Nature Publishing Group. Published originally in Ref. 83).*

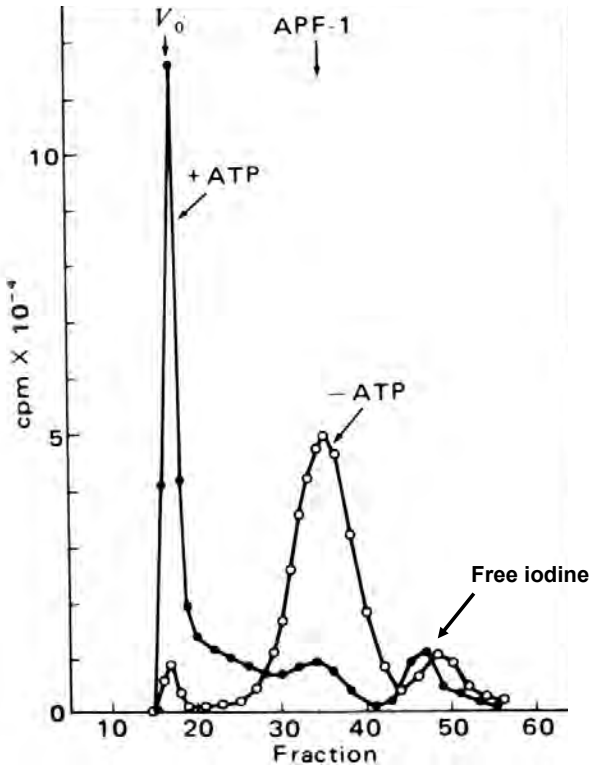


Fig. 3. *APF-1/Ubiquitin is shifted to high molecular mass compound(s) following incubation in ATP-containing crude cell extract. ^{125}I-labelled APF-1/ ubiquitin was incubated with reticulocyte crude Fraction II in the absence (open circles) or presence (closed circles) of ATP, and the reaction mixtures were resolved via gel filtration chromatography. Shown is the radioactivity measured in each fraction. As can be seen, following addition of ATP, APF-1/ubiquitin becomes covalently attached to some component(s) in fraction II, which could be another enzyme of the system or its substrate(s) (with permission from Proceedings of the National Academy of the USA; published originally in Ref. 39).*

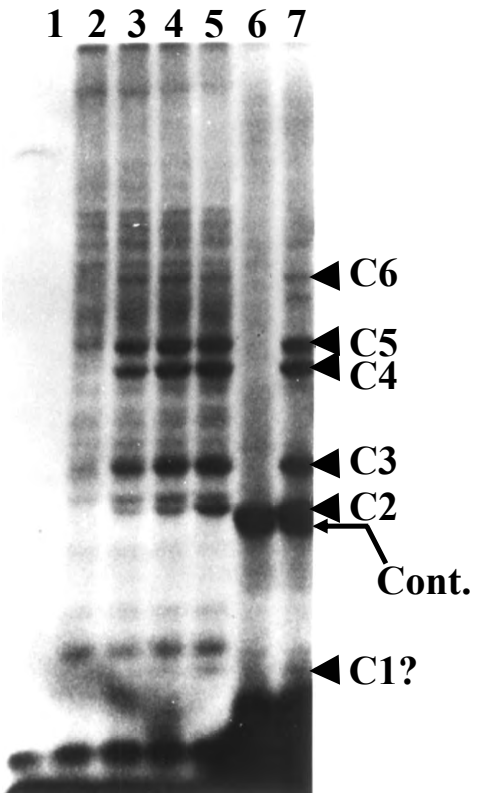


Fig. 4. *Multiple molecules of APF-1/Ubiquitin are conjugated to the proteolytic substrate, probably signalling it for degradation. To interpret the data described in the experiment depicted in Figure 2 and to test the hypothesis that APF-1 is conjugated to the target proteolytic substrate, ^{125}I-APF-1/ubiquitin was incubated along with crude Fraction II (Figure 3 and text) in the absence (lane 1) or presence (lanes 2-5) of ATP and in the absence (lanes 1, 2) or presence (lanes 3-5) of increasing concentrations of unlabeled lysozyme. Reaction mixtures resolved in lanes 6 and 7 were incubated in the absence (lane 6) or presence (lane 7) of ATP, and included unlabeled APF-1/ubiquitin and ^{125}I-labeled lysozyme. C1-C6 denote specific APF-1/ubiquitin-lysozyme adducts in which the number of APF-1/ubiquitin moieties bound to the lysozyme moiety of the adduct is increasing, probably from 1 to 6. Reactions mixtures were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized following exposure to an X-ray film (autoradiography) (with permission from Proceedings of the National Academy of the USA; published originally in Ref. 40).*

acids, KFFERQ, directs, via binding to a specific 'receptor' and along with cytosolic and lysosomal chaperones, the regulated entry of many cytosolic proteins into the lysosomal lumen. While further corroboration of this hypothesis is still required, it can only explain the mass entry of a large population of proteins that contain a homologous sequence, but not the targeting for degradation of a specific protein under defined conditions (reviewed in Majeski & Dice, 1998; Cuervo & Dice, 1998). The energy requirement for protein degradation was described as indirect, and necessary, for example, for protein transport across the lysosomal membrane (Hayashi et al, 1973) and/or for the activity of the H^+ pump and the maintenance of the low acidic intralysosomal pH that is necessary for optimal activity of the

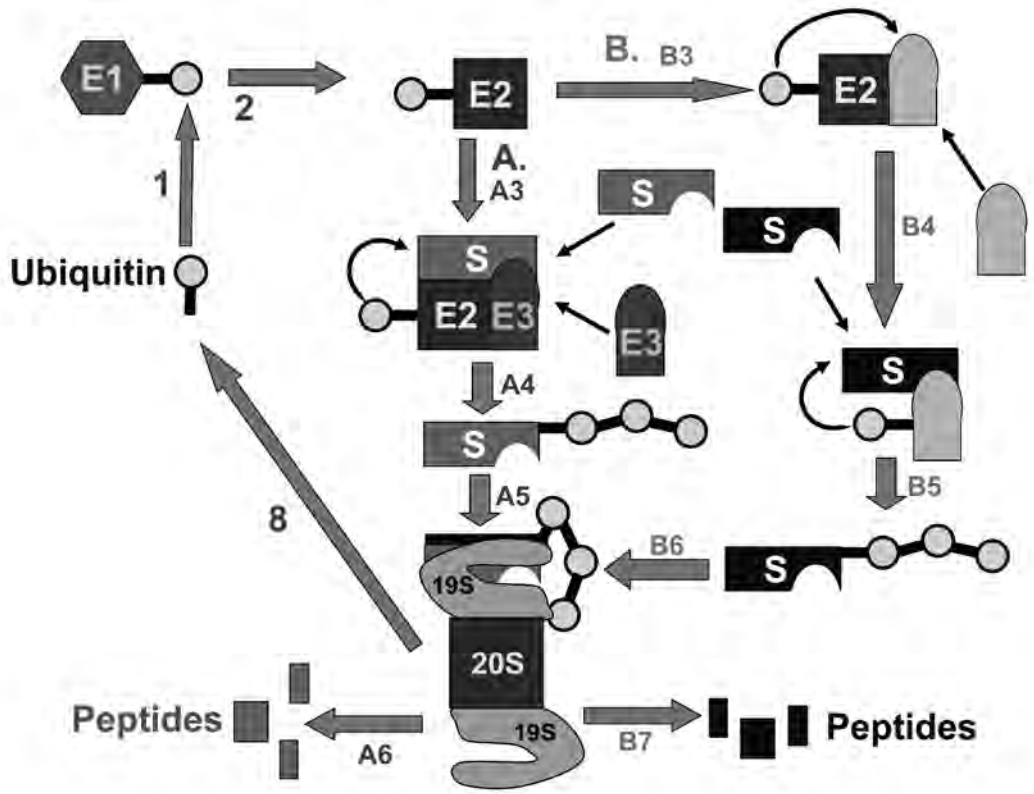


Fig. 5. *The ubiquitin-proteasome proteolytic system. Ubiquitin is activated by the ubiquitin-activating enzyme, E1 (1) followed by its transfer to a ubiquitin-carrier protein (ubiquitin-conjugating enzyme, UBC), E2 (2). E2 transfers the activated ubiquitin moieties to the protein substrate that is bound specifically to a unique ubiquitin ligase E3 (A and B). In the case of RING finger ligases, the transfer is direct (A3). Successive conjugation of ubiquitin moieties to one another generates a polyubiquitin chain (A4) that serves as the binding (A5) signal for the downstream 26S proteasome that degrades the target substrates to peptides (A6). In the case of HECT domain ligases, ubiquitin generates an additional thiol-ester intermediate on the ligase (B3), and only then is transferred to the substrate (B4). Successive conjugation of ubiquitin moieties to one another generates a polyubiquitin chain (B5) that binds to the 26S proteasome (B6) followed by degradation of the substrate to peptides (B7). Free and reusable ubiquitin is released by de-ubiquitinating enzymes (DUBs)(8).*

proteases (Schneider, 1981). We now know that both mechanisms require energy. In the absence of any alternative, and with lysosomal degradation as the most logical explanation for targeting all known classes of proteins at the time, Christian de Duve summarized his view on the subject in a review article published in the mid-1960s, saying: '*Just as extracellular digestion is successfully carried out by the concerted action of enzymes with limited individual capacities, so, we believe, is intracellular digestion'* (De Duve & Wattiaux, 1966). The problem of different sensitivities of distinct protein groups to lysosomal inhibitors has remained unsolved, and may have served as an important trigger in future quest for a non-lysosomal proteolytic system.

Progress in identifying the elusive, non-lysosomal proteolytic system(s) was hampered by the lack of a cell-free preparation that could faithfully replicate the cellular proteolytic events - i.e. degrading proteins in a specific and energy-requiring mode. An important breakthrough was made by Rabinovitz and Fisher who found that rabbit reticulocytes degrade abnormal, amino acid analogue-containing hemoglobin (Rabinovitz & Fisher, 1964). Their experiments modeled known disease states, the hemoglobinopathies. In these

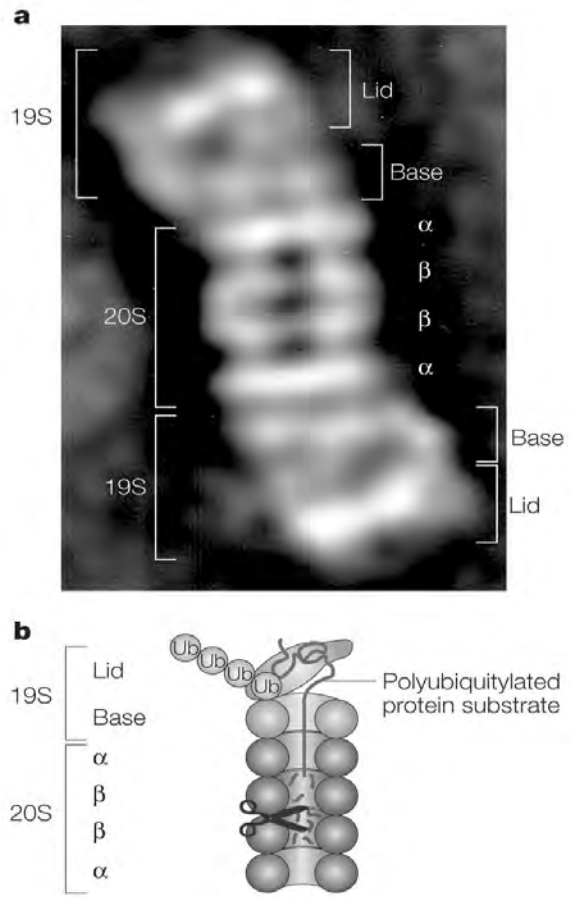


Fig. 6. *The Proteasome. The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides. It is composed of two sub-complexes: a 20S core particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure composed of four stacked rings, two identical outer a rings and two identical inner b rings. The eukaryotic a and b rings are composed each of seven distinct subunits, giving the 20S complex the general structure of* $a_{1\text{-}7}b_{1\text{-}7}b_{1\text{-}7}a_{1\text{-}7}$. *The catalytic sites are localized to some of the b subunits. Each extremity of the 20S barrel can be capped by a 19S RP each composed of 17 distinct subunits, 9 in a "base" sub-complex, and 8 in a "lid" sub-complex. One important function of the 19S RP is to recognize ubiquitinated proteins and other potential substrates of the proteasome. Several ubiquitin-binding subunits of the 19S RP have been identified, however, their biological roles mode of action have not been discerned. A second function of the 19S RP is to open an orifice in the a ring that will allow entry of the substrate into the proteolytic chamber. Also, since a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the 19S RP "base" contains six different ATPase subunits. Following degradation of the substrate, short peptides derived from the substrate are released, as well as reusable ubiquitin (with permission from Nature Publishing Group. Published originally in Ref. 83). a. Electron microscopy image of the 26S proteasome from the yeast S. cerevisiae. b. Schematic representation of the structure and function of the 26SA proteasome.*

diseases abnormal mutated hemoglobin chains (such as sickle cell hemoglobin) or excess of unassembled normal hemoglobin chains (which are synthesized normally, but also excessively in thalassemias, diseases in which the pairing chain is not synthesized at all or is mutated and rapidly degraded, and consequently the bi-heterodimeric hemoglobin complex is not assembled) are rapidly degraded in the reticulocyte (Carrell & Lehmann, 1969; Huehns and Bellingham, 1969). Reticulocytes are terminally differentiating red blood cells that do not contain lysosomes. Therefore, it was postulated that the degradation of hemoglobin in these cells was mediated by a non-lysosomal machinery. Etlinger and Goldberg (1977) were the first to isolate and characterize a cell-free proteolytic preparation from reticulocytes. The crude extract selectively degraded abnormal hemoglobin, required ATP hydrolysis, and acted optimally at a neutral pH, which further corroborated the assumption that the proteolytic activity was of a non-lysosomal origin. A similar system was isolated and characterized later by Hershko, Ciechanover, and their colleagues (1978). Additional studies by this group led subsequently to resolution, characterization, and purification of the major enzymatic components from this extracts and to the discovery of the ubiquitin signalling system (see below).

THE LYSOSOME HYPOTHESIS IS CHALLENGED:

As mentioned above, the unraveled mechanism(s) of action of the lysosome could explain only partially and at times not satisfactorily, several key emerging characteristics of intracellular protein degradation. Among them were the heterogeneous stability

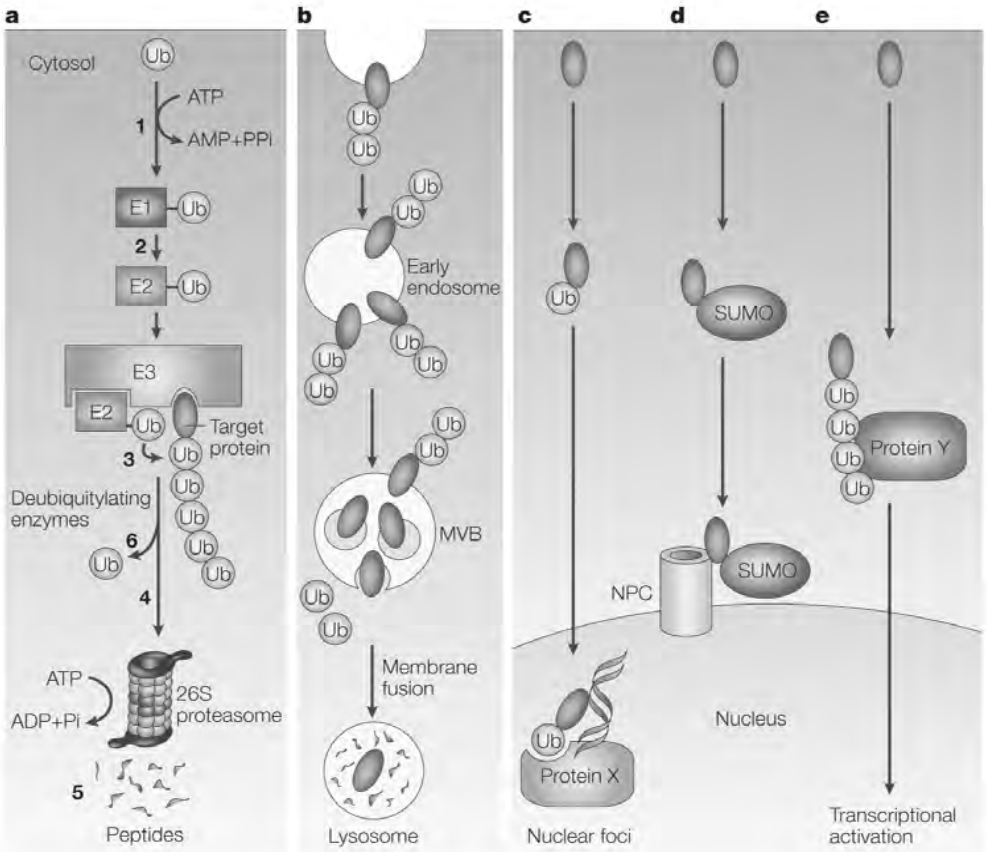


Fig. 7. *Some of the different functions of modification by ubiquitin and ubiquitin-like proteins. A. Proteasomal-dependent degradation of cellular proteins (see Figure 4). B. Mono or oligoubiquitination targets membrane proteins to degradation in the lysosome/vacuole. C. Monoubiquitination, or D. a single modification by a ubiquitin-like (UBL) protein, SUMO for example, can target proteins to different subcellular destinations such as nuclear foci or the nuclear pore complex (NPC). Modification by UBLs can serve other, non-proteolytic, functions, such as protecting proteins from ubiquitination or activation of E3 complexes. E. Generation of a Lys^{63}-based polyubiquitin chain can activate transcriptional regulators, directly or indirectly [via recruitment of other proteins (Protein Y; shown), or activation of upstream components such as kinases]. Ub denotes ubiquitin, K denotes Lys, and S denotes Cys. (with permission from Nature Publishing Group. Published originally in Ref. 83).*

of individual proteins, the effect of nutrients and hormones on their degradation, and the dependence of intracellular proteolysis on metabolic energy. The differential effect of selective inhibitors on the degradation of different classes of cellular proteins (see above but mostly below), could not be explained at all.

The evolution of methods to monitor protein kinetics in cells together with the development of specific and general lysosomal inhibitors has resulted in the identification of different classes of cellular proteins, long- and short-lived, and the discovery of the differential effects of the inhibitors on these groups (see, for example, Knowles & Ballard, 1976; Neff et al, 1979). An elegant experiment in this respect was carried out by Brian Poole and his colleagues in the Rockefeller University. Poole was studying the effect of lysosomotropic agents, weak bases such as ammonium chloride and chloroquine, which accumulate in the lysosome and dissipate its low acidic pH. It was assumed that this mechanism underlies also the anti-malarial activity of chloroquine and similar drugs where they inhibit the activity of the parasite's lysosome, 'paralyzing' its ability to digest the host's hemoglobin during the intra-erythrocytic stage of its life cycle. Poole and his colleagues

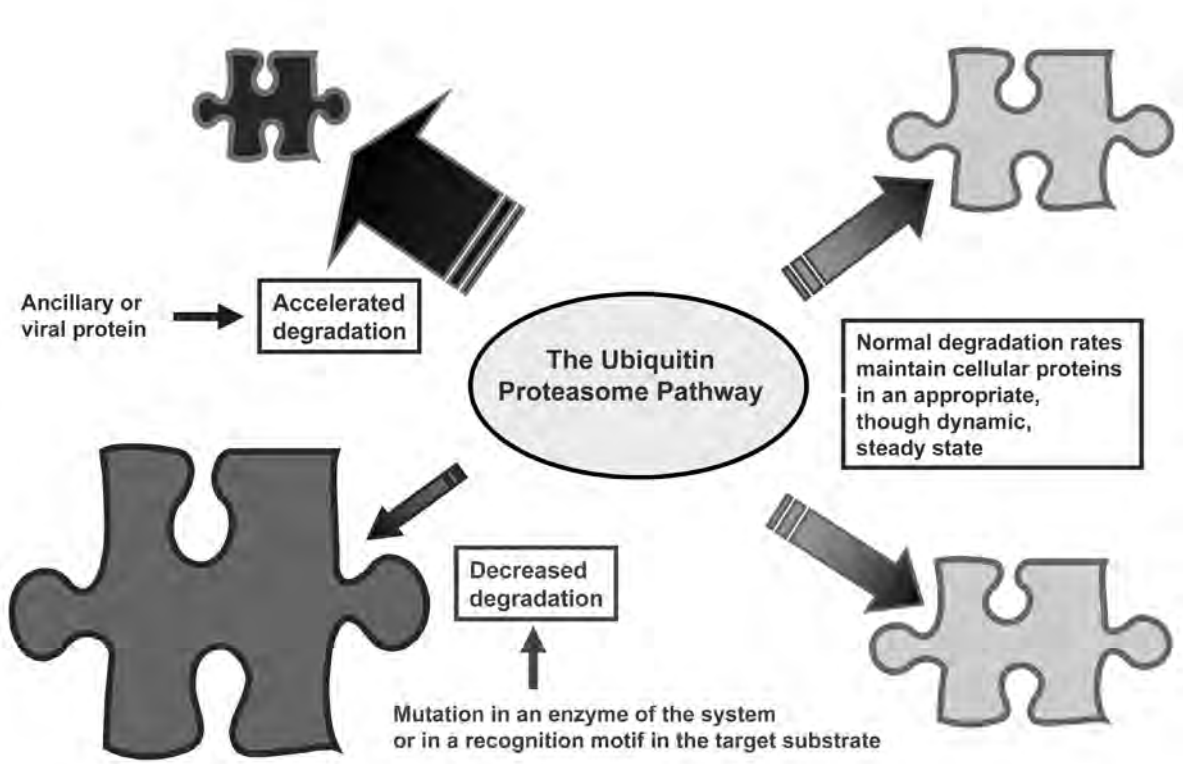


Fig. 8. *Aberrations in the ubiquitin-proteasome system and pathogenesis of human diseases. Normal degradation of cellular proteins maintains them in a steady state level, though this level may change under various pathophysiological conditions (upper and lower right side). When degradation is accelerated due an increase in the level of an E3 (Skp2 in the case of p27, for example), or overexpression of an ancillary protein that generates a complex with the protein substrate and targets it for degradation (the Human Papillomavirus E6 oncoprotein that associates with p53 and targets it for degradation by the E6-AP ligase, or the cytomegalovirus-encoded ER proteins US2 and US11 that target MHC class I molecules for ERAD), the steady state level of the protein decreases (upper left side). A mutation in a ubiquitin ligase [such as occurs in Adenomatous Polyposis Coli - APC, or in E6-AP (Angelmans' Syndrome)] or in the substrate's recognition motif (such as occurs in b-catenin or in ENaC) will result in decreased degradation and accumulation of the target substrate.*

metabolically labeled endogenous proteins in living macrophages with ^{3}H-leucine and 'fed' them with dead macrophages that had been previously labeled with ^{14}C-leucine. They assumed, apparently correctly, that the dead macrophages debris and proteins will be phagocytosed by live macrophages and targeted to the lysosome for degradation. They monitored the effect of lysosomotropic agents on the degradation of these two protein populations, In particular, they studied the effect of the weak bases chloroquine and ammonium chloride (which enter the lysosome and neutralize the H^+ ions), and the acid ionophore X537A which dissipates the H^+ gradient across the lysosomal membrane. They found that these drugs specifically inhibited the degradation of extracellular proteins, but not that of intracellular proteins (Poole et al, 1977). Poole summarized these experiments and explicitly predicted the existence of a non-lysosomal proteolytic system that degrades intracellular proteins: '*Some of the macrophages labeled with tritium were permitted to endocytise the dead macrophages labeled with ^{14}C. The cells were then washed and replaced in fresh medium. In this way we were able to measure in the same cells the digestion of macrophage proteins from two sources. The exogenous proteins will be broken down in the lysosomes, while the endogenous proteins will be broken down wherever it is that endogenous proteins*

are broken down during protein turnover' (Poole et al, 1978; the paragraph is copied verbatim; A.C.).

The requirement for metabolic energy for the degradation of both prokaryotic (Mandelstam, 1958) and eukaryotic (Simpson, 1953; Steinberg & Vaughan, 1956) proteins was difficult to explain. Proteolysis is an exergonic process and the thermodynamically paradoxical energy requirement for intracellular proteolysis made researchers believe that energy cannot be consumed directly by proteases or the proteolytic process per se, and is used indirectly. As Simpson summarized his findings (1953): '*The data can also be interpreted by postulating that the release of amino acids from protein is itself directly dependent on energy supply. A somewhat similar hypothesis, based on studies on autolysis in tissue minces, has recently been advanced, but the supporting data are very difficult to interpret. However, the fact that protein hydrolysis as catalyzed by the familiar proteases and peptidases occurs exergonically, together with the consideration that autolysis in excised organs or tissue minces continues for weeks, long after phosphorylation or oxidation ceased, renders improbable the hypothesis of the direct energy dependence of the reactions leading to protein breakdown'*. Being cautious however, and probably unsure about this unequivocal conclusion, Simpson still left a narrow orifice opened for a proteolytic process that requires energy in a direct manner: '*However, the results do not exclude the existence of two (or more) mechanisms of protein breakdown, one hydrolytic, the other energy-requiring'*. Since any proteolytic process must be at one point or another hydrolytic, the statement that makes a distinction between a hydrolytic process, and an energy-requiring, yet non-hydrolytic one, is not clear. Judging the statement from an historical point of view and knowing the mechanism of action of the ubiquitin system, where energy is required also in the pre-hydrolytic step (ubiquitin conjugation), Simpson may have thought of a two step mechanism, but did not give it a clear description. At the end of this clearly understandable, but at the same time difficult and convoluted deliberation, Simpson left us with a vague explanation linking protein degradation to protein synthesis, a process that was known to require metabolic energy: '*The fact that a supply of energy seems to be necessary for both the incorporation and the release of amino acids from protein might well mean that the two processes are interrelated. Additional data suggestive of such a view are available from other types of experiments. Early investigations on nitrogen balance by Benedict, Folin, Gamble, Smith, and others point to the fact that the rate of protein catabolism varies with the dietary protein level. Since the protein level of the diet would be expected to exert a direct influence on synthesis rather than breakdown, the altered catabolic rate could well be caused by a change in the rate of synthesis'* (Simpson, 1953). With the discovery of lysosomes in eukaryotic cells it could be argued that energy was required for the transport of substrates into the lysosome or for maintenance of the low intralysosomal pH for (see above), for example. The observation by Hershko and Tomkins that the activity of tyrosine aminotransferase (TAT) was stabilized following depletion of ATP (Hershko & Tomkins, 1971) indicated that energy could be required at an early stage of the proteolytic process, most probably before proteolysis occurs. Yet, it did not provide a clue as for the mechanism involved: energy could be used, for example, for specific modification of TAT, e.g. phosphorylation, that would sensitize it to degradation by the lysosome or by a yet unknown proteolytic mechanism, or for a modification that activates its putative protease. It could also be used for a more general lysosomal mechanism, one that involves transport of TAT into the lysosome, for example. The energy inhibitors inhibited almost completely degradation of the entire population of cell proteins, confirming previous studies (e.g. Simpson, 1953) and suggesting a general role for energy in protein catabolism. Yet, an interesting finding was that energy inhibitors had an effect that was distinct from that of protein synthesis inhibitors which affected only enhanced degradation (induced by steroid hormone depletion), but not basal degradation. This finding ruled out, at least partially, a tight linkage between protein synthesis and degradation. In bacteria, which lack lysosomes, an argument involving energy requirement for lysosomal degradation could not have been proposed, but other indirect effects of ATP hydrolysis could have affected proteolysis in *E. coli*, such as phosphorylation of substrates and/or proteolytic enzymes, or maintenance of

the 'energized membrane state'. According to this model, proteins could become susceptible to proteolysis by changing their conformation, for example, following association with the cell membrane that maintains a local, energy-dependent gradient of a certain ion. While such an effect was ruled out (Goldberg et al, 1976), and since there was no evidence for a phosphorylation mechanism (although the proteolytic machinery in prokaryotes had not been identified at that time), it seemed that at least in bacteria, energy was required directly for the proteolytic process. In any event, the requirement for metabolic energy for protein degradation in both prokaryotes and eukaryotes, a process that is exergonic thermodynamically, strongly indicated that in cells proteolysis is highly regulated, and that a similar principle/mechanism has been preserved along evolution of the two kingdoms. Implying from the possible direct requirement for ATP in degradation of proteins in bacteria, it was not too unlikely to assume a similar direct mechanism in the degradation of cellular proteins in eukaryotes. Supporting this notion was the description of the cell-free proteolytic system in reticulocytes (Etlinger & Goldberg, 1977; Hershko et al, 1978), a cell that lacks lysosomes, which indicates that energy is probably required directly for the proteolytic process, although here too, the underlying mechanisms had remained enigmatic at the time. Yet, the description of the cell-free system paved the road for detailed dissection of the underlying mechanisms involved.

THE UBIQUITIN-PROTEASOME SYSTEM

The cell-free proteolytic system from reticulocytes (Etlinger & Goldberg, 1977; Hershko et al, 1978) turned out to be an important and rich source for the purification and characterization of the enzymes that are involved in the ubiquitin-proteasome system. Initial fractionation of the crude reticulocyte cell extract on the anion-exchange resin diethylaminoethyl cellulose yielded two fractions which were both required to reconstitute the energy-dependent proteolytic activity that is found in the crude extract: The unadsorbed, flow through material was denoted fraction I, and the high salt eluate of the adsorbed proteins which was denoted fraction II (Ciechanover et al, 1978) (Table 1). This was an important observation and a lesson for the future dissection of the system. For one it suggested that the system was not composed of a single 'classical' protease that has evolved evolutionarily to acquire energy dependence [although such energy-dependent proteases, the mammalian 26S proteasome (see below) and the prokaryotic *Lon* gene product have been described later], but that it was made of at least two components. This finding of a two component, energy-dependent protease, left the researchers with no paradigm to follow, and in attempts to explain the finding, they suggested, for example, that the two fractions could represent an inhibited protease and its activator. Second, learning from this reconstitution experiment and the essential dependence between the two active components, we continued to reconstitute activity from resolved fractions whenever we encountered a loss of activity along further purification steps. This biochemical 'complementation' approach resulted in the discovery of additional enzymes of the system, all required to be present in the reaction mixture in order to catalyze the multi-step proteolysis of the target substrate. We chose first to purify the active component from fraction I. It was found to be a small, ~8.5 kDa heat stable protein that was designated ATP-dependent Proteolysis Factor 1, APF-1. APF-1 was later identified as ubiquitin (see below; I am using the term APF-1 to the point where it was identified as ubiquitin and then change terminology accordingly). In retrospect, the decision to start the purification efforts with fraction I turned out to be important, as fraction I contained only one single protein - APF-1 - that was necessary to stimulate proteolysis of the model substrate we used at the time, while fraction II turned out to contain many more. Later studies showed that fraction I contains other components necessary for the degradation of other substrates, but these were not necessary for the reconstitution of the system at that time. This enabled us not only to purify APF-1, but also to quickly decipher its mode of action. If we would have started our purification efforts with fraction II, we would have encountered a significantly bumpier road. A critically important finding that paved the way for future developments in the field was that multiple moieties of APF-1 are covalently conjugated to the target substrate when incubated in the presence of fraction II, and

the modification requires ATP (Ciechanover et al, 1980a; Hershko et al, 1980) (Figures 3 and 4). It was also found that the modification is reversible, and APF-1 could be removed from the substrate or its degradation products (Hershko et al, 1980).

The discovery that APF-1 was covalently conjugated to protein substrates and stimulates their proteolysis in the presence of ATP and crude fraction II, led in 1980 to the proposal of a model according to which protein substrate modification by multiple moieties of APF-1 targets it for degradation by a downstream, at that time an yet unidentified, protease that cannot recognize the unmodified substrate; following degradation, reusable APF-1 was released (Hershko et al, 1980). Amino-acid analysis of APF-1, along with its known molecular mass and other general characteristics raised the suspicion that APF-1 was ubiquitin (Ciechanover et al, 1980b), a known protein of previously unknown function. Indeed, Wilkinson and colleagues confirmed unequivocally that APF-1 was indeed ubiquitin (Wilkinson et al, 1980). Ubiquitin had been first described as a small, heat-stable and highly evolutionarily conserved protein of 76 residues. It was first purified during the isolation of thymopoietin (Goldstein, 1974) and was subsequently found to be ubiquitously expressed in all kingdoms of living cells, including prokaryotes (Goldstein et al, 1975). Interestingly, it was initially found to have lymphocyte-differentiating properties, a characteristic that was attributed to the stimulation of adenylate cyclase (Goldstein et al, 1975; Schlessinger et al, 1975). Accordingly, it was named UBIP for ***ub***iquitous ***i***mmunopoietic ***p***olypeptide (Goldstein et al, 1975). However, later studies showed that ubiquitin was not involved in the immune response (Low & Golstein, 1979), and that it was a contaminating endotoxin in the preparation that generated the adenylate cyclase and the T-cell differentiating activities. Furthermore, the sequence of several eubacteria and archaebacteria genomes as well as biochemical analyses in these organisms (unpublished) showed that ubiquitin was restricted only to eukaryotes. The finding of ubiquitin in bacteria (Goldstein et al, 1975) was probably due to contamination of the bacterial extract with yeast ubiquitin derived from the yeast extract in which the bacteria were grown. While in retrospect the name ubiquitin is a misnomer, as it is restricted to eukaryotes and is not ubiquitous as was previously thought, it has remained the name of the protein. The reason is probably because it was the name that was first assigned to the protein, and scientists and nomenclature committees tend, in general, to respect this tradition. Accordingly, and in order to avoid confusion, I suggest that the names of other novel enzymes and components of the ubiquitin system, but also of other systems as well, should remain as were first coined by their discoverers.

An important development in the ubiquitin research field was the discovery that a single ubiquitin moiety can be covalently conjugated to histones, particularly to histones H2A and H2B. While the function of these adducts has remained elusive until recently, their structure was unraveled in the mid 1970s. The structure of the ubiquitin conjugate of H2A (uH2A; was also designated protein A24) was deciphered by Goldknopf and Busch (1975; 1977) and by Hunt and Dayhoff (1977) who found that the two proteins are linked through a fork-like, branched isopeptide bond between the carboxy-terminal glycine of ubiquitin (Gly^{76}) and the ε-NH_2 group of an internal lysine (Lys^{119}) of the histone molecule. The isopeptide bond found in the histone-ubiquitin adduct was suggested to be identical to the bond that was found between ubiquitin and the target proteolytic substrate (Hershko et al, 1981), and between the ubiquitin moieties in the polyubiquitin chain (Hershko & Heller, 1985; Chau et al, 1989) that was synthesized on the substrate and that functions as a proteolysis recognition signal for the downstream 26S proteasome. In this particular polyubiquitin chain the linkage is between Gly^{76} of one ubiquitin moiety and internal Lys^{48} of the previously conjugated moiety. Only Lys^{48}-based ubiquitin chains are recognized by the 26S proteasome and serve as proteolytic signals. In recent years it has been shown that the first ubiquitin moiety can also be attached in a linear mode to the N-terminal residue of the proteolytic target substrate (Ciechanover & Ben-Saadon, 2004). However, the subsequent ubiquitin moieties are generating Lys^{48}-based polyubiquitin chain on the first linearly fused moiety. N-terminal ubiquitination is clearly required for targeting naturally occurring lysine-less proteins for degradation. Yet, several lysine-containing proteins have also been described that traverse this

pathway, the muscle-specific transcription factor MyoD for example. In these proteins the internal lysine residues are probably not accessible to the cognate ligases. Other types of polyubiquitin chains have also been described that are not involved in targeting the conjugated substrates for proteolysis. Thus, a Lys^{63}-based polyubiquitin chain has been described that is probably necessary to activate transcription factors (reviewed recently in Muratani & Tansey, 2003). Interestingly, the role of monoubiquitination of histones has also been identified recently, and this modification is also involved in regulation of transcription, probably via modulation of the structure of the nucleosomes (for recent reviews, see, for example, Zhang, 2003; Osley, 2004).

The identification of APF-1 as ubiquitin, and the discovery that a high-energy isopeptide bond, similar to the one that links ubiquitin to histone H2A, links it also to the target proteolytic substrate, resolved at that time the enigma of the energy requirement for intracellular proteolysis (see however below) and paved the road to the untangling of the complex mechanism of isopeptide bond formation. This process turned out to be similar to that of peptide bond formation that is catalyzed by tRNA synthetase following amino acid activation during protein synthesis or during the non-ribosomal synthesis of short peptides (Lipman, 1971). Using the unraveled mechanism of ubiquitin activation and immobilized ubiquitin as a 'covalent' affinity bait, the three enzymes that are involved in the cascade reaction of ubiquitin conjugation were purified by Ciechanover, Hershko, and their colleagues. These enzymes are: (i) E1, the ubiquitin-activating enzyme, (ii) E2, the ubiquitin-carrier protein, and (iii) E3, the ubiquitin-protein ligase (Ciechanover et al, 1982; Hershko et al, 1983). The discovery of an E3 which was a specific substrate-binding component, indicated a possible solution to the problem of the varying stabilities of different proteins – they might be specifically recognized and targeted by different ligases.

In a short period, the ubiquitin tagging hypothesis received substantial support. For example, Chin and colleagues injected into HeLa cells labeled ubiquitin and hemoglobin and denatured the injected hemoglobin by oxidizing it with phenylhydrazine. They found that ubiquitin conjugation to globin was markedly enhanced by denaturation of hemoglobin and the concentration of globin-ubiquitin conjugates was proportional to the rate of hemoglobin degradation (Chin et al, 1980). Hershko and colleagues (1982) observed a similar correlation for abnormal, amino acid analogue-containing short-lived proteins. A previously isolated cell cycle arrest mutant that loses the ubiquitin-histone H2A adduct at the permissive temperature (Matsumoto et al, 1983), was found by Finley, Ciechanover and Varshavsky to harbor a thermolabile E1 (1984). Following heat inactivation, the cells fail to degrade normal short-lived proteins (Ciechanover et al, 1984). Although the cells did not provide direct evidence for substrate ubiquitination as a destruction signal, they still provided the strongest direct linkage between ubiquitin conjugation and degradation.

At this point, the only missing link was the identification of the downstream protease that would specifically recognize ubiquitinated substrates. Tanaka and colleagues identified a second ATP-requiring step in the reticulocyte proteolytic system, which occurred after ubiquitin conjugation (Tanaka et al, 1983), and Hershko and colleagues (1984) demonstrated that the energy was required for conjugate degradation. An important advance in the field was a discovery by Hough and colleagues, who partially purified and characterized a high-molecular mass alkaline protease that degraded ubiquitin adducts of lysozyme but not untagged lysozyme, in an ATP-dependent mode (Hough et al, 1986). This protease which was later called the 26S proteasome (see below), provided all the necessary criteria for being the specific proteolytic arm of the ubiquitin system. This finding was confirmed, and the protease was further characterized by Waxman and colleagues who found that it was an unusually large, ~1.5MDa enzyme, unlike any other known protease (Waxman et al, 1987). A further advance in the field was the discovery (Hough et al, 1987) that a smaller neutral multi-subunit 20S protease complex that was discovered together with the larger 26S complex, was similar to a "multicatalytic proteinase complex" (MCP) that had been described earlier in bovine pituitary gland by Wilk and Orlowski (1980). This 20S protease was ATP-independent and has different catalytic activities, cleaving on the carboxy-terminal side of hydrophobic, basic and acidic residues.

Hough and colleagues raised the possibility - although they did not show it experimentally - that this 20S protease could be a part of the larger 26S protease that degrades the ubiquitin adducts (Hough et al, 1987). Later studies showed that indeed, the 20S complex is the core catalytic particle of the larger 26S complex (Eytan et al, 1989; Driscoll & Goldberg, 1990). However, a strong evidence that the active 'mushroom'-shaped 26S protease was generated through the assembly of two distinct sub-complexes - the catalytic 20S cylinder-like MCP and an additional 19S ball-shaped sub-complex (that was predicted to have a regulatory role) - was provided only in the early 1990s by Hoffman and colleagues (1992) who mixed the two purified particles and generated the active 26S enzyme.

The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides. It is composed of two sub-complexes: a 20S core particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure composed of four stacked rings, two identical outer α rings and two identical inner β rings. The eukaryotic α and β rings are composed each of seven distinct subunits, giving the 20S complex the general structure of $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$. The catalytic sites are localized to some of the β subunits. Each extremity of the 20S barrel can be capped by a 19S RP each composed of seventeen distinct subunits, nine in a "base" sub-complex, and eight in a "lid" sub-complex. One important function of the 19S RP is to recognize ubiquitinated proteins and other potential substrates of the proteasome. Several ubiquitin-binding subunits of the 19S RP have been identified, although their biological roles and mode of action have not been discerned. A second function of the 19S RP is to open an orifice in the α ring that will allow entry of the substrate into the proteolytic chamber. Also, since a folded protein would not be able to fit through the narrow proteasomal channel, it was assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the 19S RP "base" contains six different ATPase subunits. Following degradation of the substrate, short peptides derived from the substrate are released, as well as reusable ubiquitin (for a scheme describing the ubiquitin system, see Figure 5; for the structure of the 26S proteasome, see Figure 6).

CONCLUDING REMARKS

The evolution of proteolysis as a centrally important regulatory mechanism has served as a remarkable example for the evolution of a novel biological concept and the accompanying battles to change paradigms. The five decades journey between the early 1940s and early 1990s began with fierce discussions on whether cellular proteins are static as has been thought for a long time, or are turning over. The discovery of the dynamic state of proteins was followed by the discovery of the lysosome that was believed - between the mid-1950s and mid-1970s - to be the organelle within which intracellular proteins are destroyed. Independent lines of experimental evidence gradually eroded the lysosomal hypothesis and resulted in a new idea that the regulated degradation of intracellular proteins under basal metabolic conditions via a non-lysosomal machinery. This resulted in the discovery of the ubiquitin system in the late 1970s and early 1980s. Interestingly, modifications of different target substrates by ubiquitin and ubiquitin-like proteins are now known to be involved in all aspects of lysosomal degradation, such as in the generation of the autophagic vacuoles, and in the routing of cargo-carrying vesicles to the lysosome (see below). Modifications by ubiquitin and ubiquitin-like proteins are now viewed, much like phosphorylation, as a mechanism to generate recognition elements *in trans* on target proteins to which downstream effectors bind. In one case, generation of Lys^{48}-based polyubiquitin chains, the binding effector is the 26S proteasome that degrades the ubiquitin-tagged protein. In many other cases, different modifications serve numerous proteolytic (lysosomal) and non-proteolytic functions, such as routing of proteins to their subcellular destinations. We were fortunate at the beginning of our studies to have in mind a clear distinction between lysosomal and non-lysosomal proteolytic systems not knowing what we know nowadays that the two processes are linked to one another and are mediated via similar modifications. Had we known that, our route would

have been much more complicated.

With the identification of the reactions and enzymes that are involved in the ubiquitin-proteasome cascade, a new era in the protein degradation field began at the late 1980s and early 1990s. Studies that showed that the system was involved in targeting of key regulatory proteins - such as light-regulated proteins in plants, transcriptional factors, cell cycle regulators and tumor suppressors and promoters - started to emerge (see for example Refs. Shanklin et al, 1987; Hochstrasser & Varshavsky, 1990; Scheffner et al, 1990; Glotzer et al, 1991; Ciechanover et al, 1991). They were followed by numerous studies on the underlying mechanisms involved in the degradation of specific proteins, each with its own unique mode of recognition and regulation. The unraveling of the human genome revealed the existence of hundreds of distinct E3s, attesting to the complexity and the high specificity and selectivity of the system. Two important advances in the field were the discovery of the non-proteolytic functions of ubiquitin such as activation of transcription and routing of proteins to the vacuole, and the discovery of modification by ubiquitin-like proteins (UBLs), that are also involved in numerous non-proteolytic functions such as directing proteins to their sub-cellular destination, protecting proteins from ubiquitination, or controlling entire processes such as autophagy (see for example Mizushima et al, 1998)(for the different roles of modifications by ubiquitin and UBLs, see Figure 7). All these studies have led to the emerging realization that this novel mode of covalent conjugation plays a key role in regulating a broad array of cellular process – among them cell cycle and division, growth and differentiation, activation and silencing of transcription, apoptosis, the immune and inflammatory response, signal transduction, receptor mediated endocytosis, various metabolic pathways, and the cell quality control - through proteolytic and non-proteolytic mechanisms. The discovery that ubiquitin modification plays a role in routing proteins to the lysosome/vacuole and that modification by specific and unique ubiquitin-like proteins and modification system controls autophagy closed an exciting historical cycle, since it demonstrated that the two apparently distinct systems communicate with one another. With the many processes and substrates targeted by the ubiquitin pathway, it has not been surprising to find that aberrations in the system underlie, directly or indirectly, the pathogenesis of many diseases. While inactivation of a major enzyme such as E1 was obviously lethal, mutations in enzymes or in recognition motifs in substrates that do not affect vital pathways or that affect the involved process only partially, may result in a broad array of phenotypes. Likewise, acquired changes in the activity of the system can also evolve into certain pathologies. The pathological states associated with the ubiquitin system can be classified into two groups: **(a)** those that result from loss of function - mutation in a ubiquitin system enzyme or in the recognition motif in the target substrate that result in stabilization of certain proteins, and **(b)** those that result from gain of function - abnormal or accelerated degradation of the protein target (for aberrations in the ubiquitin system that result in disease states, see Figure 8). Studies that employ targeted inactivation of genes coding for specific ubiquitin system enzymes and substrates in animals can provide a more systematic view into the broad spectrum of pathologies that may result from aberrations in ubiquitin-mediated proteolysis. Better understanding of the processes and identification of the components involved in the degradation of key regulatory proteins will lead to the development of mechanism-based drugs that will target specifically only the involved proteins. While the first drug, a specific proteasome inhibitor is already on the market (Adams, 2003), it appears that one important hallmark of the new era we are entering now will be the discovery of novel drugs based on targeting of specific processes such as inhibiting aberrant Mdm2- or E6-AP-mediated accelerated targeting of the tumor suppressor p53 which will lead to regain of its lost function.

Many reviews have been published on different aspects of the ubiquitin system. The purpose of this article was to bring to the reader several milestones along the historical pathway along which the ubiquitin system has been evolved. For additional reading on the ubiquitin system, the reader is referred to numerous review articles written on the subject (for some older reviews, see for example Glickman & Ciechanover, 2002; Pickart & Cohen, 2004). Some parts of this review, including several Figures, are based on another published review

article (Ciechanover, 2005).

ACKNOWLEDGEMENT

Research in the laboratory of Aaron Ciechanover is supported by grants from the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF), the Israel Science Foundation (ISF), and the Deutsche-Israeli Cooperation Program (DIP). This article is based on the Nobel Lecture delivered in Stockholm on December 8, 2004, and published in: *Ciechanover, A. (2005). Les Prix Nobel.* The Nobel Foundation, Stockholm, Sweden. pp. 151-175. The article is published with permission of the Nobel Foundation©.

REFERENCES

Adams J. (2003). Potential for proteasome inhibition in the treatment of cancer. *Drug Discov. Today*. **8**, 307-315.

Ashford, T.P., and Porter, K.R. (1962). Cytoplasmic components in hepatic cell lysosomes. J. Cell Biol. 12, 198-202.

Carrell, R.W., and Lehmann, H. (1969). The unstable haemoglobin haemolytic anaemias. *Semin. Hematol*. **6**, 116-132.

Chau, V., Tobias, J. W., Bachmair, A., Mariott, D., Ecker, D., Gonda, D. K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243,** 1576-1583.

Chin, D.T., Kuehl, L., and Rechsteiner, M. (1982). Conjugation of ubiquitin to denatured hemoglobin is proportional to the rate of hemoglobin degradation in HeLa cells. *Proc. Natl. Acad. Sci. USA* **79**, 5857-5861.

Ciechanover, A. (2005). From the lysosome to ubiquitin and the proteasome. Nature Rev. Mol. Cell Biol. 6, 79-86.

Ciechanover, A., DiGiuseppe, J.A., Bercovich, B., Orian, A., Richter, J.D., Schwartz, A.L., and Brodeur, G.M. (1991). Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. *Proc. Natl. Acad. Sci. USA* **88,** 139-143.

Ciechanover, A., Elias, S., Heller, H. & Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. J. Biol. Chem. 257, 2537-2542.

Ciechanover, A., Elias, S., Heller, H., Ferber, S. & Hershko, A. (1980). Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *J. Biol. Chem.* **255**, 7525-7528 (1980).

Ciechanover, A., Finley D., and Varshavsky, A. (1984). Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell 37, 57-66.

Ciechanover A., Hod, Y., and Hershko, A. (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochem. Biophys. Res. Common.* **81**, 1100-1105.

Ciechanover, A., Heller, H., Elias, S., Haas, A.L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proc. Natl. Acad. Sci. USA*. **77**, 1365-1368 (1980).

Ciechanover, A., and Ben-Saadon R. (2004). N-terminal ubiquitination: More Protein substrates join in. *Trends Cell Biol.* **14,** 103-106

Clarke, H.T. (1958). Impressions of an organic chemist in biochemistry. *Annu. Rev. Biochem.* **27**, 1-14.

Cohn, M., and Monod, J. (1955). Studies on the induced synthesis of β-galactosidase in *Escherichia coli*: The kinetics and mechanism of sulfur incorporation. *Biochim. Biophys. Acta* **16**, 99-116.

Cuervo, A.M., and Dice, J.F. (1998). Lysosomes, a meeting point of proteins, chaperones, and proteases. *J. Mol. Med*. **76**, 6-12.

Dean, R.T. Lysosomes and protein degradation. (1977). *Acta Biol. Med. Ger.* **36**,

1815-1820.

de Duve, C., Gianetto, R., Appelmans, F., and Wattiaux, R. (1953). Enzymic content of the mitochondria fraction. *Nature (London)* **172**, 1143-1144

de Duve, C., and Wattiaux, R. Functions of lysosomes. (1966). *Annu. Rev. Physiol.* **28**, 435-492.

Driscoll, J., and Goldberg, A.L. (1990). The proteasome (multicatalytic protease) is a component of the 1,500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. *J. Biol. Chem.* **265**, 4789-4792.

Etlinger, J.D., and Goldberg, A.L. (1977). A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl. Acad. Sci. USA* **74,** 54-58.

Eytan, E., Ganoth, D., Armon, T., and Hershko, A. (1989). ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated

to ubiquitin. *Proc. Natl. Acad. Sci. USA*. **86,** 7751-7755.

Finley, D., Ciechanover, A., and Varshavsky, A. (1984). Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. *Cell* **37,** 43-55.

Gianetto, R., and de Duve, C. Tissue fractionation studies 4. (1955). Comparative study of the binding of acid phosphatase, β-glucoronidase and cathepsin by rat liver particles. *Biochem. J.* **59**, 433-438.

Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome pathway: Destruction for the sake of construction. *Physiological Reviews* **82,** 373-428.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* **349,** 132-138.

Goldberg, A.L., Kowit, J.D., and Etlinger, J.D. (1976). Studies on the selectivity and mechanisms of intracellular protein degradation. **In:** Proteolysis and physiological regulation (D.W. Ribbons & K. Brew, eds.). Academic Press, New York. pp. 313-337.

Goldberg, AL., and St. John, A.C. (1976). Intracellular protein degradation in mammalian and bacterial cells: Part 2. *Annu. Rev. Biochem.* **45**, 747-803.

Goldknopf, I.L., and Busch, H. (1975). Remarkable similarities of peptide fingerprints of histone 2A and non-histone chromosomal protein A24. *Biochem. Biophys. Res. Commun.* **65**, 951-955.

Goldknopf, I.L., and Busch, H. (1977). Isopeptide linkage between non-histone and histone 2A polypeptides of chromosome conjugate-protein A24. *Proc. Natl. Acad. Sci. USA* **74,** 864-868.

Goldstein, G. (1974). Isolation of bovine thymin, a polypeptide hormone of the thymus. *Nature (London)* **247,** 11-14.

Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D., and Boyse, E.A. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc. Natl. Acad. Sci. USA* **72**, 11-15.

Haider, M., and Segal, H.L. (1972). Some characteristics of the alanine-aminotransferase and arginase-inactivating system of lysosomes. *Arch. Biochem. Biophys.* **148,** 228-237.

Hayashi, M., Hiroi, Y., and Natori, Y. (1973). Effect of ATP on protein degradation in rat liver lysosomes. *Nature New Biol.* **242**, 163-166.

Hershko, A., Ciechanover, A., Heller, H., Haas, A.L., and Rose, I.A. (1980). Proposed role of ATP in protein breakdown: Conjugation of proteins with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proc. Natl. Acad. Sci. USA* **77**, 1783-1786.

Hershko, A., Ciechanover, A., and Rose, I.A. (1981). Identification of the active amino acid residue of the polypeptide of ATP-dependent protein breakdown. *J. Biol. Chem.* **256**, 1525-1528.

Hershko, A., Eytan, E., Ciechanover, A. and Haas, A.L. (1982). Immunochemical Analysis of the Turnover of Ubiquitin-protein Conjugates in Intact Cells: Relationship to the Breakdown of Abnormal Proteins. *J. Biol. Chem.* **257,** 13964-13970.

Hershko, A., and Heller, H. (1985). Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. *Biochem. Biophys. Res. Common.* **128,** 1079-1086.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system: Resolution, affinity purification and role in protein breakdown. *J. Biol. Chem.* **258,** 8206-8214 (1983).

Hershko, A., Heller, H., Ganoth, D., and Ciechanover, A. (1978). Mode of degradation of abnormal globin chains in rabbit reticulocytes. **In:** Protein Turnover and Lysosome Function (H.L. Segal & D.J. Doyle, eds.). Academic Press, New York. pp. 149-169. Hogness, D.S., Hershko, A., Leshinsky, E., Ganoth, D. & Heller, H. (1984). ATP-dependent degradation of ubiquitin-protein conjugates. *Proc. Natl. Acad. Sci. USA* **81,** 1619-1623.

Hershko, A., and Tomkins, G.M. (1971). Studies on the degradation of tyrosine aminotransferase in hepatoma cells in culture: Influence of the composition of the medium and adenosine triphosphate dependence. *J. Biol. Chem.* **246**, 710-714.

Hochstrasser, M., and Varshavsky, A. (1990). *In vivo* degradation of a transcriptional regulator: the yeast α2 repressor. *Cell* **61,** 697-708.

Hoffman, L., Pratt, G., and Rechsteiner, M. (1992). Multiple forms of the 20S multicatalytic and the 26S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate. *J. Biol. Chem.* **267**, 22362-22368.

Holzer, H. (1989). Proteolytic catabolite inactivation in *Saccharomyces cerevisiae*. *Revis. Biol. Celular* **21**, 305-319.

Hough, R., Pratt, G., and Rechsteiner M. (1987). Purification of two high molecular weight proteases from rabbit reticulocyte lysate. *J. Biol. Chem.* **262**, 8303-8313.

Hough, R., Pratt, G. & Rechsteiner, M. (1986).

Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *J. Biol. Chem.* **261**, 2400-2408.

Huehns, E.R., and Bellingham, A.J. (1969). Diseases of function and stability of haemoglobin. *Br. J. Haematol*. **17**, 1-10.

Hunt, L.T., and Dayhoff, M.O. (1977). Amino-terminal sequence identity of ubiquitin and the non-histone component of nuclear protein A24.

Kennedy., E.P. (2001). Hitler's gift and the era of biosynthesis. *J. Biol. Chem.* **276**, 42619-42631.

Knowles, S.E., and Ballard, F.J. (1976). Selective control of the degradation of normal and aberrant proteins in Reuber H35 hepatoma cells. *Biochem J.* **156,** 609-617.

Lipman, F. (1971). Attempts to map a process evolution of peptide biosynthesis. *Science* **173**, 875-884.

Low, T.L.K., and Goldstein, A.L. (1979). The chemistry and biology of thymosin: amino acid analysis of thymosin α1 and polypeptide β1. *J. Biol. Chem.* **254,** 987-995.

Majeski, A.E., and Dice, J.F. (2004). Mechanisms of chaperone-mediated autophagy. *Intl. J. Biochem. Cell Biol.* **36**, 2435-2444.

Mandelstam, J. (1958). Turnover of protein in growing and non-growing populations of *Escherichia coli. Biochem. J.* **69**, 110-119.

Matsumoto, Y., Yasuda, H., Marunouchi, T., and Yamada, M. (1983). Decrease in uH2A (protein A24) of a mouse temperature-sensitive mutant**.** *FEBS Lett.* **151,** 139-142

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. *Nature* **395**, 395-398.

Mortimore, G.E., and Poso, A.R. (1987). Intracellular protein catabolism and its control during nutrient deprivation and supply. *Annu. Rev. Nutr*. **7**, 539-564.

Müller, M., Müller, H, and Holzer, H. (1981). Immunochemical studies on catabolite inactivation of phosphoenolpyruvate carboxykinase in *Saccharomyces cerevisiae. J. Biol. Chem*. **256**, 723-727.

Muratani, M., and Tansey, W.P. (2003). How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* **4**, 192-201.

Neff, N.T., DeMartino, G.N., and Goldberg, A.L. (1979). The effect of protease inhibitors and decreased temperature on the degradation of different classes of proteins in cultured hepatocytes. *J. Cell Physiol.* **101,** 439-457.

Osley, M.A. (2004). H2B ubiquitylation: the end is in sight.

Biochim. Biophys. Acta. **1677,** 74-78.

Pickart, C.M., and Cohen, R.E. (2004). Proteasomes and their kin: proteases in the machine age. *Nature Rev. Mol. Cell Biol.* **5**, 177-187.

Poole, B., Ohkuma, S., and Warburton, M.J. (1977). The accumulation of weakly basic substances in lysosomes and the inhibition of intracellular protein degradation. *Acta Biol. Med. Germ.* **36**, 1777-1788.

Poole, B., Ohkuma, S. & Warburton, M.J. (1978). Some aspects of the intracellular breakdown of exogenous and endogenous proteins. **In:** Protein Turnover and Lysosome Function (H.L. Segal & D.J. Doyle, eds.). Academic Press, New York. pp. 43-58.

Rabinovitz, M., and Fisher, J.M. (1964). Characteristics of the inhibition of hemoglobin synthesis in rabbit reticulocytes by threo-α-amino-β-chlorobutyric acid. *Biochim. Biophys. Acta.* **91**, 313-322.

Ratner, S., Rittenberg, D., Keston, A.S., and Schoenheimer, R. (1940). Studies in protein metabolism: XIV. The chemical interaction of dietary glycine and body proteins in rats. *J. Biol. Chem.* **134**, 665-676.

Shanklin, J., Jaben, M., and Vierstra, R.D. (1987). Red light-induced formation of ubiquitin-phytochrome conjugates: Identification of possible intermediates of phytochrome degradation. *Proc. Natl. Acad. Sci. USA* **84**, 359-363.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63,** 1129-1136.

Schimke, R.T., and Doyle, D. (1970). Control of enzyme levels in animal tissues. *Annual Rev. Biochem.* **39**, 929-976.

Schlessinger, D.H., Goldstein, G., and Niall, H.D. (1975). The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry* **14**, 2214-2218.

Schneider, D.L. (1981). ATP-dependent acidification of intact and disrupted lysosomes: Evidence for an ATP-driven proton pump. *J. Biol. Chem.* **256,** 3858-3864.

Schoenheimer, R., Ratner, S., and Rittenberg, D. (1939). Studies in protein metabolism: VII. The metabolism of tyrosine. *J. Biol. Chem.* **127**, 333-344.

Schoenheimer, R. The Dynamic State of Body

Constituents (1942). Harvard University Press, Cambridge, Massachusetts, USA.

Segal, H.L., Winkler, J.R., and Miyagi, M.P. (1974). Relationship between degradation rates of proteins *in vivo* and their susceptibility to lysosomal proteases. *J. Biol. Chem.* **249**, 6364-6365.

Simoni, R.D., Hill, R.L., and Vaughan, M. (2002). The use of isotope tracers to study intermediary metabolism: Rudolf Schoenheimer. *J. Biol. Chem.* **277** (issue 43), e1-e3 (available on-line at: http:/www.jbc.org).

Simpson, M.V. The release of labeled amino acids from proteins in liver slices. (1953). *J. Biol. Chem*. **201**, 143-154.

Steinberg, D., and Vaughan, M. (1956). Observations on intracellular protein catabolism studied *in vitro*. *Arch. Biochem. Biophys*. **65**, 93-105.

Tanaka, K., Waxman, L., and Goldberg, A.L. (1983). ATP serves two distinct roles in protein degradation in reticulocytes, one requiring and one independent of ATP. *J. Cell Biol.* **96**, 1580-1585 (1983).

Waxman, L., Fagan, J., and Goldberg, A.L. (1987). Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates. *J. Biol. Chem.* **262**, 2451-2457.

Wilk, S., and Orlowski, M. (1980). Cation-sensitive neutral endopeptidase: isolation and specificity of the bovine pituitary enzyme. *J. Neurochem.* **35**, 1172-1182.

Wilkinson, K.D., Urban, M.K., and Haas, A.L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *J. Biol. Chem.* **255**, 7529-7532. *Biochim. Biophys. Res. Commun.* **74,** 650-655.

Zhang, Y. (2003). Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes & Dev.* **17**, 2733-2740.

LONG-TERM POTENTIATION IN ANIMAL MODELS OF ALZHEIMER'S DISEASE

R. NISTICÒ

Department of Physiology and Pharmacology, Sapienza University of Rome, and Pharmacology of Synaptic Plasticity Unit, European Brain Research Institute, Rome, Italy

The discovery of long-term potentiation (LTP) of hippocampal synaptic transmission, which represents the principal experimental model for the synaptic changes underlying learning and memory, has stimulated over the past years substantial progress in the understanding of pathogenic mechanisms underlying neurodegenerative disorders, such as Alzheimer's disease (AD). Indeed, several lines of evidence point to synaptic dysfunction not only as a core feature but also a leading cause of AD. Following intensive investigations into LTP in AD models, a variety of compounds have been found to rescue LTP impairment via numerous mechanisms. However, very few of these discoveries have been successfully translated into disease-modifying compounds in humans. This review recapitulates the main molecular mechanisms underlying LTP, the synaptic alterations across the different AD models and the disease-modifying strategies targeting amyloid β-protein (Aβ) successfully tested in experimental AD.

Before and during the 1960's there were ongoing efforts to explain how information was encoded in the brain. Such work was driven by the desire to explain the biological basis of cognition and to answer such intriguing questions as to the cellular basis of learning and memory. A major breakthrough came with the identification in Oslo of a process in the hippocampus whereby synaptic transmission was enhanced for long periods of time following brief periods of high frequency stimulation to the perforant path input into the dentate gyrus of the hippocampal formation (Lømo, 1966). This phenomenon, which is now called long-term potentiation (LTP), was originally described in two classic papers published in the Journal of Physiology (Bliss and Lømo, 1973; Bliss and Gardner-Medwin, 1973). Almost 10,000 papers have been written about LTP since its discovery in 1973. It is now widely accepted that LTP represents a fundamental cellular phenomenon underlying normal learning and memory (Bliss and Collingridge, 2003).

In the present review, I will describe (i) the main molecular mechanisms underlying LTP, (ii) how synaptic dysfunction contributes to neurodegenerative diseases, using Alzheimer's disease (AD) as a model, (iii) how LTP may serve as a tool to test the efficacy of novel compounds with disease-modifying potential. This paper will not address the full repertoire of long-term synaptic plasticity mechanisms and its pharmacological modulation; for a fuller account the reader is referred to recent work (Collingridge et al, 2010 and Nisticò et al, 2012).

MOLECULAR MECHANISMS UNDERLYING LTP

LTP has been mainly described in the Schaffer collateral-commissural pathway that provides a monosynaptic excitatory connection between CA3 and CA1 pyramidal neurons. LTP is triggered by physiological patterns of activity. Although

Key words: Long-term potentiation, synaptic plasticity, Alzheimer's disease, amyloid β-protein, hippocampus

Mailing address:
Dr. Robert Nisticò
Department of Physiology and Pharmacology
Sapienza University of Rome
P.zz.le Aldo Moro, 5 - 00185 Rome, Italy
Email: robert.nistico@uniroma1.it

2279-5855 (2013)

experimentally it is commonly induced by a period of high frequency stimulation referred to as a tetanus (e.g., 100 stimuli delivered at 100 Hz) it can be induced by very few appropriately timed stimuli (such as a single stimulus followed 200 ms later by 2-4 stimuli at 100 Hz). Such patterns of activity fall well within the physiological firing range.

In the early 80's Graham Collingridge was a postdoctoral fellow in Hugh McLennan's laboratory in Vancouver. Using recently developed glutamate receptor antagonists they observed that low frequency synaptic transmission was mediated by the activation of a class of non-NMDA receptors (now known to be AMPA receptors) and that the induction of LTP required the activation of NMDA receptors (Collingridge et al, 1983). Specifically, the NMDA receptor antagonist AP5, which had been developed by Jeff Watkins (Davies et al, 1981), was able to block the induction of LTP without affecting baseline transmission. Thus they had identified NMDA receptors as a trigger for the induction of synaptic plasticity.

However, not all forms of LTP are dependent on the activation of NMDARs. For example, it was observed that LTP at the monosynaptic connection between dentate gyrus granule cells and CA3 neurons, the mossy fibre pathway, was insensitive to NMDA receptor antagonists (Harris and Cotman, 1986). Thus, LTP is now classified according to whether it is dependent or independent on the synaptic activation of NMDA receptors.

Considering that LTP involves molecular mechanisms that are involved in cognitive processing this finding led to hypothesize that NMDA receptor antagonists should impair learning. Accordingly, this was clearly demonstrated by Richard Morris and colleagues (Morris et al, 1986) who infused AP5 into the brains of rats and observed impairment in the learning of a spatial memory task.

Several groups focused on trying to understand how the synaptic activation of NMDA receptors triggered the induction of LTP. Soon it was established that the unusual properties of the NMDA receptor voltage-dependent block by Mg^{2+} and Ca^{2+} permeability - were the key. Another key discovery was the finding that induction of LTP was prevented by the postsynaptic infusion of a Ca^{2+} chelator into CA1 neurons (Lynch et al, 1983). This clearly established the locus of induction of LTP at these synapses as postsynaptic. After Ca^{2+} enters the neuron, following the activation of NMDA receptors, it activates a complex biochemical cascade. The first such members of this cascade to be indicated were calmodulin (Finn et al, 1980), protein kinase C (Lovinger et al, 1987) and CaMKII (Malinow et al, 1989). In addition to the first Ca^{2+} sensitive proteins to be identified in LTP, there was also the first indication that Ca^{2+}-induced Ca^{2+} release might in someway be involved in the process (Obenaus et al, 1989).

The apparently simple question of whether the increase in synaptic strength during LTP is due primarily to some postsynaptic modification in AMPARs or some presynaptic change in transmitter release generated a debate that lasted for many years. It is now accepted that a fundamental mechanism for the expression of LTP involves increasing the number of AMPARs in the plasma membrane via activity-dependent changes in AMPAR trafficking (Bredt and Nicoll, 2003; Malenka and Nicoll, 1999) or via their direct phosphorylation (Benke et al, 1998; Lee et al, 2003). Present understanding of the molecular mechanisms that control the activity-dependent regulation of AMPAR trafficking to synapses has been reviewed extensively elsewhere (Bredt and Nicoll, 2003; Malinow and Malenka, 2002).

NEUROTRANSMITTERS INVOLVED IN LTP

Beyond glutamate, several other neurotransmitters can influence LTP in the hippocampus. Among these, dopamine (DA) certainly plays a central role (Frey et al, 1990). The modulation of hippocampal CA1 synaptic plasticity through activation of dopaminergic receptors is somehow surprising, given that the levels of dopamine in this area are relatively low compared to the other monoamine neurotransmitters (Lindvall and Björklund, 1978). Nevertheless, despite its low concentration in the hippocampus, the modulation of hippocampal synaptic plasticity by DA remains an intriguing phenomenon that has yet to be fully elucidated.

Beside DA, also the other catecholamine noradrenaline (NA) influences both synaptic plasticity and memory. In fact, the hippocampus receives significant noradrenergic input, and

endogenous release of NA triggers some forms of hippocampal synaptic plasticity (Frey et al, 2004; Walling and Harley, 2004). Activation of α1-adrenergic receptors mediates diverse responses in area CA1. α1-adrenergic agonists facilitate LTP induction and maintenance when paired with weak electrical tetanus (Izumi and Zorumski, 1999; Pussinen and Sirviö, 1998). Moreover, NA application prevents depotentiation of LTP by low-frequency stimulation (LFS) and this effect is mediated via α1 and β adrenergic receptors (Katsuki et al, 1997).

The data on the influence of serotonin on LTP is quite complex, possibly depending on the receptor subtype involved. Stimulation of either 5-HT_{2A} or 5-HT_4 receptors via specific agonists leads to enhancement of LTP in the hippocampus (Wang and Arvanov, 1998; Barnes and Sharp, 1999). On the other hand, agonists of the 5-HT_{1A} receptors have been described to specifically impair long-term depression (LTD) (Normann et al, 2000). Moreover, fluvoxamine, a selective serotonin reuptake inhibitor, inhibits LTP in area CA1, an effect that has been ascribed to 5-HT_{1A} receptors desensitization (Kojima et al, 2003). In addition, 5-HT_3 antagonists facilitate LTP, which suggests that 5-HT_3 receptors may inhibit LTP induction (Staubli and Xu, 1995). Notably, complete abolishment of 5-HT innervation in the hippocampus increases LTP *in vivo* (Ohashi et al, 2003), suggesting that overall serotonin exerts an inhibitory role on LTP. However, it cannot be ruled out that this effect is secondary to other changes.

Acetylcholine (ACh) has been extensively implicated in synaptic plasticity and learning and memory related to cognitive processes (Bartus et al, 1982; Buresova et al, 1964). Indeed, LTP is typically impaired following disruption of cholinergic transmission in mouse models of AD. Numerous studies have now shown that activation of nicotinic acetylcholine receptors (nAChRs) facilitates the induction of LTP *in vitro*. In particular, facilitation is observed in acute brain slices from naïve animals, in animals chronically treated with nicotine, and also in slices from aged animals in which LTP is reduced (Hamid et al, 1997; Fujii et al, 1999; Fujii and Sumikawa, 2001; Ji et al, 2001; Ge and Dani, 2005). In contrast to *in vitro* models, where nicotine converts short-term potentiation (STP) to LTP, in the intact anesthetized animal very high doses of nicotine are sufficient to induce LTP even in the absence of a conditioning train (Matsuyama et al, 2000). Besides nAChRs, the action of ACh on learning and memory is also mediated by muscarinic acetylcholine receptors (mAChRs) (Blokland, 1996). Application of mAChR agonists to hippocampal slices produces network oscillations (Huerta and Lisman, 1993; Fisahn et al, 1998, 2002), which modulate the induction of hippocampal LTP (Williams and Johnston, 1988; Huerta and Lisman, 1993; Shimoshige et al., 1997), mainly through activation of M1 receptors (Shinoe et al, 2005).

The ability of GABA inhibition to regulate the induction of LTP and to limit the synaptic activation of NMDA receptors has been firmly established. It was shown that GABA acts on autoreceptors to reduce GABA release during high frequency transmission (Davies et al, 1990). This autoreceptor was a G-protein coupled $GABA_B$ receptor and its inhibition led to a facilitation of postsynaptic $GABA_A$ receptor-mediated inhibition. The time-course of the $GABA_B$ auto-inhibition is such that it becomes particularly effective during theta patterns of activation. Indeed, GABA antagonists can prevent the induction of LTP when theta patterns of stimulation are used (Davies et al, 1991). It is, however, unclear whether long-term changes in GABAergic inhibition occur after a tetanic stimulation, and if so, how such a change may affect LTP.

Accumulating evidence has suggested that glycine is abundantly expressed in the hippocampus where it plays an important role in regulating excitability and synaptic plasticity. Accordingly, application of glycine potentiates NMDAR-mediated currents through its high-affinity binding with NMDARs and facilitates LTP at CA1 hippocampal synapses (Johnson and Ascher, 1987; Wilcox et al, 1996). These effects are likely mediated via NMDARs, since endogenous glycine has a substantially higher affinity for NMDARs rather than glycine receptors (GlyRs) (Chattipakorn and McMahon, 2002). When excessive glycine is accumulated in the synaptic cleft, it could spill over to extrasynaptic sites, hence activating GlyRs to produce an inhibitory response. Accordingly, high concentrations of a specific inhibitor of glycine transporter type 1 (GlyT1) reduce NMDAR-mediated currents with no increase in LTP magnitude (Martina et al, 2004). In addition,

high levels of glycine induced chemical LTD of EPSCs in CA1 pyramidal neurons (Chen et al, 2011). Overall, these results suggest that the synaptic levels of glycine finely regulate excitability of neurons, dictating the direction of synaptic plasticity.

Opiate drugs alter cognitive performance and affect hippocampal excitability, including LTP and seizure activity. The dentate gyrus (DG) contains both enkephalins and dynorphins, which have opposing effects on excitability and synaptic plasticity (reviewed in Drake et al, 2007). Enkephalins preferentially bind to delta- and mu-opioid receptors to increase excitability and facilitate LTP through the suppression of GABAergic inputs to the granule cell (Xie and Lewis, 1990; Bramham et al, 1991). On the other hand, dynorphins preferentially bind to kappa-opioid receptors to block the induction of LTP (Wagner et al, 1993). Functionally, dynorphins released by high-frequency stimulation act as retrograde transmitters, providing negative feedback to the presynaptic terminal (Wagner et al, 1993). Notably, exogenous opioids are capable of inhibiting hippocampus-dependent learning and memory in rodents (Decker and McGaugh, 1991).

Cannabinoid agonists generally have a disruptive effect on synaptic plasticity and hippocampus-dependent learning and memory (Akirav, 2011). CB1 receptors regulate both inhibitory and excitatory neurotransmitter release in the hippocampus, thus exerting a fine control over synaptic plasticity events. Stimulation of CB1 receptors reduces glutamate release below the level required to activate N-Methyl-d-aspartate (NMDA) receptors, in turn blocking the induction of either LTP or LTD in the hippocampal slice preparation (Shen et al, 1996; Misner and Sullivan, 1999). On the other hand, CB1 blockade favors LTP in the hippocampus (Slanina et al., 2005) and mice lacking CB1 receptors display enhanced LTP (Bohme et al., 2000).

THE SYNAPTIC BASIS OF ALZHEIMER'S DISEASE

Synaptic plasticity has certainly contributed to our understanding of various diseases that affect cognition. This is typified by work on AD. Identification of genetic mutations linked to familial AD led to the generation of several transgenic animal models. Most of these express human amyloid precursor protein (APP) and presenilin (PS1, PS2) mutations. Such models allowed the investigation of the role of age-related factors such as plaque deposition and functional deficits and provided an excellent opportunity to design novel therapies for the treatment and/or prevention of AD. Since loss of memory is one of the major hallmarks of the disorder, the phenotypic characterization of these animals has classically included electrophysiological studies to analyze synaptic transmission and LTP in the hippocampus.

Interestingly, most currently studied models show cognitive deficits and age-related disruption of synaptic markers and amyloid plaque deposition, but few strains show evidence of significant cell death (Janus et al, 2000; Chapman et al, 2001). Most studies have reported, principally, either inhibition of LTP or reduction in baseline fast excitatory transmission (Fitzjohn et al, 2001) prior to plaque deposition. However, several discrepancies emerged so far, potentially due to differences in the models and the experimental conditions used (Marchetti and Marie, 2011). Importantly, however, recent work has shown that the plasticity phenotype can be strongly influenced by the cognitive history of the animal (Middei et al, 2010). Thus, whilst LTP is normal at naive synapses it is severely impaired following training of a spatial task.

Although transgenic animals offered many advantages, it was still not possible to clearly unravel the role of APP *per se* or the different soluble and fibrillar Aβ species. The direct exogenous application of Aβ provided an alternative approach. Generally, LTP was impaired when synthetic Aβ was applied *in vitro* (Lambert et al, 1998) and *in vivo* (Cullen et al, 1997).

Good evidence that disruption of LTP is caused by Aβ oligomers was provided when naturally secreted soluble oligomers of human Aβ were injected intraventricularly into rats (Walsh et al, 2002). These studies contributed to elucidate the cellular and molecular mechanisms of Aβ action.

RESCUE OF Aβ-MEDIATED SYNAPTIC DYSFUNCTION

Understanding precisely how Aβ accumulation

and assembly compromise synaptic structure and function has become the focus of therapeutically oriented research on AD during this decade. In this frame, electrophysiological studies provided a useful tool to test novel therapeutic strategies.

Currently, the amyloid cascade hypothesis is the most important theory of AD postulating that accumulation of Aβ into plaques is the causative pathological event (Hardy and Allsop, 1991). Based on this hypothesis, interventions that reduce Aβ load in the brain would be likely to attenuate both the neuropathological changes and functional deficits characterizing AD. Indeed, several different Aβ-lowering strategies have been developed over the past years.

Among these, Aβ fibrillogenesis represents a major target for the therapeutic intervention in AD and related human β-amyloidoses (Findeis, 2002). Certain small-molecule inhibitors of synthetic Aβ fibrillogenesis (RS-0406 and RS-0466) inhibit formation of cell-derived, secreted oligomers of Aβ, and prevent the impairment of LTP induced by Aβ (Nakagami et al, 2002a,b). Importantly, this protective effect was achieved only under conditions in which they prevented new oligomer formation (Walsh et al, 2005). In fact, in order to be effective, inhibitors of fibrillogenesis need to be used at the initial stages of oligomerization thus avoiding a paradoxical enhanced neurotoxicity which may derive from active pre-fibrillar assemblies such as low-n oligomers released following inhibition of fibril formation. For these reasons, a promising strategy consisted in preventing the formation of Aβ by enhancing α-secretase activity or inhibiting either β-secretase or γ-secretase activity.

The first attempt to test the potential effects of targeting γ-secretase was conducted by Walsh et al (2002). In an intriguing study, these authors showed that the cell-penetrant γ-secretase inhibitor DAPM was able to restore LTP disruption after intracerebroventricular infusion of oligomers of human Aβ in rats. A related study showed that 3 days of oral dosing with the γ-secretase inhibitor MK-560 was sufficient to reverse LTP deficit in 6 month-old Tg2576 mice, at a stage when mice show synaptic dysfunction and behavioral changes before significant plaque deposition (Townsend et al., 2010). This study also highlights the ability of some γ-secretase inhibitors to cross the blood-brain barrier (BBB). Although inhibition of γ-secretase represents a rational pharmacological approach, serious concerns about their toxicity have been raised due to the fact that γ-secretase can cleave several other membrane proteins, the most relevant of which is the Notch protein. The discovery that some NSAIDs behave as γ-secretase modulators, thereby preventing Aβ42 production by binding to AβPP rather than to γ-secretase, suggested a way to avoid Notch toxicity. In line with this, we have recently demonstrated that oral administration of the novel γ-secretase modulator CHF5074 was able to restore synaptic plasticity in 5 month-old Tg2576 mice, and this effect was associated with reduced hyperphosphorylated tau and intraneuronal Aβ (Balducci et al, 2011). Notably, CHF5074 is currently undergoing phase II clinical trial evaluation. Beyond targeting γ-secretase, also the pharmacological inhibition of AβPP cleavage by β-secretase rescued synaptic deficits in a mouse modeling familial Danish dementia (FDD) (Tamayev et al, 2011).

One of the most promising disease-modifying therapies for AD is immunization against Aβ. Intracerebroventricular injection of naturally secreted human Aβ inhibited LTP in rat hippocampus *in vivo* but a monoclonal antibody (6E10) to Aβ completely prevented LTP impairment even when injected after Aβ. Partial protection against the block of LTP was also present in rats that were successfully actively immunized with pre-aggregated Aβ (Klyubin et al, 2008a). A later report showed that also a single intraperitoneal (i.p.) injection of the antibody 6E10 was able to rescue LTP deficit observed in Tg Arc mice (Knobloch et al, 2007). More recently, it was suggested that systemic passive immunization achieved with intracardiac injection of the monoclonal anti-Aβ antibody 4G8 was able to prevent the disruption of synaptic plasticity by Aβ dimer-containing human CSF *in vivo* (Klyubin et al, 2008b). Moreover, also gantenerumab, an investigational anti-Aβ monoclonal antibody currently undergoing phase II and III clinical trials, has proven effective in rescuing LTP deficit in a transgenic model of AD (Bohrmann et al, 2012).

Several studies suggest that reduced levels of brain derived neurotrophic factor (BDNF) and NGF are associated with AD. In particular, a deficit

in BDNF and TrkB receptors has been found in the CA1 region of the hippocampus (Ginsberg et al, 2010) and decreased levels of BDNF have been observed in mouse models of AD (Marchetti and Marie, 2009). Therefore, increasing BDNF levels was proven effective in restoring cognitive function (Nagahara et al, 2009). Also nerve growth factor (NGF) plays a critical role in regulating hippocampal plasticity and learning in rodents (Conner et al, 2009). In fact, a transgenic mouse line expressing chronic NGF deprivation displays age-dependent defects in synaptic plasticity at medial perforant path–dentate gyrus synapses, supporting the "neurotrophic unbalance" hypothesis underlying AD-related neurodegeneration (Houeland et al, 2010). Importantly, NGF application rescued DG-LTP in an AD-like mouse model (La Rosa et al, 2013). These findings suggest that neurotrophins can play a role in hippocampal function and memory also in humans, due to their modulatory role in LTP and synaptic plasticity.

CONCLUSIONS

The identification of soluble oligomers of Aβ as diffusible assemblies that are capable of interfering with synaptic function and integrity provides an important opening for understanding the basis of memory loss in AD. Importantly, similar findings in other disorders might indicate convergent mechanisms of synaptic plasticity failure in several neurodegenerative diseases. Emerging data suggest how different aggregation-prone proteins that characterize neurodegenerative diseases such as AD, Parkinson's disease, Huntington's disease, Down syndrome and prion disorders share common structural features. This might indicate that assemblies produced by different disease-causing proteins might trigger similar downstream mechanisms, raising the possibility of targeting their common structures for therapeutic treatment.

To date, however, very few preclinical findings have resulted in target validation and yet none in successful translation into disease-modifying compounds in humans. The latest clinical trial failure of bapineuzumab confirms growing concerns about how preclinical studies are conducted (see Nisticò et al, 2012). In spite of their limitations, experimental models of AD still remain the most important tool to understand the basic mechanisms underlying AD. Indeed, a more careful design of preclinical studies together with recent advances in imaging technology and biomarker discovery in mice will hopefully provide important contributions to the development of the first approved disease-modifying drug for AD and other neurodegenerative diseases.

REFERENCES

Akirav I (2011) The role of cannabinoids in modulating emotional and non-emotional memory processes in the hippocampus. *Front Behav Neurosci.* **5:**34.

Balducci C, Mehdawy B, Mare L, Giuliani A, et al. and Nisticò R (2011) The γ-secretase modulator CHF5074 restores memory and hippocampal synaptic plasticity in plaque-free Tg2576 mice. *J Alzheimers Dis.* **24:**799-816.

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. *Neuropharmacology* **38:**1083-1152.

Bartus RT, Reginald L, Dean RL, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217:**408-417.

Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. *Nature* **393:**793-797.

Bliss TVP, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361:**31-39.

Bliss TVP, Gardner-Medwin AR (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit following stimulation of the perforant path. *J Physiol (Lond)* **232:**357-374.

Bliss TVP, Lømo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the an-aesthetized rabbit following stimulation of the perforant path. *J Physiol (Lond)* **232:**331-356.

Blokland A (1996) Acetylcholine: a neurotransmitter for learning and memory? *Brain Res Rev* **21:**285-300.

Bohme GA, Laville M, Ledent C, Parmentier M, Imperato A (2000) Enhanced long-term potentiation in mice lacking cannabinoid CB1 receptors. *Neuroscience* **95:**5-7.

Bohrmann B, Baumann K, Benz J, Gerber F, et al. and Loetscher H (2012) Gantenerumab: a novel human anti-Aβ antibody demonstrates sustained cerebral amyloid-β binding and elicits cell-mediated removal of human

amyloid-β. *J Alzheimers Dis.* **28:**49-69.

Bramham CR, Milgram NW, Srebro B (1991) Delta opioid receptor activation is required to induce LTP of synaptic transmission in the lateral perforant path in vivo. *Brain Res.* **567:**42-50.

Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* **40:**361-379.

Buresova O, Bures J, Bohdanecky Z, Weiss T (1964) The effect of atropine on learning, extinction, retention and retrieval in rats. *Psychopharmacology* **5:**255-263.

Chapman PF, Falinska AM, Knevett SG, Ramsay MF (2001) Genes, models and Alzheimer's disease. *Trends Genet.* **17:**254-261.

Chattipakorn SC, McMahon LL (2002) Pharmacological characterization of glycine-gated chloride currents recorded in rat hippocampal slices. *J Neurophysiol* **87:**1515-1525.

Chen RQ, Wang SH, Yao W, Wang JJ, et al. and Lu W (2011) Role of Glycine Receptors in Glycine-Induced LTD in Hippocampal CA1 Pyramidal Neurons. *Neuropsychopharmacology* **36:**1948-1958.

Collingridge GL, Kehl SJ, McLennan H (1983) Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J Physiol* **334:**33-46.

Collingridge GL, Peineau, S, Howland, J, Wang YT (2010) Long-term depression in the CNS. *Nat Rev Neurosci* **11:**459-473.

Conner JM, Franks KM, Titterness AK, Russell K, et al. and Tuszynski MH (2009) NGF is essential for hippocampal plasticity and learning. *J Neurosci* **29:**10883-9.

Cullen WK, Suh YH, Anwyl R, Rowan MJ (1997) Block of LTP in rat hippocampus in vivo by β-amyloid precursor protein fragments. *Neuroreport* **8:**3213-3217.

Davies J, Francis AA, Jones AW, Watkins JC (1981) 2-Amino-5-phosphonovalerate (2AP5), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci Lett* **21:**77-81.

Davies CH, Davies SN, Collingridge GL (1990) Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *J Physiol* **424:**513-531.

Davies CH, Starkey SJ, Pozza MF, Collingridge GL (1991) GABA-B autoreceptors regulate the induction of LTP. *Nature* **349:**609-611.

Decker MW, McGaugh JL (1991) The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory. *Synapse* **7:**151-68.

Drake CT, Chavkin C, Milner TA (2007) Opioid systems in the dentate gyrus. *Prog Brain Res.* **163:**245-63.

Findeis MA (2002) Peptide inhibitors of beta amyloid aggregation. *Curr Top Med Chem* **2:**417-23.

Finn RC, Browning M, Lynch G (1980) Trifluoperazine inhibits hippocampal long-term potentiation and the phosphorylation of a 40,000 dalton protein. *Neurosci Lett* **19:**103-108.

Fisahn A, Pike FG, Buhl EH, Paulsen O (1998) Cholinergic induction of network oscillations at 40 Hz in the hippocampus in vitro. *Nature* **394:**186-189.

Fisahn A, Yamada M, Duttaroy A, Gan JW, et al. and Wess J (2002) Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. *Neuron* **33:**615-624.

Fitzjohn SM, Morton RA, Kuenzi F, Rosahl TW, et al. and Seabrook GR (2001) Age-related impairment of synaptic transmission but normal long-term potentiation in transgenic mice that overexpress the human APP695SWE mutant form of amyloid precursor protein. *J Neurosci* **21:**4691-4698.

Frey S, Bergado-Rosado J, Seidenbecher T, Pape HC, Frey JU (2001) *J Neurosci* **21:**3697-703.

Frey U, Schroeder H, Matthies H (1990) Dopaminergic antagonists prevent long-term maintenance of posttetanic LTP in the CA1 region of rat hippocampal slices. *Brain Res* **522:**69-75.

Fujii S, Ji Z, Morita N, Sumikawa K (1999) Acute and chronic nicotine exposure differentially facilitate the induction of LTP. *Brain Res* **846:**137-43.

Fujii S, Sumikawa K (2001) Acute and chronic nicotine exposure reverse age-related declines in the induction of long-term potentiation in the rat hippocampus. *Brain Res* **894:**347-53.

Ge S, Dani JA (2005) Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation. *J Neurosci* **25:**6084-91.

Ginsberg SD, Alldred MJ, Counts SE, Cataldo AM, et al. and Che S (2010) Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer's disease progression. Biol *Psychiatry* **68:**885-93.

Hamid S, Dawe GS, Gray JA, Stephenson JD (1997) Nicotine induces long-lasting potentiation in the dentate gyrus of nicotine-primed rats. *Neurosci Res* **29:**81-5.

Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease.

Trends Pharmacol Sci **12:**383-8.

Harris EW, Cotman CW (1986) Long-term potentiation of guinea pig mossy fiber responses in not blocked by N-methyl-D-aspartate antagonists. *Neurosci Lett* **70:**132-137.

Houeland G, Romani A, Marchetti C, Amato G, et al. and Marie H (2010) Transgenic mice with chronic NGF deprivation and Alzheimer's disease-like pathology display hippocampal region-specific impairments in short- and long-term plasticities. *J Neurosci* **30:**13089-94.

Huerta PT, Lisman JE (1993) Heightened synaptic plasticity of hippocampal CA1 neurons during a cholinergically induced rhythmic state. *Nature* **364:**723-725.

Izumi Y, Zorumski CF (1999) Norepinephrine promotes long-term potentiation in the adult rat hippocampus in vitro. *Synapse* **31:**196-202.

Janus C, Pearson J, McLaurin J, Mathews PM, et al. and Westaway D (2000) A beta peptide immunization reduces behavioral impairment and plaques in a model of Alzheimer's disease. *Nature* **408:**979-982.

Ji D, Lape R, Dani JA (2001) Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. *Neuron* **31:**131-41.

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* **325:**529-531.

Katsuki H, Izumi Y, Zorumski CF (1997) Noradrenergic regulation of synaptic plasticity in the hippocampal CA1 region. *J Neurophysiol* **77:**3013-20.

Klyubin I, Betts V, Welzel AT, Blennow K, et al. and Rowan MJ (2008b) Amyloid beta protein dimer-containing human CSF disrupts synaptic plasticity: prevention by systemic passive immunization. *J Neurosci* **28:**4231-7.

Klyubin I, Walsh DM, Lemere CA, Cullen WK, et al. and Rowan MJ (2008a) Amyloid beta protein immunotherapy neutralizes Abeta oligomers that disrupt synaptic plasticity in vivo. *Nat Med* **11:**556-61.

Knobloch M, Farinelli M, Konietzko U, Nitsch RM, Mansuy IM (2007) Abeta oligomer-mediated long-term potentiation impairment involves protein phosphatase 1-dependent mechanisms. *J Neurosci* **27:**7648-53.

Kojima T, Matsumoto M, Togashi H, Tachibana K, Kemmotsu O, Yoshioka M (2003) Fluvoxamine suppresses the long-term potentiation in the hippocampal CA1 field of anesthetized rats: an effect mediated via 5-HT1A receptors. *Brain Res* **959:**165-168.

La Rosa LR, Matrone C, Ferraina C, Panico MB, et al. and Nisticò R (2013) Age-related changes of hippocampal synaptic plasticity in AβPP-null mice are restored by NGF through p75NTR. *J Alzheimers Dis* **33:**265-72.

Lambert MP, Barlow AK, Chromy BA, Edwards C, and Klein WL (1998) Diffusible, nonfibrillar ligands derived from A beta(1–42) are potent central nervous system neurotoxins. *Proc Natl Acad Sci USA* **95:**6448-6453.

Lee HK, Takamiya K, Han JS, Man H, et al. and Huganir RL (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112:**631-643.

Lindvall O, Björklund A (1978) Anatomy of the dopaminergic neuron systems in the rat brain. *Adv Biochem Psychopharmacol* **19:**1-23.

Lømo T (1966) Frequency potentiation of excitatory synaptic activity in the dentate area of the hippocampal formation. *Acta Physiol Scand* **68:**128.

Lovinger DM, Wong KL, Murakami K, Routtenberg A (1987) Protein kinase C inhibitors eliminate hippocampal long-term potentiation. *Brain Res* **436:**177-183.

Lynch G, Larson J, Kelso S, Barrionuevo G, Schottler F (1983) Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature* **305:**719-721.

Malenka RC, Nicoll RA (1999) Long-term potentiation—a decade of progress? *Science* **285:**1870-1874.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annu. Rev. Neurosci.* **25:**103-126.

Malinow R, Schulman H, Tsien RW (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245:**862-866.

Marchetti C, Marie H (2011) Hippocampal synaptic plasticity in Alzheimer's disease: what have we learned so far from transgenic models? *Rev Neurosci.* **22:**373-402.

Martina M, Gorfinkel Y, Halman S, Lowe JA, et al. and Bergeron R (2004) Glycine transporter type 1 blockade changes NMDA receptor-mediated responses and LTP in hippocampal CA1 pyramidal cells by altering extracellular glycine levels. *J Physiol* **557:**489-500.

Matsuyama S, Matsumoto A, Enomoto T, Nishizaki T (2000) Activation of nicotinic acetylcholine receptors induces long-term potentiation in vivo in the intact mouse dentate gyrus. *Eur J Neurosci* **12:**3741-7.

Middei S, Roberto A, Berretta N, Panico MB, et al. and Nistico R (2010) Learning discloses abnormal structural and functional plasticity at hippocampal synapses in the APP23 mouse model of Alzheimer's disease. *Learn Mem* **17:**236-240.

Misner DL, Sullivan JM (1999) Mechanism of cannabinoid effects on long-term potentiation and

depression in hippocampal CA1 neurons. *J Neurosci* **19:**6795-6805.

Morris R, Anderson E, Lynch GS, Baudry M (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-d-aspartate receptor antagonist, AP5. *Nature* **319:**774-776.

Nagahara AH, Merrill DA, Coppola G, Tsukada S, et al. and Tuszynski MH (2009) Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med* **15:**331-7.

Nakagami Y, Nishimura S, Murasugi T, Kaneko I, et al. and Oda T (2002b) A novel beta-sheet breaker, RS-0406, reverses amyloid beta-induced cytotoxicity and impairment of long-term potentiation in vitro. *Br J Pharmacol* **137:**676-82.

Nakagami Y, Nishimura S, Murasugi T, Kubo T, et al. and Oda T (2002a) A novel compound RS-0466 reverses beta-amyloid-induced cytotoxicity through the Akt signaling pathway in vitro. *Eur J Pharmacol* **457:**11-7.

Nisticò R, Pignatelli M, Piccinin S, Mercuri NB, Collingridge G (2012) Targeting synaptic dysfunction in Alzheimer's disease therapy. *Mol Neurobiol.* **46:**572-87.

Normann C, Peckys D, Schulze CH, Walden J, Jonas P, Bischofberger J (2000) Associative long-term depression in the hippocampus is dependent on postsynaptic N-type Ca2+ channels. *J Neurosci* **20:**8290-8297.

Obenaus A, Mody I, Baimbridge KG (1989) Dantrolene-Na (dantrium) blocks induction of long-term potentiation in hippocampal slices. *Neurosci Lett* **98:**172-178.

Ohashi S, Matsumoto M, Togashi H, Ueno K, Yoshioka M (2003) The serotonergic modulation of synaptic plasticity in the rat hippocampo-medial prefrontal cortex pathway. *Neurosci Lett* **342:**179-182.

Pussinen R, Sirviö J (1998) Minor role for alpha1-adrenoceptors in the facilitation of induction and early maintenance of long-term potentiation in the CA1 field of the hippocampus. *J Neurosci Res* **51:**309-15.

Shen M, Piser TM, Seybold VS, Thayer SA (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J Neurosci* **16:**4322-4334.

Shimoshige Y, Maeda T, Kaneko S, Akaike A, Satoh M (1997) Involvement of M2 receptor in an enhancement of long-term potentiation by carbachol in Schaffer collateral-CA1 synapses of hippocampal slices. *Neurosci Res* **27:**175-180.

Shinoe T, Matsui M, Taketo MM, Manabe T (2005) Modulation of synaptic plasticity by physiological activation of M1 muscarinic acetylcholine receptors in the mouse hippocampus. *J Neurosci* **25:**11194-200.

Slanina KA, Roberto M, Schweitzer P (2005) Endocannabinoids restrict hippocampal long term potentiation via CB1. *Neuropharmacology* **49:**660-668.

Staubli U, Xu FB (1995) Effects of 5-HT3 receptor antagonism on hippocampal theta rhythm, memory, and LTP induction in the freely moving rat. *J Neurosci* **15:**2445-52.

Tamayev R, Matsuda S, Arancio O, D'Adamio L (2012) β- but not γ-secretase proteolysis of APP causes synaptic and memory deficits in a mouse model of dementia. *EMBO Mol Med.* **4:**171-9.

Townsend M, Qu Y, Gray A, Wu Z, et al. and Middleton RE (2010) Oral treatment with a gamma-secretase inhibitor improves long-term potentiation in a mouse model of Alzheimer's disease. *J Pharmacol Exp Ther* **333:**110-9.

Wagner JJ, Terman GW, Chavkin C (1993) Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus. *Nature* **3:**363:451-4.

Walling SG, Harley CW (2004) Locus ceruleus activation initiates delayed synaptic potentiation of perforant path input to the dentate gyrus in awake rats: a novel beta-adrenergic- and protein synthesis-dependent mammalian plasticity mechanism. *J Neurosci* **24:**598-604.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, et al. and Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416:**535-539.

Walsh DM, Klyubin I, Shankar GM, Townsend M, et al. and Selkoe DJ (2005) The role of cell-derived oligomers of Abeta in Alzheimer's disease and avenues for therapeutic intervention. *Biochem Soc Trans* **33:**1087-90.

Wang RY, Arvanov VL (1998) M100907, a highly selective 5-HT2A receptor antagonist and a potential atypical antipsychotic drug, facilitates induction of long-term potentiation in area CA1 of the rat hippocampal slice. *Brain Res* **779:**309-313.

Wilcox KS, Fitzsimonds RM, Johnson B, Dichter MA (1996) Glycine regulation of synaptic NMDA receptors in hippocampal neurons. *J Neurophysiol* **76:**3415-3424.

Williams S, Johnston D (1988) Muscarinic depression of long-term potentiation in CA3 hippocampal neurons. *Science* **242:**84-87.

Xie CW, Lewis DV (1991) Opioid-mediated facilitation of long-term potentiation at the lateral perforant path-dentate granule cell synapse. *J Pharmacol Exp Ther.* **256:**289-96.

BIOCHEMICAL AND IMAGING MARKERS FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE: AN OVERVIEW

S. LISTA[1], F. GARACI[2,3], N. TOSCHI[4], and H. HAMPEL[5]

[1]Department of Psychosomatic Medicine, University Medicine Rostock, Rostock, Germany.
[2]Department of Diagnostic Imaging, Molecular Imaging, Interventional Radiology and Radiotherapy, University of Rome "Tor Vergata", Rome, Italy.
[3]IRCCS San Raffaele Pisana, Rome, Italy.
[4]Medical Physics Section, Faculty of Medicine, University of Rome "Tor Vergata", Rome, Italy.
[5]Department of Psychiatry, Goethe-University, Frankfurt am Main, Germany.

The major neuropathological hallmarks of Alzheimer's disease (AD), the most common form of neurodegenerative disorder, are the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles. Currently, most biomarker research in AD is focused on either brain imaging or is fluid-based. In the area of fluid biomarker research, cerebrospinal fluid (CSF) has been demonstrated to be an excellent source for biomarkers. Indeed, given that CSF is in the adjacent vicinity of the brain, biochemical alterations in the cerebral tissue affect its biomarker composition. Valid CSF biochemical markers are accessible for amyloid pathology ($A\beta_{1-42}$), neurofibrillary pathology (hyperphosphorylated protein tau, p-tau), and cortical axonal degeneration/damage (total tau, t-tau). The diagnostic sensitivity and specificity of the established "core" CSF biomarkers in discriminating AD from cognitively healthy individuals and from other varieties of dementing pathologies has been satisfactorily accomplished. Notably, a combination of more than one "core" biochemical marker in the CSF is believed to provide higher diagnostic accuracy of AD. Over the last decade, besides CSF biomarkers, there has been a substantial increase of studies concerning neuroimaging techniques for AD. Many of these methods are already employed in routine clinical practice. Magnetic resonance imaging (MRI) is a tool for clinical assessment of dementia patients and offers an important aid to the clinical diagnosis and subtyping of dementia in earlier stages. The main characterized structural MRI biomarker in AD is represented by cortical diffuse atrophy. Diffusion tensor imaging (DTI), investigating white matter microarchitecture and integrity, has been employed in several AD studies. Furthermore, more advanced protocols have been fruitfully utilized in AD. The introduction of machine learning algorithms has made practicable to manage images from different modalities simultaneously and to classify a single subject into a predefined group. These techniques have been identified as promising tools in neuroimaging data analysis. In the future, machine learning algorithms are expected to be integrated into scanner softwares along with all the pre-processing steps necessary to assist the radiological expert in the semi-automated detection of prodromal AD stages based on pattern recognition strategies.

Key words: Alzheimer's disease; mild cognitive impairment; cerebrospinal fluid; amyloid beta peptides; total tau; hyperphosphorylated protein tau; CSF biomarkers; magnetic resonance imaging; cortical diffuse atrophy; voxel based morphometry; diffusion tensor imaging; white matter integrity; machine learning algorithms; support vector machines.

Corresponding Author:
Simone Lista, PhD
Department of Psychosomatic Medicine
University Medicine Rostock
Gehlsheimer Straße 20, 18147 – Rostock Germany
Phone: +49-15251904006
Email: slista@libero.it (S. Lista)

2279-5855 (2013)

Basic and clinical research progresses over the past decades provided a growing familiarity with molecular/cellular mechanisms as well as clinical development of Alzheimer's disease (AD), the most common neurodegenerative disorder in the elderly. Current estimates have clearly demonstrated that there are around 5.3 million people with AD in the USA (Wimo and Prince, 2010). In light of this – regardless of fluctuations on estimates of the burden of AD in Europe, North America, Japan, and elsewhere in the world – the number of people with AD is expected to rise drastically as the global population ages, unless ways for disease prevention or treatment are swiftly introduced (Trojanowski and Hampel, 2011). AD recently displaced diabetes as the 6th leading cause of death in the USA. By 2030, as many as 7.7 million individuals may be affected by AD in the USA only; this number could grow to about 11-16 million people within the year 2050. It is presently estimated that AD costs in the USA exceed US $150 billion annually, and this devastating pathological condition will strike the economies of other populations, including developing countries, to a similar extent (Wimo and Prince, 2010).

THE PATHOGENESIS OF ALZHEIMER´S DISEASE

From the time AD was first described more than a century ago and through to present times, a definite diagnosis of AD has needed neuropathological examination based on the visualization of pathologic alterations of senile plaques (SPs) and neuronal neurofibrillary tangles (NFTs). In this connection, there have been significant progresses in the elucidation of the neuropathological basis of the disease. Most studies indicate that AD lesions accumulate progressively, starting with the extensive accumulation of SPs followed by NFTs (Braak and Braak, 1991; Jack et al, 2010); however, the exact sequence is still questioned (Braak and Del Tredici, 2011).

Extracellular amyloid beta (Aβ) deposits of SPs and intraneuronal NFTs have been largely recognized as the two histological hallmarks of AD. SPs consist of fibrillar amyloid material that contains Aβ peptide. Aβ is synthetized by the anomalous proteolytic cleavage of amyloid precursor protein (APP), a membrane-bound protein that is physiologically cleaved by α-secretase to produce non-amyloidogenic fragments. In AD, the cleavage of Aβ by β- and γ-secretases leads to the release of 37-43 amino acid long Aβ peptides (Haass and Selkoe, 1993). The predominant forms of the peptides are insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ fibrils. The former is the most abundant whereas the latter, highly hydrophobic, forms oligomers and fibrils accumulating in extracellular plaques (Andreasen and Zetterberg, 2008). Plaques have been frequently reported to be "sticky" with other molecules that are collected within the plaques including Apolipoprotein E (ApoE), interleukins, and proteins of the complement system (Thal et al, 2006).

The structural unit of NFTs is represented by the tau protein. This cytoplasmic microtubule-associated protein (MAP), synthesized in all neurons and found in the glia as well, is implicated in the assembly and stability of microtubules in the nerve cells and in axoplasmatic transport. The microtubule connection is modulated by a complex interaction between tau expression and phosphorylation (Perl et al, 2010). Under physiological conditions, both phosphorylated and dephosphorylated forms of tau are in equilibrium. In the AD brain, tau is hyperphosphorylated (phospho tau, p-tau) at several amino acid residues. As a result of this aberrant phosphorylation, p-tau separates from axonal microtubules and aggregates into paired helical filaments (PHFs) in the neuronal perikarya in order to generate NFTs, and in the neuronal dendrites to produce neuropil threads (Ballatore et al, 2007; Braak and Braak, 1988). Such pathological mechanisms lead to disrupt the axonal transport and intracellular organelles activities, such as mitochondria (Reddy, 2011).

Among all various tau post-translational modifications suggested to promote tau aggregation, tau phosphorylation (including 85 putative phosphorylation sites especially at the level of serine (Ser) and threonine (Thr) amino acids) is the most prominent. In light of this, disturbance of the equilibrium between tau kinase and phosphatase activities has been proposed to be at the basis of anomalous tau phosphorylation and aggregation (Martin et al, 2013a,b). A number of protein kinases have been associated with the genesis of p-tau, namely glycogen synthase kinase-3β (GSK3β),

cyclin-dependent protein kinase-5 (CDK5), and mitogen activated protein kinases (MAPK) such as p38, Erk1/2 and JNK1/2/3). Tau phosphatases belong to phosphoprotein phosphatase PPP group (PP1, PP2A, PP2B and PP5) and protein tyrosine phosphatase (PTP) group. In AD brains, total phosphatase activity is diminished by half (Liu et al, 2005) with PP2A, PP1 and PP5 activities reduced by 50%, 20%, and 20%, respectively, hence indicating that distinct tau phosphatases account for AD process (Gong et al, 1993; 1995).

In aged brains and in those presenting AD pathology, NFTs have been described in a distinctive regional depiction. In this regard, a peculiar distribution and progression of NFTs in the brains of elderly – that encompasses six stages, beginning in the transentorhinal and entorhinal layers and progressing advancing to the neocortex – has been reported (Braak and Braak, 1991).

Mutations in *APP* or in the presenilin genes (*PSEN1* or *PSEN2*) encoding proteins that participate to APP metabolism have been demonstrated to induce familial forms of AD (Blennow et al, 2006). Based on these mutations, Aβ – specifically the $A\beta_{1-42}$ peptide – has been assumed to be the key constituent in the disease mechanism. Actually, the *"amyloid cascade hypothesis"* (Hardy and Selkoe, 2002) speculates that an imbalance between the production and clearance of Aβ is the opening event in AD, with the increase in Aβ load finally accounting for tau pathology, neuronal degeneration, and dementia.

Besides SPs and NFTs, there are other typical neuropathological and neurochemical features of AD. These consist of selective death of neurons, loss of synapses, and reductions in markers for some neurotransmitters. Nerve cells primarily susceptible in AD comprise those in layer II of the entorhinal cortex, the pyramidal layers of the hippocampus, and some regions of the temporal, parietal, and frontal cortices. Although the majority of neurons exposed to AD utilize glutamate as neurotransmitter, there is also depletion and/or dysfunction of specific subcortical neurons, for instance: noradrenergic cells in the *locus coeruleus* and central cholinergic neurons in the basal forebrain (BF) (Holtzman et al, 2011). It is well-known that cholinergic cells release acetylcholine (ACh), which can bind two categories of receptors: the nicotinic ACh (nAChRs) and the muscarinic ACh receptors (mAChRs). Numerous lines of evidence relate nicotine neurotransmission to AD pathogenesis (Pakaski and Kalman, 2008). Besides cell loss (Whitehouse et al, 1981; 1982), the degenerating BF exhibits neuronal shrinkage (Vogels et al, 1990) and depletion of cortical cholinergic markers, including a substantial decrease of ACh levels, choline acetyltransferase (ChAT) and acetylcholine esterase (AChE) enzymatic activities, in AD (Perry et al, 1978; Gil-Bea et al, 2005). Intriguingly, since both the impact of APP and Aβ toxicity on the viability of cholinergic neurons and the effects of the modulation of cholinergic receptors or enzymes on APP metabolism/Aβ production have been constantly reported (see reviews by Pakaski and Kalman, 2008 and by Schliebs and Arendt, 2006), the link between Aβ and the components of the cholinergic system is expected to be bidirectional.

THE CLINICAL DIAGNOSIS OF ALZHEIMER´S DISEASE

AD clinical diagnostic criteria have been reported almost 30 years ago by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al, 1984). As stated by these criteria, AD diagnosis is based completely on the assessment of clinical signs. In addition, both the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) and the International Classification of Diseases 10 (ICD-10) criteria for AD also need that a patient has dementia before a diagnosis of AD can be implemented. These recommendations designated a neuropathologically advanced disease, i.e. the AD cases were disclosed late during the disease process. Although the study of dementia has more than 100-year history, systematic efforts to address scientific and clinical challenges to differential diagnosis of AD syndrome are quite recent (Khachaturian, 2006). Subsequently, a number of complications had to be overcome during the period between the 1984 NINCDS-ADRDA criteria and 2011 (Khachaturian, 2011). Moreover, substantial efforts have been made to summarize recommendations for the clinical stage preceding dementia. In this connection, the introduction of the paradigm of mild cognitive

impairment (MCI) (Petersen et al, 1999) as a potential precursor or "prodrome" of the disease has represented a major landmark (Khachaturian, 2006).

Unlike previous recommendations, the recently established new diagnostic criteria for AD – both the International Working Group for New Research Criteria for the Diagnosis of AD (Dubois et al, 2007; 2010) and the National Institute on Aging and Alzheimer's Association initiative (NIA-AA) (McKhann et al, 2011; Albert et al, 2011; Sperling et al, 2011) – have pointed out the integration of the use of biomarkers as crucial supportive criteria for preclinical stages of AD (Sperling et al, 2011).

"CORE" NEUROCHEMICAL BIOMARKERS IN ALZHEIMER´S DISEASE

The present evolving status of multimodal core biomarker discovery, development, and validation integrating neurobiochemical/neurogenetic as well as structural/functional/metabolic neuroimaging studies in AD has been comprehensively scrutinized providing the converging viewpoints of industry stakeholders and regulatory bodies (Hampel et al, 2012; Weiner et al, 2012; Hampel et al, 2010a; Blennow et al, 2010; Hampel et al, 2008; Teipel et al, 2013; Hampel and Lista, 2012; Ewers et al, 2011).

Basically, the most relevant markers under inspection and their correlation with AD pathological mechanisms can be categorized as follows: (I) core markers that mirror crucial neuropathological processes in AD such as deregulated metabolism of Aβ and APP; (II) downstream markers that reflect secondary changes to brain structure and function, i.e. volumetric and metabolic alterations to temporo-medial structures (Forlenza et al, 2010).

Cerebrospinal fluid (CSF) is presently considered the best source for biomarker discovery and development as it is in direct contact with the extracellular space of the brain and can reproduce biochemical modifications occurring within the brain. Apprehension due to lumbar puncture-associated side-effects should not impede CSF examination, since complications are very uncommon in the elderly, on condition that systematic well-known precautions to any qualified physician are taken (Blennow et al, 1993; Peskind et al, 2005).

A lot of examinations have been performed in order to validate CSF levels of $A\beta_{1-42}$, p-tau, and total tau (t-tau, i.e. all isoforms of tau regardless of phosphorylation state) as biomarkers reflecting key features of the fundamental AD processes.

- Low CSF concentrations of $\mathbf{A\beta_{1-42}}$ are indicators of the aggregation of Aβ into SPs and, consequently, the peptide retention in the brain parenchyma (Strozyk et al, 2003; Fagan et al, 2006; Forsberg et al, 2008; Grimmer et al, 2009). This biochemical modification is assumed to be the initial step during the advancement of AD (Fagan et al, 2009). The most abundant CSF Aβ isoform is Aβ1-40. Unlike $A\beta_{1-42}$, there are no major changes in $A\beta_{1-40}$ CSF levels in AD. Notably, there is a marked decline in the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio in AD and MCI that seems to be more evident than the reduction observed in Aβ1-42 alone (Hansson et al, 2007). This ratio might be more important to the AD neurobiology compared to the absolute concentration of $A\beta_{1-42}$ (Hampel et al, 2010b).

- High CSF amounts of **p-tau** are the most accurate parameter indicating an enduring AD process and reflect the formation of NFTs within the brain (Hampel et al, 2010c). Actually, measurements of p-tau in CSF specimens, collected both during life and at autopsy, disclosed associations for p-tau phosphorylated at Thr181 ($p\text{-}tau_{181}$) or Thr231 ($p\text{-}tau_{231}$) with neocortical NFT pathology and with the rate of hippocampal atrophy in the brain (Buerger et al, 2006; Hampel et al, 2005).

- Pathophysiologic mechanisms damaging axons in the cortex and disrupting their function lead to growing CSF levels of **t-tau**. This is an established biomarker of the intensity of neuronal/axonal injury/degeneration regardless of pathognomonic aetiology (Samgard et al, 2010).

Overall, these established "core" biomarkers are able to detect and predict AD pathophysiology in prodromal MCI with a sensitivity and specificity of 75% to 95% (Blennow et al, 2010). The combined use of t-tau, p-tau, and $A\beta_{1-42}$ demonstrates an enhanced predictive value to detect subjects of prodromal AD in MCI subjects (Mattsson et al, 2009). The elevated diagnostic performance of these "gold standard" CSF biomarkers has been further reasserted in large multinational multi-center validation studies (1) such as the Worldwide Alzheimer's Disease Neuroimaging Initiative (WW-ADNI) (Weiner et al,

2012), the DESCRIPA (Development of screening guidelines and criteria for predementia Alzheimer's disease) study (Visser et al, 2009), and the Swedish Brain Power (SBP) initiative (Blennow et al, 2010).

Biomarker studies have stressed a reappraisal of the time course of AD (Cummings, 2012). Numerous studies of conducted in the CSF demonstrate that Aβ concentration start decreasing as well as t-tau and p-tau levels start growing many years prior to the onset of AD dementia (Buchhave et al, 2012). Likewise, amyloid imaging studies prove that amyloid is deposited in the brain a number of years previous to the onset of dementia in AD (Aizenstein et al, 2008). Brain atrophy detected by magnetic resonance imaging (MRI), hypometabolism on fluorodeoxyglucose (FDG) positron emission tomography (PET), and hypoperfusion on single-photon emission computed tomography (SPECT) display aberrations before the onset of dementia and parallel or are in anticipation of the beginning of cognitive impairment (Westman et al, 2012; Ossenkoppele et al, 2012; Alegret et al, 2012).

In summary, valid CSF biomarkers are accessible for amyloid pathology ($A\beta_{1-42}$), neurofibrillary pathology (p-tau), and cortical axonal degeneration/ damage (t-tau). However, the biochemical data should be interpreted with caution in the framework of the whole clinical picture that should also incorporate results from neuroimaging examinations as well as neuropsychological testings (Zetterberg et al, 2010).

NEUROIMAGING MARKERS

Over the last decade there has been a considerable increase of studies regarding neuroimaging techniques for AD. Many of these techniques are already used in routine clinical practice. In particular structural MRI is an important tool for clinical assessment of dementia patients providing a valuable aid to the clinical diagnosis and subtyping of dementia in earlier stages. In fact, in the 2011 revision of the NINCDS-ADRDA criteria structural and functional biomarkers are included to provide evidence of AD (McKhann et al, 2011).

Shrinkage and atrophy of the brain is one of the pathologic hallmarks of AD, based on the neuron death and assonal loss. The role of structural MRI consists in the morphometrical and volumetric evaluation of the brain structures in order to provide biomarkers that may not only exclude disease but also help in early diagnosis. Several studies have demonstrated the reliability and reproducibility of these MRI measurements in repeated examinations with minimal interscan variability (Byrum et al, 1996; Giedd et al, 1995) once minimal criteria for scanner quality are met.

Mainly there are two methods to assess cerebral atrophy: a qualitative method which consists of visual assessment for the presence and severity of cerebral atrophy, and a quantitative method which measures the volume of brain structures. Methods of measuring brain regions from MR images include both manual and semi-automated methods.

The main structural MRI biomarker in AD is cortical diffuse atrophy which is caused by neuronal degeneration involving in particular the temporal and parietal lobes with the most severity in the hippocampus. The reported sensitivity and specificity of hippocampal measurements in the diagnosis of AD have been reported to be 85% and 88% respectively (Laakso et al, 1998) Furthermore it has been reported that MRI volumetric measures demonstrate significant atrophy of the hippocampal formation even during prodromal stages of AD and predict later conversion to AD with about 80% accuracy (Jack et al, 1999).

Bobinski and colleagues (2000) showed that very accurate volumetric measurements of the hippocampus could be obtained by using MRI, demonstrating positive correlations between the MRI and the histological volume. Furthermore, the same authors of this study pointed out a very strong correlation between the MRI volume and the calculated total numbers of neurons in the hippocampus, confirming the relationship between volume reductions and neuronal loss (Bobinski et al, 2000).

Different approaches have been suggested for the evaluation of atrophy. The most used and validated method is the visual score of Global Cortical Atrophy (GCA-scale) and Medial Temporal lobe Atrophy (MTA-Scale). GCA is the mean score for cortical atrophy of the whole cerebrum, but because atrophy is common to different kind of dementias this method may be aspecific for AD. This scale ranges ranges from 0 to 3, with 0 representing no

cortical atrophy, 1 mild atrophy (widening of sulci), 2 moderate atrophy with loss of gyri and 3 severe atrophy.

Several techniques are available for the assessment of MTA: volumetric assessment, linear assessment and visual qualitative rating. The latter is the most clinically accessible and therefore most used in clinical routine. This method is based on a visual rating scale, developed by Scheltens and colleagues, evaluating the width of the choroid fissure, the temporal horn and the height of the hippocampus assessed through a specific coronal image passing through the corpus of the hippocampus at the level of the anterior pons (Scheltens et al, 1995). This scale ranges from 0 to 4 with 0 representing no atrophy, 1 the widening of only the choroid fissure, 2 the widening of the temporal horn and reduction of the hippocampal height as well, 3 moderate-severe atrophy with an increased widening of the choroid fissure and temporal horn and further reduction of the hippocampal height compared to grade 2, grade 4 represents end-stage atrophy with severe volume loss of hippocampus. Scores 0 and 1 are normal for individuals younger than 75 years and, while 2 is an acceptable score for individuals of age beyond this threshold, 3 and 4 are always abnormal values.

MTA, just as GCA, needs to be rated in both hemispheres separately in order to assess the eventual presence and degree of asymmetry which is usually not found in AD patients but may affect individuals with an early stage disease. Shi and colleagues performed a meta-analysis of MRI studies evaluating hippocampal volume and asymmetry in MCI and AD, and evaluated hippocampal asymmetry. To compare the asymmetry of the left and right hippocampi, meta-analyses were performed in controls from 29 studies for a total of 915 subjects, 365 individuals affected by MCI from 14 selected studies, and 700 AD patients from 23. The analysis pointed out a left-less-than-right asymmetry in all three groups but with different extent but least in AD patients with a decreased prevalence with disease progression (Shi et al, 2009).

In AD the most important structural imaging feature is progression of atrophy which is estimated to be 2.5 times greater in normal subjects with a strong relationship between memory loss and hippocampal damage, making serial evaluations mandatory. For this purpose visual manual volumetric assessment of regional brain atrophy is obtained using multiple Regions of Interests (ROIs) on a series of adjacent MRI images. Because of the complexity of the evaluated regions (mainly the hippocampus) and the uncertain relationships between cytoarchitectonic areas and anatomical landmarks which makes this kind of assessment subject to interindividual variability and therefore poorly reproducible, EADC (European Alzheimer's Disease Consortium) and ADNI harmonize the available protocols for the manual tracing of the hippocampus in order to create a standard protocol. Furthermore, it is understandable how this approach is laborious and time consuming, and for these reasons the ROIs studied in a single investigation is generally limited making this method not well suited for a comprehensive assessment of the whole brain.

For these reasons automated methods would be useful in clinical practice. The most widely published is voxel-based morphometry (VBM) which allows the assessment of focal differences in brain anatomy. VBM is based segmentation, spatial normalization and smoothing of MRI images in order to evaluate local gray matter concentration and perform a comparison between groups of subjects. This can be obtained through different workflows and performed using different algorithms.

Several cross-sectional studies have employed VBM to compare MCI subjects to healthy controls, reporting in the first group prevalent atrophy of the medial temporal lobe, temporal neocortex, thalamus and the cingulate gyrus (Chetelat et al, 2002; Pennanen et al, 2005) whereas another study demonstrated that aging affects the gray matter density in the prefrontal, medial temporal and striatal cortices (Tisserand et al, 2004). In this latter study, Tisserand and colleagues also reported that longitudinal cognitive decline is related to posterior parietal, medial temporal and prefrontal cortices (Tisserand et al, 2004). VBM has also been used in longitudinal studies to investigate the evolution of atrophy between MCI non-converter patients and MCI converters (Chetelat et al, 2002; Bozzali et al, 2006). The results of these studies showed that in MCI converters atrophy is prevalent both in frontoparietal and MTL areas as well as in the posterior medial parietal cortices. The same data were later confirmed by Hämäläinen and colleagues

that demonstrated the presence of widespread cortical atrophy already two and a half years before a clinical diagnosis of dementia could be set and showed the utility of neuroimaging combined with neuropsychological testing in potentially predicting the development of dementia

Another MRI technique that has been proposed in AD is Magnetization Transfer Imaging which demonstrated a correlation between defined demyelination and axon loss and the Magnetization Transfer Ratio (MTR) as reported by Dousset and colleagues (1998) suggesting that this technique can provide information on brain structure on a microscopic scale. A recent longitudinal study assessed MTR in 28 mild to moderate AD patients at 6 and 12 months, and 19 healthy individuals. In this study, reported a significant reduction in all global MTR metrics in subjects affected by AD in comparison to healthy controls was reported (Ropele et al, 2012). The biggest difference was recorded in the hippocampal region where MTR values were significantly lower than in the control group while no significant differences were seen in the putamen, caudate nucleus, and thalamus. MRI is also used to assess other biomarkers such as the presence of microbleeds which are seen at high frequency in patients with AD and are considered to be a manifestation of cerebral amyloid angiopathy, one of the hypothesize pathological mechanism behind AD. For this reason when present in a lobar subcortical distribution, they represent a helpful finding in the differential diagnosis of dementia. Structural MRI may also detect white matter lesions (using the Fazekas scale) which may suggest a small vessel disease.

Advanced Imaging and Processing Techniques

Diffusion Tensor Imaging (DTI) (Basser and Jones 2002) is a leading technique in investigating white matter microarchitecture and integrity, and has been employed in numerous studies investigating AD and MCI (Bozzali and Cherubini, 2007; Chua et al, 2008; Hess, 2009). Usually one observes a decrease in Fractional Anisotropy (FA), commonly accompanied by an increase in mean diffusivity (MD) in AD and MCI (Liu et al, 2011; Medina et al, 2006; O'Dwyer et al, 2011), which points to unspecific bundle degeneration, and correlations between WM integrity and disease severity have also been reported in AD (Fjell et al, 2009; Heo et al, 2009). While early (MCI stage) WM alterations have been detected within the parahippocampus, hippocampus, posterior cingulate fibers, and splenium (Chua et al, 2009; Takahashi et al, 2002; Zhang et al, 2007; Zhuang et al, 2010), most DTI-based investigations of both MCI and AD consistently report alterations in the uncinate fasciculus, the entire corpus callosum and the cingulum tract. In contrast, a recent study on AD and MCI patients (Douaud et al, 2011) revealed an increase in FA in MCI as well as an increase of anisotropy mode (MO) (Ennis and Kindlmann, 2006). In the regions where an FA increase was detected, the accompanying increase in MO allowed to deduce a selective degeneration of only one of two crossing fibers. In addition, DTI has been able to track the age-related advance in AD in terms of progressive white matter degeneration and, in agreement with the retrogenesis hypothesis, white matter changes seem to appear earliest in specific brain regions such as prefrontal cortex white matter, the inferior longitudinal fasciculus and temporo-parietal areas (Chua et al, 2008; Chua et al, 2009; Stricker et al, 2009; Teipel et al, 2007). It should be noted that DTI employs extremely noisy Echo-Planar Imaging sequences, demanding strict quality control and quality assurance routines (Giannelli et al, 2011), as indicated by a recent meta-analysis (Sexton et al, 2011) and a recent European multicenter study, the European DTI Study in Dementia (EDSD) (Teipel et al, 2012), which demostrated a significant center-related effect in DTI-derived measures. Also, more advanced protocols such as Diffusion Spectrum Imaging (Wedeen et al, 2008), Diffusional Kurtosis Imaging (Fieremans et al, 2011; Giannelli et al, 2012; Hui et al, 2008; Jensen and Helpern, 2010), higher order tensor models (Alexander, 2005), compartment models (Assaf and Basser, 2005; Assaf et al, 2008) and anomalous diffusion (Alexander et al, 2010; De Santis et al, 2011a), which can be optimized in order to enhance their suitability in a clinical setting (De Santis et al, 2011b), have been already been successfully employed in several pathologies including AD (Falangola et al, 2008; Iraji et al, 2011; Mintun et al, 2006; Wang et al, 2011).

Several functional MRI (fMRI) studies have detected functional alterations which precede AD

symptoms or AD-related structural neurodegeneration (Bookheimer et al, 2000; Borghesani et al, 2008; Filippini et al, 2009). Task-based fMRI has been used to study memory-related activation in the hippocampus and MTL, usually reporting a decrease in hippocampal or parahippocampal activity during information encoding (Golby et al, 2005; Gron et al, 2001; Hamalainen et al, 2007; Sperling, 2007; Sperling et al, 2010). Also, several studies have reported a decreased activation in the MTL in patients with MCI (Celone et al, 2006; Johnson et al, 2006; Petrella et al, 2007). A number of fMRI studies have focused on the "default mode network" (DMN), i.e. the interaction between a set cortical regions and the hippocampal memory system (Satterthwaite et al, 2007), and several investigators have reported dysfunctional modulation of encoding-related network activity in AD (Celone et al, 2006; Fleisher et al, 2009; Pihlajamäki et al, 2011; Pihlajamäki et al, 2008; Pihlajamäki et al, 2010) or altered default mode activity in AD patients (Greicius et al, 2004; Greicius et al, 2009; Sorg et al, 2007; Wang et al, 2007; Wermke et al, 2008). Also, fMRI can detect drug-induced modulation of memory-related networks (Kukolja et al, 2009), however only a few studies have demonstrated altered activation after (e.g.) long-term treatment with cholinesterase inhibitors in MCI and AD (Goekoop et al, 2006; Rombouts et al, 2002; Saykin et al, 2004; Shanks et al, 2007).

While modern scanners allow multimodal imaging, which can be optimized to simultaneously target the assessment of brain structure and function within a scanning time compatible with clinical constraints (Landman et al, 2011), it should be noted that multimodal investigations necessitate analysis tool which are able to integrate information from different protocols into the same processing pipeline. This approach is expected to aid in better discrimination and staging of AD (Casanova et al, 2007; Ewers et al, 2011; Oakes et al, 2007; Yang et al, 2011), however conventional analysis techniques are not able to classify a single subject into a predefined group or predict disease evolution and/or drug response using a baseline MR examination alone. In contrast, machine learning algorithms (e.g. support vector machines) are able to manage images from different modalities simultaneously and, importantly, to classify a single subject into a predefined group (e.g. diseased or control group). Such techniques have recently been identified as promising tools in neuroimaging data analysis (Orru et al, 2012), and it has recently been shown that their performance is not significantly degraded if same-modality data is collected in different centers (Orru et al, 2012). In the context of atrophy quantification, machine learning algorithms can be trained to recognize spatial patterns of brain atrophy which best distinguish between AD patients and controls, and pattern recognition strategies can detect prodromal AD pathology in MCI subjects with accuracies which can reach 80% (Diciotti et al, 2012; Misra et al, 2009) as well as assess an individual´s risk for future cognitive decline (Davatzikos et al, 2009). Also, preliminary applications in multimodal neuroimaging have succeeded in discriminating AD and MCI patients (Zhang et al, 2012a; Zhang et al, 2012b). In the foreseeable future, machine learning algorithms will be incorporated into scanner software along with all the preprocessing step necessary to assist the radiological expert in the semi-automated detection of prodromal AD stages based on pattern recognition strategies.

CONCLUSIONS

Major progresses have occurred in the exploration and development of disease markers for AD in the past two decades. Longitudinal studies have proven that in subjects who ultimately develop AD dementia, the cognitive signs begin insidiously and, in most cases, evolve slowly. The existence of AD typical pathological process might be documented several years preceding the onset of AD dementia. As a result, the dementia form of AD denotes a late stage of the biological process.

In light of the findings from neurobiochemical and neuroimaging markers, AD can be re-conceptualized as a progressive disorder that evolves from biological alterations in the brain with any associated memory defects, to a condition of cognitive decay with biomarker fluctuations linked to AD, to mild, moderate, severe stages of AD dementia.

ACKNOWLEDGEMENTS

H.H. was supported by grants of the Katharina-

Hardt-Foundation, Bad Homburg vor der Höhe, Germany.

We thank Dr. Valeria Cozzolino from University of Rome "Tor Vergata", Rome, Italy, for her helpful inputs and feedbacks.

REFERENCES

Aizenstein HJ, Nebes RD, Saxton JA, Price JC et al. and Klunk WE (2008) Frequent amyloid deposition without significant cognitive impairment among the elderly. *Arch Neurol* **65**:1509-1517.

Albert MS, DeKosky ST, Dickson D, Dubois B et al and Phelps CH (2011) The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* **7**:270-279.

Alegret M, Cuberas-Borrós G, Vinyes-Junqué G, Espinosa A et al and Boada M (2012) A two-year follow-up of cognitive deficits and brain perfusion in mild cognitive impairment and mild Alzheimer's disease. *J Alzheimers Dis* **30**:109-120.

Alexander DC (2005) Multiple-fiber reconstruction algorithms for diffusion MRI. *Ann N Y Acad Sci* **1064**:113-133.

Alexander DC, Hubbard PL, Hall MG, Moore EA, Ptito M, Parker GJ, Dyrby TB (2010) Orientationally invariant indices of axon diameter and density from diffusion MRI. *Neuroimage* **52**:1374-89.

Andreasen N, Zetterberg H (2008) Amyloid-related biomarkers for Alzheimer's disease. *Curr Med Chem* **15**:766-771.

Assaf Y, Basser PJ (2005) Composite hindered and restricted model of diffusion (CHARMED) MR imaging of the human brain. *Neuroimage* **27**:48-58.

Assaf Y, Blumenfeld-Katzir T, Yovel Y, Basser PJ (2008) AxCaliber: a method for measuring axon diameter distribution from diffusion MRI. *Magn Reson Med* **59**:1347-1354.

Ballatore C, Lee VM, Trojanowski JQ (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci* **8**;663-672.

Bartzokis G (2004) Age related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. *Neurobiol Aging* **25**:5-18.

Bartzokis G, Sultzer D, Lu PH, Nuechterlein KH, Mintz J, Cummings JL (2004) Heterogeneous age related breakdown of white matter structural integrity: implications for cortical "disconnection in aging and Alzheimer's disease. *Neurobiol Aging* **25**:843-851.

Basser PJ, Jones DK (2002) Diffusion-tensor MRI: theory, experimental design and data analysis - a technical review. *NMR Biomed* **15**:456-467.

Beaulieu C (2002) The basis of anisotropic water diffusion in the nervous system - a technical review. *NMR Biomed* **15**:435-455.

Blennow K, de Leon MJ, Zetterberg, H (2006) Alzheimer's disease. *Lancet* **368**:387-403.

Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nat Rev Neurol* **6**:131-144.

Blennow K, Wallin A, Hager O (1993) Low frequency of post-lumbar puncture headache in demented patients. *Acta Neurol Scand* **88**: 221-223.

Bobinski M, de Leon MJ, Wegiel J, Desanti S et al and Wisniewski HM (2000) The histological validation of post mortem magnetic resonance imaging-determined hippocampal volume in Alzheimer's disease. *Neuroscience* **95**:721-725.

Bookheimer SY, Strojwas MH, Cohen MS, Saunders AM, Pericak-Vance MA, Mazziotta JC, Small GW. (2000) Patterns of brain activation in people at risk for Alzheimer's disease. *N Engl J Med* **343**:450-456.

Borghesani PR, Johnson LC, Shelton AL, Peskind ER, Aylward EH, Schellenberg GD, Cherrier MM (2008) Altered medial temporal lobe responses during visuospatial encoding in healthy APOE*4 carriers. *Neurobiol Aging* **29**:981-991.

Bozzali M, Cherubini A (2007) Diffusion tensor MRI to investigate dementias: a brief review. *Magn Reson Imaging* **25**:969-77.

Bozzali M, Falini A, Franceschi M, Cercignani M et al and Filippi M (2002) White matter damage in Alzheimer's disease assessed in vivo using diffusion tensor magnetic resonance imaging. *J Neurol Neurosurg Psychiatry* **72**:742-746.

Bozzali M, Filippi M, Magnani G, Cercignani M, et al and Falini A (2006) The contribution of voxel-based morphometry in staging patients with mild cognitive impairment. *Neurology* **67**:453-460.

Braak H, Braak E (1988) Neuropil threads occur in dendrites of tangle-bearing nerve cells. *Neuropathol Appl Neurobiol* **14**;39-44.

Braak H, Braak E (1991) Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol (Berl)* **82**:239-259.

Braak H, Del Tredici K (2011) The pathological process underlying Alzheimer's disease in individuals under thirty. *Acta Neuropathol* **121**:171-181.

Buchhave P, Minthon L, Zetterberg H, Wallin AK, Blennow K, Hansson O (2012) Cerebrospinal fluid levels of β-amyloid 1-42, but not of tau, are fully changed already 5 to 10 years before the onset of Alzheimer dementia. *Arch Gen Psychiatry* **69**:98-106.

Buerger K, Ewers M, Pirttilä T, Zinkowski R et al and Hampel H (2006). CSF phosphorylated tau protein correlates with neocortical neurofibrillary pathology in Alzheimer's disease. *Brain* **129**:3035-3041.

Byrum CE, MacFall JR, Charles HC, Chitilla VR et al and Krishnan R (1996) Accuracy and reproducibility of brain and tissue volumes using a magnetic resonance segmentation method. *Psychiatry Res* **67**:215-234 .

Casanova R, Srikanth R, Baer A, Laurienti PJ at al. and Maldjian JA (2007) Biological parametric mapping: A statistical toolbox for multimodality brain image analysis. *Neuroimage* **34**:137-143.

Celone KA, Calhoun VD, Dickerson BC, Atri A, at al. and Sperling R (2006) Alterations in memory networks in mild cognitive impairment and Alzheimer's disease: an independent component analysis. *J Neurosci* **26**:10222-10231.

Chételat G, Desgranges B, De La Sayette V, Viader F, Eustache F, Baron JC (2002) Mapping gray matter loss with voxel-based morphometry in mild cognitive impairment. *Neuroreport* **13**:1939-1943.

Chua TC, Wen W, Chen X, Kochan N, Slavin MJ, Trollor JN, Brodaty H, Sachdev PS (2009) Diffusion tensor imaging of the posterior cingulate is a useful biomarker of mild cognitive impairment. *Am J Geriatr Psychiatry* **17**:602-613.

Chua TC, Wen W, Slavin MJ, Sachdev PS (2008) Diffusion tensor imaging in mild cognitive impairment and Alzheimer's disease: a review. *Curr Opin Neurol* **21(1)**:83-92.

Cummings J (2012) Alzheimer's disease diagnostic criteria: practical applications. *Alzheimers Res Ther* **4**:35.

Davatzikos C, Xu F, An Y, Fan Y, Resnick SM (2009) Longitudinal progression of Alzheimer's-like patterns of atrophy in normal older adults: the SPARE-AD index. *Brain* **132**:2026-2035.

De Santis S, Gabrielli A, Bozzali M, Maraviglia B, Macaluso E, Capuani S (2011) Anisotropic anomalous diffusion assessed in the human brain by scalar invariant indices. *Magn Reson Med* **65**:1043-1052.

De Santis S, Gabrielli A, Palombo M, Maraviglia B, Capuani S. (2011) Non-Gaussian diffusion imaging: a brief practical review. *Magn Reson Imaging* **29**:1410-1416.

Diciotti S, Ginestroni A, Bessi V, Giannelli M et al and Toschi N (2012) Identification of Mild Alzheimer's Disease through automated classification of structural MRI features. *Conf Proc IEEE Eng Med Biol Soc* **2012**:428-431.

Douaud G, Jbabdi S, Behrens TE, Menke RA at al. and Smith S (2011) DTI measures in crossing-fibre areas: increased diffusion anisotropy reveals early white matter alteration in MCI and mild Alzheimer's disease. *Neuroimage* **55**:880-890.

Dousset V, Gayou A, Brochet B, Caille JM (1998) Early structural changes in acute MS lesions assessed by serial magnetization transfer studies. *Neurology* **51**:1150-1155.

Dubois B, Feldman HH, Jacova C, Cummings JL et al and Scheltens P (2010) Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol* **9**:1118-1127.

Dubois B, Feldman HH, Jacova C, Dekosky ST et al and Scheltens P (2007) Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. *Lancet* Neurol 6:734-746.

Ennis DB, Kindlmann G (2006) Orthogonal tensor invariants and the analysis of diffusion tensor magnetic resonance images. *Magn Reson Med* **55**:136-146.

Ewers M, Frisoni GB, Teipel SJ, Grinberg LT, Amaro E Jr, Heinsen H, Thompson PM, Hampel H (2011) Staging Alzheimer's disease progression with multimodality neuroimaging. *Prog Neurobiol* **95**:535-546.

Ewers M, Sperling RA, Klunk WE, Weiner MW, Hampel H (2011) Neuroimaging markers for the prediction and early diagnosis of Alzheimer's disease dementia. *Trends Neurosci* **34**:430-442.

Fagan AM, Head D, Shah AR, Marcus D, Mintun M, Morris JC, Holtzman DM (2009) Decreased cerebrospinal fluid Abeta(42) correlates with brain atrophy in cognitively normal elderly. *Ann Neurol* 65:176-183.

Fagan AM, Mintun MA, Mach RH, Lee SY et al and Holtzman DM (2006) Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid Abeta42 in humans. *Ann Neurol* **59**:512-519.

Falangola MF, Jensen JH, Babb JS, Hu C, Castellanos FX, Di Martino A, Ferris SH, Helpern JA. (2008) Age-related non-Gaussian diffusion patterns in the prefrontal brain. *J Magn Reson Imaging* **28**:1345-1350.

Fieremans E, Jensen JH, Helpern JA (2011) White

matter characterization with diffusional kurtosis imaging. *Neuroimage* **58**:177-188.

Filippini N, MacIntosh BJ, Hough MG, Goodwin GM et al and Mackay CE. (2009) Distinct patterns of brain activity in young carriers of the APOE-epsilon4 allele. *Proc Natl Acad Sci U S A* **106**:7209-7214.

Fjell AM, Amlien IK, Westlye LT, Walhovd KB (2009) Mini-mental state examination is sensitive to brain atrophy in Alzheimer's disease. *Dement Geriatr Cogn Disord* **28**:252-258.

Fleisher AS, Sherzai A, Taylor C, Langbaum JB, Chen K, Buxton RB (2009) Resting-state BOLD networks versus task-associated functional MRI for distinguishing Alzheimer's disease risk groups. *Neuroimage* **47**:1678-1690.

Forlenza OV, Diniz BS, Gattaz WF (2010) Diagnosis and biomarkers of predementia in Alzheimer's disease. *BMC Med* **8**:89.

Forsberg A, Engler H, Almkvist O, Blomquist G et al and Nordberg A (2008) PET imaging of amyloid deposition in patients with mild cognitive impairment. *Neurobiol Aging* 29:1456-1465.

Giannelli M, Belmonte G, Toschi N, Pesaresi I et al and Cosottini M (2011) Technical note: DTI measurements of fractional anisotropy and mean diffusivity at 1.5 T: comparison of two radiofrequency head coils with different functional designs and sensitivities. *Med Phys* **38**:3205-3211.

Giannelli M, Toschi N, Passamonti L, Mascalchi M, Diciotti S, Tessa C (2012) Diffusion kurtosis and diffusion-tensor MR imaging in Parkinson disease. *Radiology* **265**:645-646.

Giedd JN, Kozuch P, Kaysen D, Vaituzis AC, Hamburger SD, Bartko JJ, Rapoport JL (1995) Reliability of cerebral measures in repeated examinations with magnetic resonance imaging. *Psychiatry Res* **61**:113-119.

Gil-Bea FJ, Garcia-Alloza M, Dominguez J, Marcos B, Ramirez MJ (2005) Evaluation of cholinergic markers in Alzheimer's disease and in a model of cholinergic deficit. *Neurosci Lett* **375**:37-41.

Goekoop R, Scheltens P, Barkhof F, Rombouts SA (2006) Cholinergic challenge in Alzheimer patients and mild cognitive impairment differentially affects hippocampal activation--a pharmacological fMRI study. *Brain* **129**:141-157.

Golby A, Silverberg G, Race E, Gabrieli S et al and Gabrieli J (2005) Memory encoding in Alzheimer's disease: an fMRI study of explicit and implicit memory. *Brain* **128**:773-787.

Gong CX, Shaikh S, Wang JZ, Zaidi T, Grundke-Iqbal I, Iqbal K (1995) Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J Neurochem* **65**: 732-738.

Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K (1993) Phosphoprotein phosphatase activities in Alzheimer disease brain. *J Neurochem* **61**:921-927.

Greicius MD, Srivastava G, Reiss AL, Menon V (2004) Default-mode network activity distinguishes Alzheimer's disease from healthy aging: evidence from functional MRI. *Proc Natl Acad Sci U S A* **101**:4637-4642.

Greicius MD, Supekar K, Menon V, Dougherty RF (2009) Resting-state functional connectivity reflects structural connectivity in the default mode network. *Cereb Cortex* **19**:72-78.

Grimmer T, Riemenschneider M, Förstl H, Henriksen G et al and Drzezga A (2009) Beta amyloid in Alzheimer's disease: increased deposition in brain is reflected in reduced concentration in cerebrospinal fluid. *Biol Psychiatry* 65:927-934.

Grön G, Bittner D, Schmitz B, Wunderlich AP, Tomczak R, Riepe MW (2001) Hippocampal activations during repetitive learning and recall of geometric patterns. *Learn Mem* **8**:336-345.

Haass C, Selkoe DJ (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. *Cell* **75**:1039-1042.

Hämäläinen A, Pihlajamäki M, Tanila H, Hänninen T at al. and Soininen H (2007) Increased fMRI responses during encoding in mild cognitive impairment. *Neurobiol Aging* **28**:1889-1903.

Hampel H, Blennow K, Shaw LM, Hoessler YC, Zetterberg H, Trojanowski JQ (2010c) Total and phosphorylated tau protein as biological markers of Alzheimer's disease. *Exp Gerontol* **45**:30-40.

Hampel H, Bürger K, Pruessner JC, Zinkowski R et al and Teipel SJ (2005) Correlation of cerebrospinal fluid levels of tau protein phosphorylated at threonine 231 with rates of hippocampal atrophy in Alzheimer disease. *Arch Neurol* 62:770-773.

Hampel H, Bürger K, Teipel SJ, Bokde AL, Zetterberg H, Blennow K (2008) Core candidate neurochemical and imaging biomarkers of Alzheimer's disease. *Alzheimers Dement* **4**:38-48.

Hampel H, Frank R, Broich K, Teipel SJ et al and Blennow K (2010a) Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. *Nat Rev Drug Discov* **9**:560-574.

Hampel H, Lista S (2012) Alzheimer disease: From

inherited to sporadic AD-crossing the biomarker bridge. *Nat Rev Neurol* **8**:598-600.

Hampel H, Lista S, Khachaturian ZS (2012) Development of biomarkers to chart all Alzheimer's disease stages: the royal road to cutting the therapeutic Gordian Knot. *Alzheimers Dement* **8**:312-336.

Hampel H, Shen Y, Walsh DM, Aisen P et al and Blennow K (2010b) Biological markers of amyloid beta-related mechanisms in Alzheimer's disease. *Exp Neurol* **223**:334-346.

Hansson O, Zetterberg H, Buchhave P, Andreasson U, Londos E, Minthon L, Blennow K (2007) Prediction of Alzheimer's disease using the CSF Aβ42/Aβ40 ratio in patients with mild cognitive impairment. *Dement Geriatr Cogn Disord* **23**:316-320.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**:353-356.

Head D, Buckner RL, Shimony JS, Williams LE et al and Snyder AZ (2004) Differential vulnerability of anterior white matter in nondemented aging with minimal acceleration in dementia of the Alzheimer type: evidence from diffusion tensor imaging. *Cerebral Cortex* **14**:410-423.

Heo JH, Lee ST, Kon Chu, Park HJ, Shim JY, Kim M (2009) White matter hyperintensities and cognitive dysfunction in Alzheimer disease. *J Geriatr Psychiatry Neurol* **22**:207-212.

Hess CP (2009) Update on diffusion tensor imaging in Alzheimer's disease. *Magn Reson Imaging Clin N Am* **17**:215-224.

Holtzman DM, Morris JC, Goate AM (2011) Alzheimer's disease: the challenge of the second century. *Sci Transl Med* **3**:77sr1.

Hui ES, Cheung MM, Qi L, Wu EX (2008) Advanced MR diffusion characterization of neural tissue using directional diffusion kurtosis analysis. *Conf Proc IEEE Eng Med Biol Soc* **2008**:3941-3944.

Iraji A, Davoodi-Bojd E, Soltanian-Zadeh H, Hossein-Zadeh GA, Jiang Q (2011) Diffusion kurtosis imaging discriminates patients with white matter lesions from healthy subjects. *Conf Proc IEEE Eng Med Biol Soc* **2011**:2796-2799.

Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM et al and Trojanowski JQ (2010) Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol* **9**:119-112.

Jack CR Jr, Petersen RC, Xu YC, O'Brien PC at al. and Kokmen E (1999) Prediction of AD with MRI-based hippocampal volume in mild cognitive impairment. *Neurology* **52**:1397-1403.

Jensen JH, Helpern JA (2010) MRI quantification of non-Gaussian water diffusion by kurtosis analysis. *NMR Biomed* **23**:698-710.

Johnson SC, Schmitz TW, Moritz CH, Meyerand ME et al and Alexander GE (2006) Activation of brain regions vulnerable to Alzheimer's disease: the effect of mild cognitive impairment. *Neurobiol Aging* **27**:1604-1612.

Khachaturian ZS (2006) Diagnosis of Alzheimer's disease: two-decades of progress. *J Alzheimers Dis* **9**:409-415.

Khachaturian ZS (2011) Revised criteria for diagnosis of Alzheimer's disease: National Institute on Aging-Alzheimer's Association diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* **7**:253-256.

Kukolja J, Thiel CM, Fink GR (2009) Cholinergic stimulation enhances neural activity associated with encoding but reduces neural activity associated with retrieval in humans. *J Neurosci* **29**:8119-8128.

Laakso MP, Soininen H, Partanen K, Lehtovirta M at al. and Riekkinen PJ Sr (1998) MRI of the hippocampus in Alzheimer's disease: sensitivity, specificity, and analysis of the incorrectly classified subjects. *Neurobiol Aging* **19**:23-31.

Landman BA, Huang AJ, Gifford A, Vikram DS et al and van Zijl PC (2011) Multi-parametric neuroimaging reproducibility: a 3-T resource study. *Neuroimage* **54**:2854-2866.

Liu F, Grundke-Iqbal I, Iqbal K, Gong CX (2005) Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *Eur J Neurosci* **22**:1942-1950.

Liu Y, Spulber G, Lehtimäki KK, Könönen M et al and Soininen H (2011) Diffusion tensor imaging and tract-based spatial statistics in Alzheimer's disease and mild cognitive impairment. *Neurobiol Aging* **32**:1558-1571.

Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin ML, Terro F (2013a) Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A. *Ageing Res Rev* **12**:39-49.

Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin ML, Yardin C, Terro F (2013b) Tau protein kinases: involvement in Alzheimer's disease. *Ageing Res Rev* **12**:289-309.

Mattsson N, Zetterberg H, Hansson O, Andreasen N et al and Blennow K (2009) CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive

impairment. *JAMA* **302**:385-393.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology* **34**:939-944.

McKhann GM, Knopman DS, Chertkow H, Hyman BT at al. and Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* **7**:263-269.

Medina D, DeToledo-Morrell L, Urresta F, Gabrieli JD et al and Stebbins GT (2006) White matter changes in mild cognitive impairment and AD: A diffusion tensor imaging study. *Neurobiol Aging* **27**:663-672.

Mintun MA, Larossa GN, Sheline YI, Dence CS et al and Morris JC (2006) [11C]PIB in a nondemented population: potential antecedent marker of Alzheimer disease. *Neurology* **67**:446-452.

Misra C, Fan Y , Davatzikos C (2009) Baseline and longitudinal patterns of brain atrophy in MCI patients, and their use in prediction of short-term conversion to AD: results from ADNI. *Neuroimage* **44**:1415-1422.

Oakes, TR, Fox AS, Johnstone T, Chung MK, Kalin N, Davidson RJ (2007) Integrating VBM into the General Linear Model with voxelwise anatomical covariates. *Neuroimage* **34**:500-508.

O'Dwyer L, Lamberton F, Bokde AL, Ewers M at al and Hampel H (2011) Multiple indices of diffusion identifies white matter damage in mild cognitive impairment and Alzheimer's disease. *PLoS One* **6**:e21745.

Orrù G, Pettersson-Yeo W, Marquand AF, Sartori G, Mechelli A.(2012) Using Support Vector Machine to identify imaging biomarkers of neurological and psychiatric disease: a critical review. *Neurosci Biobehav Rev* **36**:1140-1152.

Ossenkoppele R, Tolboom N, Foster-Dingley JC, Adriaanse SF et al and van Berckel BN (2012) Longitudinal imaging of Alzheimer pathology using [(11)C]PIB, [(18) F]FDDNP and [(18)F]FDG PET. *Eur J Nucl Med Mol Imaging* **39**:990-1000.

Pákáski M, Kálmán J (2008) Interactions between the amyloid and cholinergic mechanisms in Alzheimer's disease. *Neurochem Int* **53**:103-111.

Pennanen C, Testa C, Laakso MP, Hallikainen M, et al and Soininen H (2005) A voxel based morphometry study on mild cognitive impairment. *J Neurol Neurosurg Psychiatry* **76**:11-14.

Perl DP (2010) Neuropathology of Alzheimer's disease. *Mt Sinai J Med* **77**:32-42.

Perry EK, Tomlinson BE, Blessed G, Bergmann K, Gibson PH, Perry RH (1978) Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *Br Med J* **2**:1457-1459.

Peskind ER, Riekse R, Quinn JF, Kaye J et al and Galasko D (2005) Safety and acceptability of the research lumbar puncture. *Alzheimer Dis Assoc Disord* **19**:220-225.

Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E (1999) Mild cognitive impairment: clinical characterization and outcome. *Arch Neurol* **56**:303-308.

Petrella JR, Wang L, Krishnan S, Slavin MJ, Prince SE, Tran TT, Doraiswamy PM. (2007) Cortical deactivation in mild cognitive impairment: high-field-strength functional MR imaging. *Radiology* **245**:224-35.

Pihlajamäki M, DePeau KM, Blacker D, Sperling RA (2008) Impaired medial temporal repetition suppression is related to failure of parietal deactivation in Alzheimer disease. *Am J Geriatr Psychiatry* **16**:283-292.

Pihlajamäki M, O' Keefe K, Bertram L, Tanzi RE at al. and Sperling RA. (2010) Evidence of altered posteromedial cortical FMRI activity in subjects at risk for Alzheimer disease. *Alzheimer Dis Assoc Disord* **24**:28-36.

Pihlajamäki M, O'Keefe K, O'Brien J, Blacker D, Sperling RA. (2011) Failure of repetition suppression and memory encoding in aging and Alzheimer's disease. *Brain Imaging Behav* **5**:36-44.

Reddy PH (2011) Abnormal tau, mitochondrial dysfunction, impaired axonal transport of mitochondria, and synaptic deprivation in Alzheimer's disease. *Brain Res* **1415**;136-148.

Reisberg B, Franssen EH, Souren LE, Auer SR, Akram I, Kenowsky S (2002) Evidence and mechanisms of retrogenesis in Alzheimer's and other dementias: management and treatment import. *Am J Alzheimers Dis Other Demen* **17**:202-212.

Rombouts SA, Barkhof F, Van Meel CS, Scheltens P (2002) Alterations in brain activation during cholinergic enhancement with rivastigmine in Alzheimer's disease. *J Neurol Neurosurg Psychiatry* **73**:665-671.

Ropele S, Schmidt R, Enzinger C, Windisch M, Martinez NP, Fazekas F (2012) Longitudinal magnetization transfer imaging in mild to severe Alzheimer disease. *AJNR Am J Neuroradiol* **33**:570-575.

Rose SE, McMahon KL, Janke AL, O'Dowd B,

de Zubicaray G, Strudwick MW, Chalk JB (2006) Diffusion indices on magnetic resonance imaging and neuropsychological performance in amnestic mild cognitive impairment. *J Neurol Neurosurg Psychiatry* **77**:1122-1128.

Samgard K, Zetterberg H, Blennow K, Hansson O, Minthon L, Londos E (2010) Cerebrospinal fluid total tau as a marker of Alzheimer's disease intensity. *Int J Geriatr Psychiatry* **25**:403-410.

Satterthwaite TD, Green L, Myerson J, Parker J, Ramaratnam M, Buckner RL (2007) Dissociable but inter-related systems of cognitive control and reward during decision making: evidence from pupillometry and event-related fMRI. *Neuroimage* **37**:1017-1031.

Saykin AJ, Wishart HA, Rabin LA, Flashman LA, McHugh TL, Mamourian AC, Santulli RB (2004) Cholinergic enhancement of frontal lobe activity in mild cognitive impairment. *Brain* **127**:1574-1583.

Scheltens P, Launer LJ, Barkhof F, Weinstein HC, van Gool WA (1995) Visual assessment of medial temporal lobe atrophy on magnetic resonance imaging: interobserver reliability. *J Neurol* **242**:557-560.

Schliebs R, Arendt T (2006) The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. *J Neural Transm* **113**:1625-1644.

Sexton CE, Kalu UG, Filippini N, Mackay CE, Ebmeier KP (2011) A meta-analysis of diffusion tensor imaging in mild cognitive impairment and Alzheimer's disease. *Neurobiol Aging* **32**:2322 e5-e18.

Shanks MF, McGeown WJ, Forbes-McKay KE, Waiter GD, Ries M, Venneri A (2007) Regional brain activity after prolonged cholinergic enhancement in early Alzheimer's disease. *Magn Reson Imaging* **25**:848-859.

Shi F, Liu B, Zhou Y, Yu C, Jiang T (2009) Hippocampal volume and asymmetry in mild cognitive impairment and Alzheimer's disease: Meta-analyses of MRI studies. *Hippocampus* **19**:1055-1064.

Sorg C, Riedl V, Mühlau M, Calhoun VD at al. and Wohlschläger AM (2007) Selective changes of resting-state networks in individuals at risk for Alzheimer's disease. *Proc Natl Acad Sci U S A* **104**:18760-18765.

Sperling R (2007) Functional MRI studies of associative encoding in normal aging, mild cognitive impairment, and Alzheimer's disease. *Ann N Y Acad Sci* **1097**:146-155.

Sperling RA, Aisen PS, Beckett LA, Bennett DA et al and Phelps CH (2011) Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* **7**:280-292.

Sperling RA, Dickerson BC, Pihlajamaki M, Vannini P et al and Johnson KA (2010) Functional alterations in memory networks in early Alzheimer's disease. *Neuromolecular Med* **12**:27-43.

Stahl R, Dietrich O, Teipel SJ, Hampel H, Reiser MF, Schoenberg SO (2007) White matter damage in Alzheimer's disease and mild cognitive impairment: assessment with diffusion-tensor MR imaging and parallel imaging techniques. *Radiology* **243**:482-492.

Stricker NH, Schweinsburg BC, Delano-Wood L, Wierenga CE at al. and Bondi MW (2009) Decreased white matter integrity in late-myelinating fiber pathways in Alzheimer's disease supports retrogenesis. *Neuroimage* **45**:10-16.

Strozyk D, Blennow K, White LR, Launer LJ (2003) CSF Abeta 42 levels correlate with amyloid-neuropathology in a population-based autopsy study. *Neurology* 60:652-656.

Takahashi S, Yonezawa H, Takahashi J, Kudo M, Inoue T, Tohgi H (2002) Selective reduction of diffusion anisotropy in white matter of Alzheimer disease brains measured by 3.0 Tesla magnetic resonance imaging. *Neurosci Lett* **332**:45-48.

Teipel SJ, Sabri O, Grothe M, Barthel H et al and Hampel H (2013) Perspectives for multimodal neurochemical and imaging biomarkers in Alzheimer's disease. *J Alzheimers Dis* **33**:S329-S347.

Teipel SJ, Stahl R, Dietrich O, Schoenberg SO at al. and Hampel H (2007) Multivariate network analysis of fiber tract integrity in Alzheimer's disease. *Neuroimage* **34**:985-995.

Teipel SJ, Wegrzyn M, Meindl T, Frisoni G at al. and Ewers M (2012) Anatomical MRI and DTI in the Diagnosis of Alzheimer's Disease: A European Multicenter Study. *J Alzheimers Dis* **31**:S33-S47.

Thal DR, Capetillo-Zarate E, Del Tredici K, Braak H (2006) The development of amyloid- β protein deposits in the aged brain. *Sci Aging Knowledge Environ* **2006**:re1.

Tisserand DJ, van Boxtel MP, Pruessner JC, Hofman P, Evans AC, Jolles J (2004) A voxel-based morphometric study to determine individual differences in gray matter density associated with age and cognitive change over time. *Cereb Cortex* **14**:966-973.

Trojanowski JQ, Hampel H (2011) Neurodegenerative disease biomarkers: guideposts for disease prevention through early diagnosis and intervention. *Prog Neurobiol* **95**:491-495.

Visser PJ, Verhey F, Knol DL, Scheltens P et al and Blennow K (2009) Prevalence and prognostic value of CSF markers of Alzheimer's disease pathology in patients with subjective cognitive impairment or mild cognitive impairment in the DESCRIPA study: a prospective cohort study. *Lancet Neurol* **8**:619-627.

Vogels OJ, Broere CA, ter Laak HJ, ten Donkelaar HJ, Nieuwenhuys R, Schulte BP (1990) Cell loss and shrinkage in the nucleus basalis Meynert complex in Alzheimer's disease. *Neurobiol Aging* **11**:3-13.

Wahlund LO, Julin P, Johansson SE, Scheltens P (2000) Visual rating and volumetry of the medial temporal lobe on magnetic resonance imaging in dementia: a comparative study. *J Neurol Neurosurg Psychiatry* **69**:630-635.

Wang JJ, Lin WY, Lu CS, Weng YH at al. and Wai YY (2011) Parkinson disease: diagnostic utility of diffusion kurtosis imaging. *Radiology* **261**:210-217.

Wang K, Liang M, Wang L, Tian L, Zhang X, Li K, Jiang T (2007) Altered functional connectivity in early Alzheimer's disease: a resting-state fMRI study. *Hum Brain Mapp* **28**:967-978.

Wedeen VJ, Wang RP, Schmahmann JD, Benner T at al. and de Crespigny AJ. (2008) Diffusion spectrum magnetic resonance imaging (DSI) tractography of crossing fibers. *Neuroimage* **41**:1267-1277.

Weiner MW, Veitch DP, Aisen PS, Beckett LA et al and Trojanowski JQ (2012) The Alzheimer's Disease Neuroimaging Initiative: a review of papers published since its inception. *Alzheimers D*ement **8**:S1-S68.

Wermke M, Sorg C, Wohlschläger AM, Drzezga A (2008) A new integrative model of cerebral activation, deactivation and default mode function in Alzheimer's disease. *Eur J Nucl Med Mol Imaging* **35**:S12-S24.

Westman E, Muehlboeck JS, Simmons A (2012) Combining MRI and CSF measures for classification of Alzheimer's disease and prediction of mild cognitive impairment conversion. *Neuroimage* **62**:229-238.

Whitehouse PJ, Price DL, Clark AW, Coyle JT, DeLong MR (1981) Alzheimer disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann Neurol* **10**:122-126.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science **215**:1237-1239.

Wimo A, Prince M. Alzheimer's Disease International. World Alzheimer Report (2010). The Global Economic Impact of Dementia. http://www.alz.co.uk/research/files/WorldAlzheimerReport2010.pdf.

Yang X, Beason-Held L, Resnick SM, Landman BA (2011) Biological parametric mapping with robust and non-parametric statistics. *Neuroimage* **57**:423-430.

Zetterberg H, Mattsson N, Blennow K (2010) Cerebrospinal fluid analysis should be considered in patients with cognitive problems. *Int J Alzheimers Dis* **2010**:163065.

Zhang L, Dean D, Liu JZ, Sahgal V, Wang X, Yue GH (2007) Quantifying degeneration of white matter in normal aging using fractal dimension. *Neurobiol Aging* **28**:1543-1555.

Zhang Y, Schuff N, Jahng GH, Bayne W, et al and Weiner MW (2007) Diffusion tensor imaging of cingulum fibers in mild cognitive impairment and Alzheimer's disease. *Neurology* **68**:13-19.

Zhang, D, Shen D, Initiative Alzheimer's Disease Neuroimaging (2012) Multi-modal multi-task learning for joint prediction of multiple regression and classification variables in Alzheimer's disease. *Neuroimage* **59**:895-907.

Zhang, D, Shen D, Initiative Alzheimer's Disease Neuroimaging (2012) Predicting future clinical changes of MCI patients using longitudinal and multimodal biomarkers. *PLoS One* **7**:e33182.

Zhou Y, Dougherty JH Jr, Hubner KF, Bai B, Cannon RL, Hutson RK (2008) Abnormal connectivity in the posterior cingulate and hippocampus in early Alzheimer's disease and mild cognitive impairment. *Alzheimers Dement* **4**:265-270.

Zhuang L, Wen W, Zhu W, Trollor J, Kochan N, Crawford J, Reppermund S, Brodaty H, Sachdev P (2010) White matter integrity in mild cognitive impairment: a tract-based spatial statistics study. *Neuroimage* **53**:16-25.

HUMAN UMBILICAL CORD BLOOD-DERIVED MONONUCLEAR, HEMATOPOIETIC AND NGF-RESPONSIVE STEM CELLS CONFER NEUROPROTECTION IN ISCHEMIC MODELS

G. GINCBERG[1], H. ARIEN-ZAKAY[1], A. NAGLER[2], U. ELCHALAL[3], P. I LELKES[4], and P. LAZAROVICI[1]

[1]School of Pharmacy Institute for Drug Research , Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel.
[2]Division of Hematology and Cord Blood Bank, Chaim Sheba Medical Center, Tel Hashomer, Israel.
[3]Department of Obstetrics and Gynecology, Hadassah University Hospital, Jerusalem 91120, Israel.
[4]Department of Bioengineering and Temple Institute for Regenerative Medicine and Engineering (TIME), Temple University, Philadelphia, PA, USA.

Human umbilical cord blood (HUCB), which is a valuable source for cell therapy of hematologic diseases, was recently found to harbor a population of cells that confer neuroprotection in animal models of brain injury. To explore the neuroprotective properties of cord blood we investigated HUCB-derived mononuclear cells (MNC), $CD45^+$ hematopoietic cells as well as a unique MNC population of a collagen-adherent cells which in the presence of nerve growth factor (NGF) and interferon-gamma (IFN-γ) differentiate in vitro into a neuronal phenotype (termed HUCBNP). In vitro co-culture of MNC with oxygen-glucose-deprived PC12 cells resulted in about 30% neuroprotection, as assessed by decreased LDH release and inhibition of caspase 3 activity, and yielded a decrease by 95% in the level of free radicals, concomitant with the secretion of antioxidants and growth factors, such as NGF into the culture medium. Using a closed head injury (CHI) brain trauma mouse model, we also compared neuroprotective effects of HUCB-derived MNC and $CD45^+$ cells upon either brain intraventricular (icv) or intravenous (iv) transplantation. The in vivo experiments in which iv implantation of MNC or $CD45^+$ cells in mice with TBI indicated a fast 2 h homing of the cells to the brain lesion followed by a progressive neuroprotective effect maintained up to 3 weeks. In conclusion, our studies are in line with other reports and propose that either HUCB-derived MNC or $CD45^+$ fraction may represent a valuable source of neuroprotective cells such as NGF-responsive progenitors for therapy of ischemic disorders such as stroke, brain trauma and myocardial infarct.

Over the last decade, as some of the complex molecular mechanisms of neuronal cell death following traumatic brain injury have been addressed (Kazantsev, 2007), a plethora of drugs with putative neuroprotective effects have been evaluated. However, despite impressive progress, a clinically proven neuroprotective therapy, which would effectively ameliorate brain injury and prevent neurodegeneration, still does not exist (Bordet et al, 2007). The emerging consensus suggests that an approach based on the use of a single neuroprotective agent may not be sufficient for neuronal rescue (Lo,

Key words: Human umbilical cord blood, mononuclear fraction, CD45 positive hematopoietic progenitors, neuronal progenitors, NGF, neuroprotection, ischemia, oxygen-glucose-deprivation, brain trauma

Corresponding Author: Philip Lazarovici, Ph.D.,
School of Pharmacy Institute for Drug Research, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel.
Phone: +972-2-6758729, +972-2-6758767;
Fax: +972-2-6757490.
Email: philipl@ekmd.huji.ac.il

2279-5855 (2013)

2008). Rather, novel therapeutic approaches are required targeting multiple biochemical pathways involved in ischemic cell death.

Traumatic brain injury (TBI) represents a major health care problem and a significant socioeconomic challenge worldwide. According to the Center for Disease Control, at least 1.4 million patients suffer TBI each year in the United States alone. The mortality of severe TBI remains as high as 35–40% (Beauchamp et al, 2008). Posttraumatic brain damage is determined by a combination of primary and secondary insults (Bramlett and Dietrich, 2007). Primary damage results from the mechanical forces applied at the time of impact, while secondary brain damage evolves over time, and shares similar mechanisms with those which occur after cerebral ischemia (Leker and Shohami, 2002). The closed head injury (CHI) model of TBI is induced by a standardized weight-drop device generating a blunt focal brain injury across an intact skull (Chen et al, 1996; Flierl et al, 2009). The resulting mechanical impact triggers, with high consistency and reproducibility, a profound neuroinflammatory response within the brain leading to neurological and cognitive impairment and breakdown of the blood-brain barrier (Beauchamp et al, 2008). Although cytokines released in the brain after ischemia exhibit both pro- and anti-inflammatory effects, the overall balance of the initial post-ischemic inflammatory reaction appears to enhance cell death, neuronal loss, and finally, expansion of the infarct zone (Stoll and Jander, 1999). Attraction of hematopoietic cells from the blood circulation and from the spleen toward the ischemic lesion site includes recruitment of bone marrow-derived mesenchymal and hematopoietic stem cells that are involved in the "natural healing" of the damaged brain tissue (Stumm et al, 2002), which in most cases is slow and incomplete. Since these endogenous stem cells (SC) do participate in regenerative process, but not with high efficacy, the use of exogenous SC offers an attractive complementary cell therapy approach in experimental models of TBI and cerebral ischemia. For example, local injection into the ischemic boundary zone or systemic iv injection of bone marrow (BM) cells resulted in functional improvement and attenuated brain damage after middle cerebral artery occlusion and stroke models in rats (Chen et al, 2000; Chen et al, 2003; Zhang et al, 2006). Regarding TBI, Han and colleagues found that injection of BM cells into the injury site after TBI have limited therapeutic potential with respect to neuroprotection. However, injection on day 7 after TBI produced greater functional improvements in neurobehavioral tests and more effectively suppressed astroglial activation than an injection on post-injury day 1 (Han et al, 2013).

Human umbilical cord blood (HUCB), once a discarded material, has been shown both in the laboratory and clinical to be valuable source for (stem) cell transplantation, in addition to BM. HUCB cell transplantation made its clinical appearance in 1988, when it was used to successfully treat a 5-year-old child afflicted with Fanconi anemia (Gluckman and Rocha, 2005). To date, with the aid of cord blood banks, more than 20,000 HUCB transplants have been performed worldwide, many of them with unrelated allogeneic donor blood (Tse and Laughlin, 2005; Stanevsky et al, 2010; Shahrokhi et al, 2012). Similar to BM, the indication for using HUCB has been limited to hematopoietic malignancies, marrow failure, and immunodeficiency disorders (Goldstein et al, 2007). Current studies, however, suggests that certain HUCB derivatives may be a much more versatile and powerful clinical resource and tool. By comparison to SC from adult sources, the relative immaturity of HUCB cells, which are fetal in origin, suggests a high potential for cellular plasticity. The use of HUCB cells as an alternative to whole BM transplantation continues to grow as research better defines their composition, mechanisms of action, safety and broad therapeutic capacity. For example, like BM, HUCB cells confer neuroprotection in experimental cerebral ischemia (Willing et al, 2003; Vendrame et al, 2005). Although functional improvement and attenuation of brain damage were demonstrated after transplantation of HUCB cells it is yet unclear how and which HUCB-derived populations mediate neuroprotection in ischemic brain lesion.

HUMAN UMBILICAL CORD BLOOD (HUCB)

The interest in the clinical potential of HUCB began when it was found that it contains a much larges population of hematopoietic stem cells

(HSC) than do adult sources (Harris, 2008). Moreover, HUCB provides an easily accessible, low immunogenic source of multipotent stem-cell-like progenitor cells that reportedly have the capability to become many type of cells in the body under specific differentiation conditions (Arien-Zakay et al, 2010a). In general, the level of maturity of a cell is identified by the expression (or lack thereof) of a combination of specific cell surface antigens. For example, the $CD34^+$ population in HUCB can be defined as more primitive than those found in BM, because a higher proportion of them are negative for CD38, which is a marker for pre-lymphoid cells (Broxmeyer et al, 1989; Cardoso et al, 1993; Conrad and Emerson, 1998). $CD133^+$ cells have also been identified in fetal brain and are in this context considered to be neural stem cells (NSC) (Conrad and Emerson, 1998; Tamaki et al, 2002). However, it is not yet known whether the $CD133^+$ cells found in HUCB are phenotypically and functionally identical to the NSC found in fetal brain.

Mesenchymal stem cells (MSC)

MSC have also been found in HUCB, however in much lower numbers than in BM ranging from 0.05-2.8 or 2-5 mesenchymal stem cells per one million mononuclear cells of either cord blood or bone marrow, respectively (Goodwin et al, 2001; Wexler et al, 2003). MSC can give rise to diverse lineages such as osteoblasts, chondroblasts and adipocytes (Pittenger et al, 1999) and, presumably, also neural cells, such as (astrocytes and neurons) (Goodwin et al, 2001). Other differentiation pathways for MSC also have been documented *in vitro* with varying degrees of rigor. It is clear that cultured MSC may spontaneously, or by induction, co-express genes characteristic of multiple lineages even on single-cell analysis raising doubts about the validity of gene expression/lineage assumptions made *in vitro*. Several investigators have claimed muscle, tendon, central nervous system (CNS) and other potentialities for MSC, supporting their claims by morphologic, gene expression, and/or phenotypic criterion. However, the reproducibility of these experiments has been variable and, in general, functional criteria have not been satisfied (Javazon et al, 2004). Unequivocal identification of MSC populations is challenging because they currently lack a definitive phenotype as well as consensus on exactly which surface antigens are unique markers for these cells. For example, Robinson et al. suggested that MSC are positive for the markers CD13, CD29, CD44, CD73, CD90, CD105, and CD166 and negative for the markers CD14, CD31, CD34, CD45, CD51/61, CD64, CD80, CD106 (Wexler et al, 2003; Robinson et al, 2005; Sueblinvong et al, 2008; Lee et al, 2010). MSC used in recent clinical trials have been characterized by the expression of the following markers CD29, CD54, CD73, CD90 and CD106 (Kranz et al, 2010). Some form of agreement on the phenotype of these cells needs to be reached before their true abundance in HUCB can be established unambiguously.

Mononuclear cells (MNC)

In the first step of isolation of progenitor cells from HUCB, the whole blood, collected and stored in an appropriate blood bag, is layered over a cushion of Ficoll to allow centrifugation and separation of progenitor cells, which localize in the interphase between the Ficoll and serum, while the erythrocytes sediment to the bottom of the separation tube (Jaatinen and Laine, 2007). It is estimated that the MNC fraction of HUCB contains a heterogeneous mixture of SC, which then can be further separated according to a variety of hematopoietic and non-hematopoietic biomarkers (Ali and Bahbahani, 2010). In recent clinical trials the use of HUCB-derived MNC was approved, similar to whole HUCB transplants, for treating a variety of illnesses, such as chronic spinal cord injury, ischemic stroke and acquired hearing loss in children (NCT01354483, NCT01673932, and NCT01343394 ClinicalTrials.gov, respectively).

CD45 positive hematopoietic stem cells ($CD45^+$)

The HUCB contains multiple populations of pluripotent SC, capable of giving rise to hematopoietic, epithelial, endothelial, myotubes and neural cells (Arien-Zakay et al, 2010b). These cell populations can be selected based on the expression of various cell-type specific markers and may be divided into hematopoietic and non-hematopoietic SC, according to the expression of hematopoietic-related biomarkers such as CD34, CD133 and CD45. PTP, a cell membrane protein tyrosine phosphatase (also known as CD45) is abundantly expressed on all

nucleated hematopoietic cells and is critical for the classical antigen receptor signaling, as indicated by the arrested development of B and T cells in CD45-deficient mice (Huntington and Tarlinton, 2004). In a recent study we demonstrated that $CD45^+$ but not $CD45^-$ cells are responsible for the therapeutic effect after brain trauma (Gincberg et al, 2013). The pan leukocyte CD45 phosphatase plays an essential role in trafficking and repopulation of the BM by $CD34^+$ HSC. Inhibition of CD45 negatively affected development of hematopoietic progenitors *ex vivo* and their recovery in transplanted recipients *in vivo*, supporting the central role of CD45 in the regulation of hematopoiesis (Shivtiel et al, 2011). Therefore, the ability of HUCB to improve neurological deficits in the CHI mice may be explained by a direct effect of $CD45^+$ cells homing to the brain. Minimal manipulation is necessary for $CD45^+$ isolation from HUCB. This property, together with the ability to reduce neurological deficits even when transplanted 8 days after the insult and their capacity for fast homing to the brain, proposed that $CD45^+$ should be considered for translational therapy in treating patients with brain trauma (Gincberg et al, 2013).

Neuronal progenitors (HUCBNP)

Buzanska et al. used the $CD34^-/CD45^-$ non-hematopoietic MNC fraction of HUCB to obtain a population of what appeared to be neural stem-cell-like cells (Buzanska et al, 2002). Upon differentiation of nestin-positive HUCB cells, either by direct treatment with differentiation-promoting media or by plating clone-forming cells onto poly-L-lysine-coated coverslips both with the addition of retinoic acid (RA) or in combination with brain derived neurotrophic factor (BDNF), 40% of the cells expressed markers of more advanced neuronal differentiation, β-tubulin III and microtubule associated protein (MAP-2), while 30% the astrocytic markers glial fibrillary acidic protein (GFAP) and S100β and 11% expressed the oligodendrocytic marker galactosylceramide. More recently, Jurga et al. showed that treatment of the HUCB-derived MNC fraction for 7 days with RA and BDNF also induced neural differentiation, with 80% of the cells expressing β-tubulin III and 64% co-expressing MAP-2 (Jurga et al, 2006). Xiao and colleagues isolated a line of cells from HUCB that they termed non-hematopoietic umbilical cord blood SC (nh-UCBSC) (Xiao et al, 2005). After intravenously (iv) injection of these cells into a rat model of ischemic brain injury, a 50% reduction in infarct volume was observed compared to control group. Some labeled human nuclei cells were also positive for NeuN, indicating the expression of a neuronal phenotype of transplanted cells. However, these cells were rather insignificant in the brain, suggesting effect through other mechanisms than cell replacement. Sanchez-Ramos et al. used HUCB-derived MNC cultured with RA and nerve growth factor (NGF) and measured the expression of established neural markers Musashi-1, β-tubulin III and GFAP in those cells (Sanchez-Ramos et al, 2001). Furthermore, Zigova et al. showed that MNC fraction of HUCB treated with RA and NGF survived in the subventricular zone of the rat neonatal forebrain for a month and was positive for TuJ1 and GFAP (Zigova et al, 2002). Characterization of cell populations within cultured HUCB indicated the expression of neurotrophin receptors TrkB and TrkC in both the non-adherent and adherent cellular fractions (Chen et al, 2005). McGuckin et al. induced neural differentiation of HUCB using a three-step protocol (cell isolation, expansion, and differentiation). Their isolation procedure yielded a purified fraction of small ($CD7^-$, $CD33^-$, $CD45^-$, $CD235a^-$) putative SC that co-express embryonic SC markers Oct4 and Sox2 and termed embryonic-like stem cells. Initial expansion was performed in high density cultures (5–10 million cells per 1 ml) in which the cells were grown on fibronectin and collagen type IV coated surfaces and treated with epidermal growth factor. Subsequent neural differentiation as assessed by the expression of neural markers such as nestin, GFAP, MAP-2 and TUJI, required sequential treatment with RA, BDNF and cyclic AMP (McGuckin et al, 2008).

Our laboratory described the isolation of a neuronal population from the MNC fraction of HUCB using yet another approach (Arien-Zakay et al, 2007). Selection of HUCB-contained neuronal progenitors (HUCBNP) was based on cell adherence to type I and IV collagens. Collagen-adherent MNC were also nestin-positive progenitors expressing α_1 (CD49a) and α_2 (CD49b) integrin receptors and survived in conventional monolayer culture for more than 14 days. *In vitro* differentiation of HUCBNP

was induced by treatment with 10% human SH-SY5Y neuroblastoma cell-conditioned media (CM) supplemented with 10 ng/ml NGF. A majority of the surviving progenitors (83±8.2%) acquired a neuronal-like morphology, i.e. they developed neurite-like cellular outgrowths of different lengths. About 35±6% of the differentiating HUCBNP had outgrowths with a length/cell diameter ratio greater than 2, typical of developing neurons. The majority of these progenitors expressed common neuronal markers such as MAP-2, NGF-neurotrophin receptor TrkA, neurofilament-160 (NF-160), β-tubulin III, and neuron-specific enolase (NSE). The identity of the progenitors was further established by assessing gene expression using Affymetrix microarray technology. The "naïve" progenitors were negative for the hematopoietic markers: CD34, CD49c, CD49d, CD62e, CD62p, CD106, CD117, CD133, CD235a, HLA-DRB4, HAS1, and positive for the mesenchymal markers: CD13, CD29, CD44, CD49a,b, CD49e, CD73, CD105 and vimentin (Arien-Zakay et al, 2009a). Combined treatment with CM and NGF induced constitutive activation of the mitogen-activated protein kinases ERK2, p38α and p38β, most likely related to survival and/or differentiation (Arien-Zakay et al, 2007). Affimatrix™ DNA microarray analysis of gene expression in HUCBNP committed towards neural differentiation clearly indicated that 28% of the induced genes were related to neural differentiation, while 35% of suppressed genes were related to hematopoiesis. This genomic analysis focused on the significant transcriptome shift of the human cord blood neuronal progenitors from hematopoiesis to neural differentiation upon cell adhesion to collagens and treatment with CM and NGF (Figure 1).

A key component of in the CM used for the induction of differentiation of HUCBNP was interferon gamma (IFN-γ). A neutralizing antibody against IFN-γ significantly inhibited either IFN-γ or CM-induced differentiation. Analysis of the transcriptome in CM-differentiated HUCBNP, identified 25 genes that were induced by IFN-γ, such as 2,5-oligoadenylate synthetase 1 and 2 and STAT1 (IFN-γ-receptor signal transducer and activator of transcription), hallmark genes under IFN-γ regulation (Saha et al, 2010). Treatment of HUCBNP with human recombinant IFN-γ, inhibited cell proliferation in a dose-dependent manner and enhanced neuronal differentiation, as assessed by neurite outgrowth and increased expression of the neuronal markers β-tubulin III, MAP-2, neuronal nuclei (NeuN), neurofilament-M (NF-M) and NSE. IFN-γ additively cooperated with NGF to induce the differentiation of HUCBNP. These findings indicate that IFN-γ and NGF are two well-defined factors that promote neuronal differentiation of HUCB-derived progenitors, suggesting their use in future protocols towards neuronal progenitor/SC-based therapies (Arien-Zakay et al, 2009a).

Divya and colleagues recently reported that HUCB-derived MSC contain a unique population of progenitor cells co-expressing mesenchymal and neuronal markers. Interestingly, these progenitors were capable of instantaneous neuronal differentiation, suggesting the commitment of early neuronal progenitors within pluripotent MSC populations (Divya et al, 2012), as also proposed earlier by us (Arien-Zakay et al, 2009a).

In order to further optimize the survival and neuronal differentiation of HUCBNP, we cultured these neuronal progenitors in three-dimensional (3D) collagen constructs (Bercu et al, 2012). In contrast to conventional two-dimensional (2D) culture conditions in which their life-span was limited up to 20 days (Arien-Zakay et al, 2007) the cells survived in 3D for more than 2 months (Bercu et al, 2012). Importantly, under 3D conditions, HUCBNP underwent spontaneous neuronal differentiation, which was further enhanced by treatment of the constructs with CM and NGF. Neurite outgrowth was significantly enhanced under 3D conditions in the presence of CM/NGF, concomitant with a reduced expression of the early neuronal marker nestin, and increased levels of mature neuronal markers such as MAP-2, β-tubulin III and NSE and the appearance of the synaptic marker synaptophysin. To assess the feasibility for clinical usage, we also isolated HUCBNP from frozen HUCB samples, (stored for up to 6 months) and cultured the cells under 3D conditions. Our data suggest that the neurotrophic (survival) and neurotropic (neurite outgrowth) properties are essentially fully preserved in frozen cells. We believe that 3D culture conditions are essential for both maintenance of HUCB neuronal progenitors *in vitro* and for investigating specific

features of neuronal differentiation and that these constructs may be an indispensible step towards future use in regenerative therapy (Bercu et al, 2012).

As yet unknown is whether these *in vitro*-produced, HUCB-derived neuronal cells function similar to their *in vivo*-derived counterparts. Each laboratory may be using phenotypically distinct NSC, since these cells have been differentiated by different sets of neurotrophins or chemicals and/or cultured on different ECM proteins (Jurga et al, 2011; Zychowicz et al, 2011), therefore differing in the expression of neuronal markers, and potentially in their identity and functionality. Recently, an interesting attempt was made to reverse the HUCB-NSC phenotype to the pluripotent state by using small molecules representing epigenetic modulators (histone deacetylase inhibitor Trichostatin A and DNA methyltransferase inhibitor RG-108) in conjunction with "classical" reprogramming factors (OCT4, SOX2 and KLF4) to generate induced pluripotent stem cells (iPS). This study indicates the plasticity of HUCB neural progenitors and their amenability for manipulation in differentiation directions (Szablowska-Gadomska et al, 2012).

IN VITRO NEUROPROTECTIVE EFFECTS OF HUCB, HUCB-DERIVED SC AND OF NGF

We used oxygen-glucose-deprivation (OGD)/reoxygenation-induced injury in rat PC12 pheochromocytoma as an established ischemia model (Tabakman et al, 2002; Tabakman et al, 2004a; Tabakman et al, 2004b; Tabakman et al, 2005), to test for the neuroprotective effect of both HUCB cells and HUCBNP. Using both undifferentiated (dopaminergic) or NGF-differentiated (adrenergic) PC12 cells, we have employed this model in the past to measure the neuroprotective effects of the anti-Parkinsonian drug Rasagiline (Azilect) (Abu-Raya et al, 1999), as well as those of antioxidants, such as Carnosine, Homocarnosine and Tempol (Tabakman et al, 2002). Subsequently, we refined this model to measure cell–cell-induced neuroprotection, using a double chamber approach, enabling the addition of HUCBNP with spatial separation from the PC12 cells at any time during the insult. With the ability to remove HUCBNP from the system we can assess apoptotic/necrotic cell death in OGD/reoxygenation-insulted PC12 cells by respectively measuring caspase-3 activity and LDH release. In our pharmacologically validated model (Tabakman et al, 2005) the HUCBNP conferred neuroprotection upon mild insult (20–50% cell death), independently of their state of differentiation and irrespective of whether or not they were in direct contact with the insulted PC12 cells. The data strongly suggest that the observed neuroprotection is mediated by soluble factors, rather than by cell-cell contact (Arien-Zakay et al, 2009b). The neuroprotective effect of HUCBNP in OGD/reoxygenation-insulted PC12 cells was similar to the neuroprotective effect of the HUCB-derived MNC, from which the HUCBNP were isolated. This was shown in primary rat cortical neuronal cultures exposed to hypoxia, in hippocampal slice cultures exposed to OGD/reoxygenation (Vendrame et al, 2005), as well as in differentiated neuroblastoma SH-SY5Y cells exposed to hypoxia *in vitro* (Hau et al, 2008).

HUCBNP-induced neuroprotection was correlated with suppression of reactive oxygen species (ROS) in the OGD/ reoxygenation-insulted PC12 cells and the secretion of antioxidant(s) and NGF into the culture medium. ELISA assays with different specificities for rat or human NGFs clearly indicated that the NGF in the medium was derived from both the OGD/reoxygenation-insulted PC12 and the neuroprotective human HUCBNP cells. The CM of the co-cultures also induced neuroprotection, most probably due to NGF presence, as evident from the inhibitory effect of K252a, an NGF receptor antagonist (Arien-Zakay et al, 2009b).

NGF is the prototype of the neurotrophin family of growth factor molecules. NGF regulates the growth, development, and plasticity of cholinergic neuronal populations in the nervous system (Arien-Zakay et al, 2011). Neuronal NGF expression *in vivo* is markedly upregulated by seizure, forebrain ischemia, marked hypoglycemia and tissue injury (Gall and Isackson, 1989; Zafra et al, 1991; Lindvall et al, 1994). Studies *in vivo* and *in vitro* indicate that cerebral insults modulate NGF gene expression via excitatory amino acid receptors, as well as through other pathways (Lindvall et al, 1994). The functional consequences of the increased production of NGF and other neurotrophic factors following brain injury might represent part of the intrinsic neuroprotective

signals (Lindvall et al, 1994; Arien-Zakay et al, 2011). Among its different functions, NGF signaling appears to play important roles in neural repair in response to injury or disease (Arien-Zakay et al, 2011). NGF induced neuroprotection in the PC12 ischemic model supports the role of NGF as a neuroprotective agent in the nervous system as previously documented in variety of other neuronal insults (Tabakman et al, 2004c) and in the neuroprotective effect of HUCB cells (Arien-Zakay et al, 2011).

NGF is known to be released from PC12 cells by autocrine regulation (Gill et al, 1998) and to confer neuroprotection in OGD/reoxygenation-insulted PC12 cells (Boniece and Wagner, 1993), a phenomenon, which might be related to the attenuation of MAPK isoforms (c-Jun amino-terminal kinase 1 and stress-activated kinases p38α and p38β) (Tabakman et al, 2004b). One of the potential mechanisms for its neuroprotective effect in our *in vitro* ischemia model as well as in an *in vivo* stroke model (Andsberg et al, 2002), may be ability of NGF to suppress intracellular ROS accumulation by rapid activation of antioxidants defenses in neurons (Kirkland et al, 2007).

NEUROPROTECTIVE EFFECTS OF HUCB IN A BRAIN TRAUMA MOUSE MODEL

Following ischemic insult, a small fraction of iv administered HUCB-derived MNC, is able to migrate to/survive at the site of injury in the brain and improve functional recovery (Meier et al, 2006; Gincberg et al, 2013). Since HUCB cells are readily available and have been used clinically for hematopoietic-related illnesses, they or some of their derivatives, such as HUCBNP, that confer neuroprotection *in vitro*, may represent a suitable source of cells for the treatment of ischemic brain injury. We hypothesized that the release of neurotrophic and angiogenic factors (Cho et al, 2006; Alexanian et al, 2008) and/or antioxidants *in vivo*, as described for the *in vitro* scenario, would be highly specific and locally limited to the damaged brain area contributing to the observed neuroprotection conferred by HUCB-derived MNC. To test this hypothesis, first we implanted unpurified HUCB-derived MNC locally into the lesion cavity of mice brains 1- or 8-days after CHI brain trauma. We found significant improvement in functional outcome in terms of the motor and modified neurological severity score (mNSS) in the animals receiving HUCB-derived MNC (Gincberg et al, 2013). In order to identify the active population in the MNC fraction, we separated the cells according to their CD45 expression, for hematopoietic CD45-positive and non-hematopoietic CD45-negative fractions. Only the CD45-positive fraction conferred neuroprotection and improvement in the mNSS (Gincberg et al, 2013).

NGF INDUCED PROTECTION ON MYOCARDIAL ISCHEMIC REPERFUSION INFARCT

In addition to its neuroprotective role in the CNS (following ischemic stroke and brain trauma) it is important to emphasize that NGF also supports the survival and differentiation of sympathetic neurons in the heart. Increased NGF expression after myocardial infarct was correlated with cardiac sympathetic hyper-innervation and arrhythmias (Govoni et al, 2011). We recently reported that NGF protects the rat heart after exposure to ischemia/reperfusion injury (IRI) (Strande et al, 2013). NGF was administered iv 15 min before ischemia, at different concentrations (0.015-15 μg/kg body weight) in the absence or presence of inhibitors of phosphatidylinositol-3 kinase (PI3K) or nitric oxide synthase (NOS) in rats with left coronary occlusion for 30 min followed by 120 min reperfusion. At an optimal dose (0.15 μg/kg) NGF treatment significantly reduced the infarct size tantamount to 60% cardioprotection. This beneficial effect of NGF was partially abolished by the inhibition of PI3K and NOS using wortmannin and *N* (G)-monomethyl-l-arginine, respectively. These findings extend the neuroprotective role of NGF in the CNS (ischemic stroke and TBI) to the potential for neuroprotection outside of CNS, for example following myocardial infarction.

CONCLUSIONS

In regenerative medicine to date, MSC (Trounson et al, 2011), including HUCB-derived MSC (Zanier et al, 2011) represent the most active SC population for pre-clinical and clinical therapeutic trials of

brain injury. While the majority of clinical trials using MSC have proven safety in phase I, many of the phase II clinical trials, addressing efficacy, are still ongoing. The translation of basic research data to clinical application in TBI patients has invariably failed, and results from prospective clinical trials are disappointing. This is in part due to the short temporal window of opportunity for intervention and failure of many candidate drugs to cross the blood-brain barrier (Menon, 2009). To date, single and double HUCB units are used in the clinics for therapy of hematopoietic-related disorders, however there is no FDA approval, yet, for the clinical utilization of HUCB-derived isolated populations such as MSC, since minimally manipulated, unrelated allogeneic placental/HUCB are intended for hematopoietic reconstitution for specified indications (FDA Draft HPC-C IND Guidance).

Recent studies from a number of laboratories, including ours, showed that HUCB-derived MNC can act as a neuroprotective agent in brain trauma and other neurodegenerative disorders, such as stroke (Meier et al, 2006; Arien-Zakay et al, 2011; Zanier et al, 2011; Gincberg et al, 2013). As of today, only a limited narrow therapeutic window of 4-6 h has been established for experimental clinical intervention and no pharmacologically agent was found to demonstrate therapeutic effect beyond this window. A unique and novel finding from our laboratory extends the window up to 8 days post-trauma by given locally, in the damaged brain area, HUCB-derived MNC 8 days following the onset of TBI. Moreover, profound behavioral improvement was observed in this model upon systemic iv infusion of HUCB-derived hematopoietic ($CD45^+$) fraction (Gincberg et al, 2013). The HUCB-derived $CD45^+$ is being considered by medical community for therapy of hematopoietic and non-hematopoietic disorders, since it can be easily isolated and is the most abundant in the mononuclear fraction. We suggest that this particular fraction has promising therapeutic potential for treating TBI. The administration of HUCB-derived populations most likely inhibits the apoptotic cascade and/or locally modulates the immune/inflammatory response to the injury, providing neuroprotection (Figure 2).

Hence, HUCB-derived populations provide neuroprotection most probably through a "bystander effect", i.e. by secretion of soluble factors, such as NGF and other anti-inflammatory growth factors (Arien-Zakay et al, 2009b; Gincberg et al, 2012; Gincberg et al, 2013; Figure 2). Most likely, HUCB cells provide, in addition to NGF, a complex arsenal of therapeutic growth factors (Arien-Zakay et al, 2009b; Neuhoff et al, 2007) that no single pharmacological agent could mimic. The ability of HUCB-derived populations to synthesize and secrete NGF and other neurotrophins in an ischemic environment provides circumstantial evidence for the important neuroprotective role of NGF. If indeed this is the case, adjuvant therapy with human recombinant NGF and other growth factors may be also considered in order to reach an effective dose for neuroprotection. NGF has an important therapeutic role in the treatment of both stroke and myocardial infarct (Strande et al, 2013; Figure 2), a possibility which deserves further pre-clinical and clinical investigation.

There is a growing agreement regarding the neuroprotective potential of HUCB-derived populations in ischemic disorders (Figure 2). Establishing their mechanism of action *in vitro* concomitant with ascertaining *in vivo* efficacy, safety and conditions for transplantation will be crucial before HUCB-derived populations can be utilized for therapy of ischemic diseases in a clinical setting.

ACKNOWLEDGMENTS

This review is dedicated to the memory of Prof. Rita Levi-Montalcini, who will always be remembered for her seminal scientific contribution to the field of neurotrophins as well as for her intellectual insights, integrity, and friendship. This review is part of a PhD thesis to be submitted by G.G. to The Hebrew University of Jerusalem. P.L. holds the Jacob Gitlin Chair in Physiology and is affiliated with and partially supported by David R. Bloom Center for Pharmacy and the Dr. Adolf and Klara Brettler Center for Research in Molecular Pharmacology and Therapeutics at the Hebrew University of Jerusalem, Israel. PIL is the Laura H. Carnell Professor and Chair, Dept. Bioengineering and Director of the Institute for Regenerative Medicine and Engineering at Temple University Philadelphia, PA.

REFERENCES

Abu-Raya S, Blaugrund E, Trembovler V, Shilderman-Bloch E, Shohami E, Lazarovici P (1999) Rasagiline, a monoamine oxidase-B inhibitor, protects NGF-differentiated PC12 cells against oxygen-glucose deprivation. *J Neurosci Res* **58**: 456-463.

Alexanian AR, Maiman DJ, Kurpad SN, Gennarelli TA (2008) In vitro and in vivo characterization of neurally modified mesenchymal stem cells induced by epigenetic modifiers and neural stem cell environment. *Stem Cells Dev* **17**: 1123-1130.

Ali H, Bahbahani H (2010) Umbilical cord blood stem cells – potential therapeutic tool for neural injuries and disorders. *Acta Neurobiol Exp* **70**: 316–324

Andsberg G, Kokaia Z, Klein RL, Muzyczka N, Lindvall O, Mandel RJ (2002) Neuropathological and behavioral consequences of adeno-associated viral vector-mediated continuous intrastriatal neurotrophin delivery in a focal ischemia model in rats. *Neurobiol Dis* **9**: 187-204.

Arien-Zakay H, Nagler A, Galski H, Lazarovici P (2007) Neuronal conditioning medium and nerve growth factor induce neuronal differentiation of collagen-adherent progenitors derived from human umbilical cord blood. *J Mol Neurosci* **32**: 179-191.

Arien-Zakay H, Lecht S, Bercu MM, Amariglio N et al. and Nagler A (2009a) Interferon-gamma-induced neuronal differentiation of human umbilical cord blood-derived progenitors. *Leukemia* **23**: 1790-1800.

Arien-Zakay H, Lecht S, Bercu MM, Tabakman R et al. and Lazarovici P (2009b) Neuroprotection by cord blood neural progenitors involves antioxidants, neurotrophic and angiogenic factors. *Exp Neurol* **216**: 83-94.

Arien-Zakay H, Lazarovici P, Nagler A (2010a) Tissue regeneration potential in human umbilical cord blood. *Best Pract Res Clin Haematol* **23**: 291-303.

Arien-Zakay H, Lecht S, Nagler A, Lazarovici P (2010b) Human umbilical cord blood stem cells: rational for use as a neuroprotectant in ischemic brain disease. *Int J Mol Sci* **11**: 3513-28.

Arien-Zakay H, Lecht S, Nagler A, Lazarovici P (2011) Neuroprotection by human umbilical cord blood-derived progenitors in ischemic brain injuries. *Arch Ital Biol* **149**: 233-45.

Beauchamp K, Mutlak H, Smith WR, Shohami E, Stahel PF (2008) Pharmacology of traumatic brain injury: where is the 'golden bullet'? *Mol Med* **14**: 731–740.

Bercu MM, Arien-Zakay H, Stoler D, Lecht S et al. and Elchalal U (2012) Enhanced Survival and Neurite Network Formation of Human Umbilical Cord Blood Neuronal Progenitors in Three-Dimensional Collagen Constructs. *J Mol Neurosci* [Epub ahead of print].

Broxmeyer HE, Douglas GW, Hangoc G Cooper S et al. and Boyse EA (1989) Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. *Proc Natl Acad Sci U S A* **86** :3828–3832.

Boniece IR, Wagner JA (1993) Growth factors protect PC12 cells against ischemia by a mechanism that is independent of PKA, PKC, and protein synthesis. *J Neurosci* **13**: 4220-4228.

Bordet R, Lestage P, Onteniente B (2007) Neuroprotection: Present and future. *Therapie* **62**: 473-476.

Bramlett HM, Dietrich WD (2007) Progressive damage after brain and spinal cord injury: pathomechanisms and treatment strategies. *Prog Brain Res* **161**: 125-141.

Buzańska L, Machaj EK, Zabłocka B, Pojda Z, Domańska-Janik K (2002) Human cord blood-derived cells attain neuronal and glial features in vitro. *J Cell Sci* **115**: 2131-2138.

Cardoso AA, Li ML, Batard P, Hatzfeld A et al. and Hatzfeld J (1993) Release from quiescence of CD34+ CD38- human umbilical cord blood cells reveals their potentiality to engraft adults. *Proc Natl Acad Sci U S A* **90**: 8707-8711.

Chen J, Li Y, Chopp M (2000) Intracerebral transplantation of bone marrow with BDNF after MCAO in rat. *Neuropharmacology* **39**: 711-716.

Chen J, Li Y, Katakowski M, Chen X et al. and Chopp M (2003) Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res* **73**: 778-786.

Chen N, Hudson JE, Walczak P, Misiuta I et al. and Willing AE (2005) Human umbilical cord blood progenitors: the potential of these hematopoietic cells to become neural. *Stem Cells* **23**: 1560-1570.

Chen Y, Constantini S, Trembovler V, Weinstock M, Shohami E (1996) An Experimental model of closed head injury in mice: Pathophysiology, histopathology and cognitive deficits. *J Neurotrauma* **13**: 557-568.

Cho SW, Gwak SJ, Kang SW, Bhang SH et al. and Kim BS (2006) Enhancement of angiogenic efficacy of human cord blood cell transplantation. *Tissue Eng* **12**: 1651-1661.

Conrad PD, Emerson SG (1998) Ex vivo expansion of hematopoietic cells from umbilical cord blood for clinical transplantation. *J Leukoc Biol* **64**: 147-155.

Divya MS, Roshin GE, Divya TS, Rasheed VA et al. and Pillai RM (2012) Umbilical cord blood-derived mesenchymal stem cells consist of a unique population of progenitors co-expressing mesenchymal stem cell and neuronal markers capable of instantaneous neuronal differentiation. *Stem Cell Res Ther* **3**: 57.

Flierl MA, Stahel PF, Beauchamp KM, Morgan SJ, Smith WR, Shohami E (2009) Mouse closed head injury model induced by a weight-drop device. *Nat Protoc* **4**: 1328-1337.

Gall CM, Isackson PJ (1989) Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. *Science* **245**: 758-761.

Gill JS, Schenone AE, Podratz JL, Windebank AJ (1998) Autocrine regulation of neurite outgrowth from PC12 cells by nerve growth factor. *Brain Res Mol Brain Res* **57**: 123-131.

Gincberg G, Arien-Zakay H, Lazarovici P, Lelkes PI (2012) Neural stem cells: therapeutic potential for neurodegenerative diseases. *Br Med Bull* **104**:7-19.

Gincberg G, Arien-Zakay H, Liraz-Zaltsman S, Nagler A et al. and Philip Lazarovici (2013) Human cord blood $CD45^+$ cells protect mice brain after closed head injury. Submitted for publication.

Gluckman E, Rocha V (2005) History of the clinical use of umbilical cord blood hematopoietic cells. *Cytotherapy* **7**: 219-227.

Goldstein G, Toren A, Nagler A (2007) Transplantation and other uses of human umbilical cord blood and stem cells. *Curr Pharm Des* **13**: 1363-73.

Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA (2001) Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. *Biol Blood Marrow Transplant* **7**: 581-8.

Govoni S, Pascale A, Amadio M, Calvillo L et al. and Vanoli E (2011) NGF and heart: is there a role in heart disease? *Pharmacol Res* **63**: 266 – 77.

Han EY, Chun MH, Kim ST, Lim DP (2013) Injection time-dependent effect of adult human bone marrow stromal cell transplantation in a rat model of severe traumatic brain injury. *Curr Stem Cell Res Ther* **8**: 172-81.

Harris DT (2008) Cord blood stem cells: a review of potential neurological applications. *Stem Cell Rev* **4**: 269-74.

Hau S, Reich DM, Scholz M, Naumann W, Emmrich F, Kamprad M, Boltze J (2008) Evidence for neuroprotective properties of human umbilical cord blood cells after neuronal hypoxia in vitro. *BMC Neurosci* **9**: 30.

Huntington ND, Tarlinton DM (2004) CD45: direct and indirect government of immune regulation. *Immunol Lett* **94**: 167-174.

Jaatinen T, Laine J (2007) Isolation of mononuclear cells from human cord blood by Ficoll-Paque density gradient. *Curr Protoc Stem Cell Biol*. **Chapter 2**: Unit 2A.1.

Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* **32**: 414-425.

Jurga M, Markiewicz I, Sarnowska A, Habich A et al. and Domanska-Janik K (2006) Neurogenic potential of human umbilical cord blood: neural-like stem cells depend on previous long-term culture conditions. *J Neurosci Res* **83**: 627-637.

Jurga M, Dainiak MB, Sarnowska A, Jablonska A et al. and McGuckin CP (2011) The performance of laminin-containing cryogel scaffolds in neural tissue regeneration. *Biomaterials* **32**: 3423-34.

Kazantsev AG (2007) Cellular pathways leading to neuronal dysfunction and degeneration. *Drug News Perspect* **20**: 501-509.

Kirkland RA, Saavedra GM, Franklin JL (2007) Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal apoptosis: evidence for a role in cytochrome c redistribution. *J Neurosci* **27**: 11315-11326.

Kranz A, Wagner DC, Kamprad M, Scholz M et al. and Boltze J (2010) Transplantation of placenta-derived mesenchymal stromal cells upon experimental stroke in rats. *Brain Res* **1315**: 128-36.

Lee MW, Jang IK, Yoo KH, Sung KW, Koo HH (2010) Stem and progenitor cells in human umbilical cord blood. *Int J Hematol* **92**: 45-51.

Leker RR, Shohami E (2002) Cerebral ischemia and trauma-different etiologies yet similar mechanisms: neuroprotective opportunities. *Brain Res Brain Res Rev* **39**: 55-73.

Lindvall O, Kokaia Z, Bengzon J, Elmér E, Kokaia M (1994) Neurotrophins and brain insults. *Trends Neurosci* **17**: 490-496.

Lo EH (2008) Experimental models, neurovascular mechanisms and translational issues in stroke research. *Br J Pharmacol* **1**: S396-405.

McGuckin C, Jurga M, Ali H, Strbad M, Forraz N (2008) Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. *Nat Protoc* **3**: 1046-1055.

Meier C, Middelanis J, Wasielewski B, Neuhoff S et al. and Jensen A (2006) Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatr Res* **59**: 244-249.

Menon DK (2009) Unique challenges in clinical trials in traumatic brain injury. *Crit Care Med* **37**: S129-135.

Neuhoff S, Moers J, Rieks M, Grunwald T, Jensen A, Dermietzel R, Meier C (2007) Proliferation, differentiation, and cytokine secretion of human umbilical cord blood-derived mononuclear cells in vitro. *Exp Hematol* **35**: 1119-1131.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK et al. and Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143-7.

Robinson S, Niu T, de Lima M, Ng J et al. and Shpall E (2005) Ex vivo expansion of umbilical cord blood. *Cytotherapy* **7**: 243-250.

Saha B, Jyothi Prasanna S, Chandrasekar B, Nandi D (2010) Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine* **50**: 1-14.

Sanchez-Ramos JR, Song S, Kamath SG, Zigova T et al. and Sanberg PR (2001) Expression of neural markers in human umbilical cord blood. *Exp Neurol* **171**: 109-115.

Shahrokhi S, Menaa F, Alimoghaddam K, McGuckin C, Ebtekar M (2012) Insights and hopes in umbilical cord blood stem cell transplantations. *J Biomed Biotechnol* **2012**: 572821.

Shivtiel S, Lapid K, Kalchenko V, Avigdor A et al. and Lapidot T (2011) CD45 regulates homing and engraftment of immature normal and leukemic human cells in transplanted immunodeficient mice. Exp Hematol. **39**: 1161-1170.

Stanevsky A, Shimoni A, Yerushalmi R, Nagler A (2010) Double umbilical cord blood transplant: more than a cell dose? *Leuk Lymphoma* **51**: 975-982.

Stoll G, Jander S (1999) The role of microglia and macrophages in the pathophysiology of the CNS. *Prog Neurobiol* **58**: 233-247.

Strande JL, Routhu KV, Lecht S, Lazarovici P (2013) Nerve growth factor reduces myocardial ischemia/reperfusion injury in rat hearts. *J Basic Clin Physiol Pharmacol* **24**: 81-4.

Stumm RK, Rummel J, Junker V, Culmsee C et al. and Schulz S (2002) A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. *J Neurosci* **22**: 5865-5878.

Sueblinvong V, Loi R, Eisenhauer PL, Bernstein IM, Suratt BT, Spees JL, Weiss DJ (2008) Derivation of Lung Epithelium from Human Cord Blood–derived Mesenchymal Stem Cells. *Am J Respir Crit Care Med* **177**: 701-11.

Szablowska-Gadomska I, Sypecka J, Zayat V, Podobinska M, Pastwinska A, Pienkowska-Grela B, Buzanska L (2012) Treatment with small molecules is an important milestone towards the induction of pluripotency in neural stem cells derived from human cord blood. *Acta Neurobiol Exp (Wars)* **72**: 337-50.

Tabakman R, Lazarovici P, Kohen R (2002) Neuroprotective effects of carnosine and homocarnosine on pheochromocytoma PC12 cells exposed to ischemia. *J Neurosci Res* **68**: 463-9.

Tabakman R, Jiang H, Levine RA, Kohen R, Lazarovici P (2004a) Apoptotic characteristics of cell death and the neuroprotective effect of homocarnosine on pheochromocytoma PC12 cells exposed to ischemia. *J Neurosci Res* **75**: 499-507.

Tabakman R, Jiang H, Schaefer E, Levine RA, Lazarovici P (2004b) Nerve growth factor pretreatment attenuates oxygen and glucose deprivation-induced c-Jun amino-terminal kinase 1 and stress-activated kinases p38alpha and p38beta activation and confers neuroprotection in the pheochromocytoma PC12 Model. *J Mol Neurosci* **22**: 237-50.

Tabakman R, Lecht S, Sephanova S, Arien-Zakay H, Lazarovici P (2004c) Interactions between the cells of the immune and nervous system: neurotrophins as neuroprotection mediators in CNS injury. *Prog Brain Res* **146**: 387-401.

Tabakman R, Jiang H, Shahar I, Arien-Zakay H, Levine RA, Lazarovici P (2005) Neuroprotection by NGF in the PC12 in vitro OGD model: involvement of mitogen-activated protein kinases and gene expression. *Ann N Y Acad Sci* **1053**: 84-96.

Tamaki S, Eckert K, He D, Sutton R et al. and Uchida N (2002) Engraftment of sorted/expanded human central nervous system stem cells from fetal brain. *J Neurosci Res* **69**: 976-986.

Trounson A, Thakar RG, Lomax G, Gibbons D (2011) Clinical trials for stem cell therapies. *BMC Med* **9**:52.

Tse W, Laughlin MJ (2005) Umbilical cord blood transplantation: a new alternative option. *Hematology Am Soc Hematol Educ Program* **2005**: 377-383.

Vendrame M, Gemma C, de Mesquita D, Collier L et al. and Willing AE (2005) Anti-inflammatory effects of human cord blood cells in a rat model of stroke. *Stem Cells Dev* **14**: 595-604.

Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM (2003) Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol* **121**: 368-374.

Willing AE, Lixian J, Milliken M, Poulos S et al. and Sanberg PR (2003) Intravenous versus intrastriatal cord blood administration in a rodent model of stroke. *J Neurosci Res* **73**: 296-307.

Xiao J, Nan Z, Motooka Y, Low WC (2005) Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury. *Stem Cells Dev* **14**: 722-733.

Zafra F, Castrén E, Thoenen H, Lindholm D (1991) Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proc Natl Acad Sci U S A* **88**: 10037-10041.

Zanier ER, Montinaro M, Vigano M, Villa P et al. and De Simoni MG (2011) Human umbilical cord blood mesenchymal stem cells protect mice brain after trauma. *Crit Care Med* **39**: 2501-2510.

Zhang C, Li Y, Chen J, Gao Q, Zacharek A, Kapke A, Chopp M (2006) Bone marrow stromal cells upregulate expression of bone morphogenetic proteins 2 and 4, gap junction protein connexin-43 and synaptophysin after stroke in rats. *Neuroscience* **141**: 687-695.

Zigova T, Song S, Willing AE, Hudson JE et al. and Sanberg PR (2002) Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. *Cell Transplant* **11**: 265-274.

Zychowicz M, Mehn D, Ruiz A, Colpo P et al. and Buzanska L (2011) Proliferation capacity of cord blood derived neural stem cell line on different micro-scale biofunctional domains. *Acta Neurobiol Exp (Wars)* **71**: 12-23.

TAKING PAIN OUT OF NGF: PAINLESS NGF FOR ALZHEIMER'S DISEASE THERAPY

A. CATTANEO[1,2] and F. MALERBA[1,2]

[1]*European Brain Research Institute, Rome, Italy;* [2]*Scuola Normale Superiore, Pisa, Italy*

Recent data from different lines of research point to an imbalance in the homeostasis of the NGF system as an upstream driver for Alzheimer's neurodegenerations. In this framework, the first therapeutic choice would be to use NGF itself as a drug. However, it is a challenge to deliver NGF into the brain, in a safe and efficient manner and a clinical application of NGF requires solving two major problems: effective CNS delivery, since NGF does not readily cross the blood-brain-barrier, and, second, the pronociceptive actions of NGF, that is a potent pain sensitizing agent. Inspired by a genetic mutation in the NGF gene, that is found in patients suffering from a rare genetic congenital form of insensitivity to pain (HSAN V), we have engineered painless NGF, a recombinant form of NGF that is traceable against endogenous NGF, has equal neurotrophic potency to NGF but has a 10 fold lower nociceptive activity with respect to NGF. The painless hNGF P61 R100 traceable NGF molecule represents a candidate drug that has the potential for being developed not only into a disease-modifying non invasive therapy for Alzheimer's Disease, as well as for other therapeutic indications.

The case for NGF and Alzheimer's disease: the classical connection (cholinergic and retrograde transport)

NGF (Levi-Montalcini and Hamburger, 1951) has been classically connected to Alzheimer's Disease (AD) on a purely correlative basis, because of the selective vulnerability of basal forebrain cholinergic neurons (BFCNs) in AD (cholinergic connection) and of the retrograde transport of NGF in these neurons (retrograde transport connection). BFCNs provide major projections to the cerebral cortex and the hippocampus, subserving cognitive functions and memory. NGF is BFCN principal target-derived neurotrophic factor, protecting these neurons from various insults and from their aging dependent atrophy (Mobley et al, 1986; Li et al, 1995; Holtzman et al, 1993). Accordingly, a decreased trophic support, due to a reduced amount of NGF available to BFCNs, could contribute to the cholinergic cells loss observed in AD (Mufson et al, 1999).

NGF is produced in the cortex and hippocampus and retrogradely transported to BFCN (Mufson et al, 1999). NGF expression is not altered in AD. Several findings collectively support the view that a diminished retrograde transport of NGF can determine a reduced neurotrophic support to BFCNs (Mufson et al, 1999). A reduced capacity of TrkA-dependent retrograde transport of NGF may lead to the loss of BFCNs observed in early AD and explains the seemingly paradoxical observation that, in AD postmortem brains, NGF and proNGF proteins are increased in the cortex and hippocampus and are diminished in basal forebrain (Mufson et al, 2000; Fahnestock et al, 1996). Indeed, cytoskeletal transport dysfunctions, and a reduced axonal transport of NGF, represent a common link between NGF trophic deficits, cholinergic dysfunction, and neurodegeneration (Salehi et al, 2003; Schindowski et al, 2008), so that neurons in AD cannot take full advantage of NGF, since both APP and Tau are

Corresponding Author:
Antonino Cattaneo
European Brain Research Institute
Via del Fosso di Fiorano, 64-65 – 00143 Rome
Phone: +39 (0)650170 3118
Email: a.cattaneo@ebri.it

2279-5855 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

involved in axonal transport. However, according to this view, a reduced neurotrophic support would be downstream of APP (or Tau) alterations.

The case for NGF and Alzheimer's disease: NGF deficits as an upstream driver for neurodegeneration

Recent evidence from independent laboratories and different experimental lines point to a mechanistic role for deficits in the homeostasis of the NGF system as an upstream driver for AD neurodegeneration (Capsoni and Cattaneo, 2006).

The principal lines of evidence in support of this view are:

l) the neurodegeneration phenotype observed in AD11 mice, expressing an anti NGF antibody transgene (Capsoni et al, 2002a).

2) cell death of hippocampal neurons, in vitro, induced by removal of NGF (Matrone et al, 2008).

3) the homeostasis of the NGF system and the emerging complexity of the proNGF/NGF equilibrium.

Anti-NGF AD11 Mice as a Comprehensive Model for Sporadic Alzheimer's disease

The first demonstration that NGF deficits could have consequences beyond a direct interference with the cholinergic system came from studies in the anti-NGF AD11 mouse model (Capsoni et al, 2000; Ruberti et al, 2000). These mice express a recombinant, highly specific anti-NGF antibody in the adult brain and allow studying the effects of a chronic neutralization of NGF in the adult brain, after normal development. Quite unexpectedly, these mice progressively develop a comprehensive AD-like neurodegeneration, more severe than the expected cholinergic deficit per se, with functional and behavioral impairments, encompassing several features of human AD. The progressive impairment of working memory, revealed by a number of behavioural tasks (Berardi et al, 2007; De Rosa et al, 2005) is accompanied by synaptic plasticity deficits in the cortex (Origlia et al, 2006) and the hippocampus (Sola et al, 2006). At the neuropathological level, besides the expected deficit in BFCNs, AD11 mice show an intracellular and extracellular accumulation of β-amyloid in the hippocampus and a progressive neuronal expression of hyperphosphorylated and truncated Tau (Capsoni et al, 2002a). The neurodegeneration in AD11 mice is NGF dependent, since it can be fully reverted by NGF administration (Capsoni et al, 2002b; De Rosa et al, 2005), demonstrating that NGF sequestration by antibodies results in Alzheimer's neurodegeneration. Cholinergic drugs fail to substantially revert neurodegeneration in AD11 mice, confirming that the cholinergic neurotransmission deficit is not a primary event of the neurodegeneration cascade in AD11. Breeding AD11 mice under environmental enrichment (EE) conditions rescues their memory and neuropathological deficits (Berardi et al, 2007).

Route	Dosage (mg /kg)	% of NGF in the brain	NGF amount in the brain
Intranasal	**0.96**	**0.023**	**0.22**
Endovenous	**0.96**	**0.001**	**0.0096**

Table I. *NGF has a great potential for the treatment of AD, but the therapeutic administration of NGF represents a significant challenge, due to the difficulty to deliver relevant doses to the brain, in a safe and non-invasive way. An efficient and non-invasive delivery of NGF to the brain in animal models, is represented by the intranasal route. In the table a comparison between intranasal and intravenous routes is showed. The dose through endovenous route, needed to reach the same amount of NGF in the brain, must be 23 times higher, i.e. = 22.8 μg/kg. The dose that determined pain in humans is 1 μg/kg*

TrkA				
	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)	KA (M^{-1})
hNGF	$1.23\ 10^6$	$1.16\ 10^{-3}$	0.94	$1.06\ 10^9$
hNGFR100E	$1.34\ 10^6$	$1.93\ 10^{-3}$	1.44	$6.9\ 10^8$
hNGFP61S	$0.79\ 10^6$	$1.09\ 10^{-3}$	1.38	$7.26\ 10^8$
hNGFP61S/R100E	$1.07\ 10^6$	$1.45\ 10^{-3}$	1.35	$7.41\ 10^8$

p75NTR				
	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)	KA (M^{-1})
hNGF	$3.05\ 10^6$	$4.68\ 10^{-3}$	1.53	$6.52\ 10^8$
hNGFR100E	$4.51\ 10^4$	$5.62\ 10^{-3}$	125	$8.02\ 10^6$
hNGFP61S	$3.92\ 10^6$	$8.83\ 10^{-3}$	2.25	$4.44\ 10^8$
hNGFP61S/R100E	$5.43\ 10^4$	$10.9\ 10^{-3}$	200	$4.98\ 10^6$

Table II. *SPR analysis. Summary of the derived kinetic and equilibrium binding constants of hproNGF and hNGF and the muteins P61S and P61S/R100E towards TrkA and p75 receptors.*

EE is a complex stimulus, and its effectiveness in rescuing AD11 neurodegeneration confirms this neurodegeneration to be a multifactorial process.

In summary, AD11 mice display a comprehensive and progressive neurodegeneration reminiscent of LOAD (Late Onset Alzheimer's Disease). Unlike other transgenic AD models, and similarly to LOAD, in AD11 mice beta-amyloid pathology arises from endogenous APP, in the absence of a mutation in APP/APP processing genes. The phenotype of AD11 mice has uncovered a new mechanism whereby neurotrophic deficits are an upstream driver, causally linked to altered APP processing and Tau pathology, in addition to determining a cholinergic deficit (Capsoni and Cattaneo, 2006; Cattaneo et al, 2008).

Linking directly NGF Deficits to the Activation of the Amyloidogenic cascade

The AD11 model establishes direct links between alterations in NGF signaling and aberrant APP/Tau processing. Further independent evidence supporting this concept came from subsequent studies in cultured neurons, which allowed dissecting the precise mechanisms whereby deficits in NGF signaling determine the activation of the amyloidogenic cascade (Matrone et al, 2008). These studies also uncover a different and novel role for NGF induced TrkA signaling, in concert with APP. First, NGF-differentiated PC12 cells, deprived of NGF, were shown to undergo apoptosis by a mechanism involving activation of APP amyloidogenesis, with release and accumulation of secreted Abeta peptide, with prevalence of Aβ1-42 over Aβ1-40 peptides. The down-regulation of amyloidogenic APP processing with selective – beta and gamma -secretase inhibitors or with anti Aβ antibodies, significantly reduced the apoptotic death of NGF-deprived PC12 neurons, providing further evidence linking neuronal death

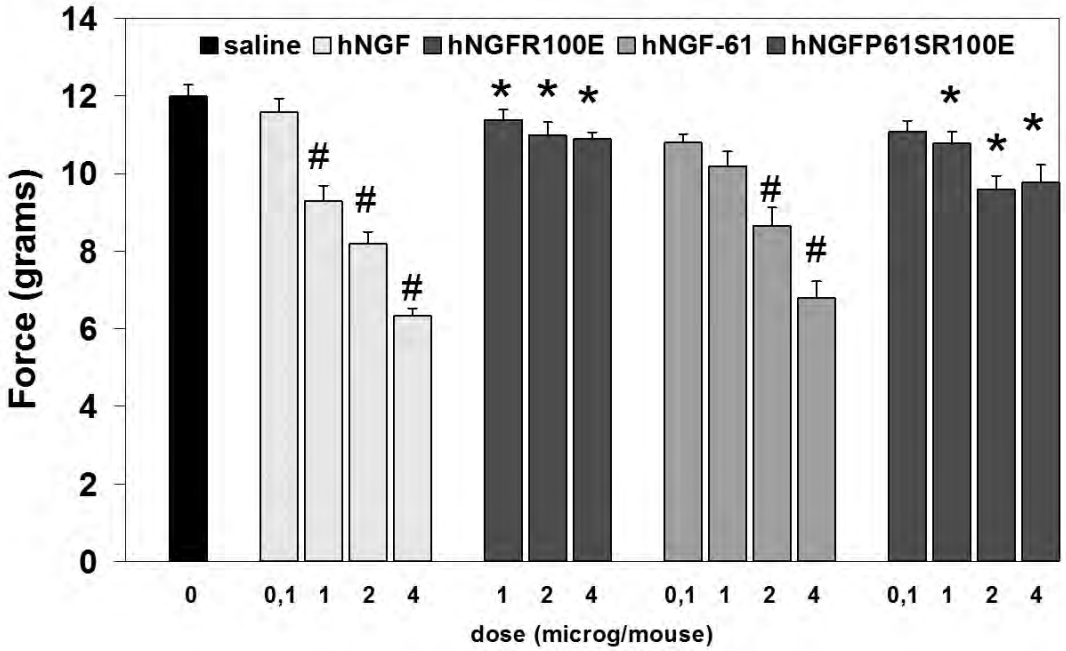


Fig. 1. *Reduced pro-nociceptive responses of hNGF muteins (mechanical allodynia): dose-response allodynic effects of hNGFR100E, hNGF P61S and hNGF P61S R100E compared to hNGF, 5 hours after intraplantar injection in the hindpaw. The allodynic response was reduced for hNGF R100E and hNGF P61S R100E compared to hNGF. (Modified from Capsoni et al. PLOS 2011)*

and amyloidogenic APP metabolism. The link between NGF deprivation and amyloidogenesis was further corroborated in NGF-deprived primary hippocampal cultures (Matrone et al, 2008). Thus, also in NGF-deprived hippocampal neurons, it was observed that: i) removal of NGF activates aberrant APP processing, with the released Abeta peptides creating a neurotoxic loop, ii) beta and gamma - secretase inhibitors - and Aβ antibodies, largely prevented not only the Aβ intra- and extra-cellular production but also neuronal death; iii) silencing the mRNA coding for APP correspondingly reduced the extent of cell death (Matrone et al, 2008).

The overproduction of Aβ in NGF deprived hippocampal neurons correlates with early, specific and temporally-restricted phosphorylation state changes of tau protein. Indeed, similar NGF-dependent modifications of site-specific tau phosphorylation have been found in the AD11 animal model, in correlation with the temporal appearance of Aβ peptide species (Capsoni et al, 2002a).

Interestingly, 24 h after NGF withdrawal, the NGF receptor TrkA, in the apoptotic hippocampal primary neurons, regained its phosphorylated state. This paradoxical, NGF-independent, delayed TrkA phosphorylation appears to represent an intracellular signaling linked to Aβ increase. Incubation of NGF-deprived neurons with TrkA antagonists, largely protect hippocampal neurons from apoptotic death. Furthermore, an identical anomalous TrkA phosphorylation was evoked in neurons by exogenously added synthetic Aβ peptide, and TrkA mRNA silencing also paradoxically favored neuronal survival, suggesting that the NGF receptor may switch from pro-survival to pro- apoptotic action in the absence of its specific ligand. Relevantly, in p75-silenced neurons, TrkA did not appear phosphorylated following NGF removal (Matrone et al, 2009), supporting previous suggestions about a p75 role as an early mediator of the toxic Aβ effect (Coulson et al, 2009). Thus, the same TrkA membrane receptor may switch from pro-survival to pro-apoptotic action, in the presence or absence of its physiological cognate ligand, suggesting a causal relationship between NGF withdrawal, amyloidogenic APP metabolism and NGF independent TrkA phosphorylation (Longo and Massa, 2005). This conclusion is further supported by evidence on a direct interaction between TrkA and APP,

crucially involving phosphorylation of APP residue Y682 (Matrone et al, 2011). These studies suggest that NGF and APP functions are strictly interconnected, probably via some sort of direct interaction, and that the NGF/TrkA signaling pathway could indeed be causally involved in AD pathogenesis.

Thus, one of the consequences of trophic factor deprivation (or other NGF signaling and processing alterations, such as proNGF/NGF imbalance, see below) of BFCN *in vivo*, could be a local action on their synaptic axonal terminals, involving the activation of the aberrant APP processing cascade.

The Emerging Complexity of proNGF/NGF Equilibrium

The emerging complexity of the proNGF/NGF system adds another element to the picture of the NGF connection to AD mechanisms.

NGF is translated as a pre-pro-protein, which is cleaved intracellularly or extracellularly by furin or plasmin and matrix metalloprotease-7, respectively (Bruno and Cuello, 2006). ProNGF and NGF have distinct biological activities (Lee et al, 2001): proNGF has a higher affinity for p75NTR and a lower one for TrkA compared to mature NGF and induces p75NTR-dependent apoptosis. ProNGF can also induce TrkA-dependent neuronal survival, although less effectively than NGF (Fahnestock et al, 2004). The prodomain of NGF interacts with sortilin, a neuronal type-1 VPS10-domain receptor, a coreceptor with p75NTR for proNGF (Goate et al, 1991). The levels of proNGF and its coreceptor sortilin increase in mild cognitive impairment and early AD brains (Counts et al, 2004; Fahnestock et al, 2001), paralleling the progressive decline in TrkA receptors and a diminished conversion of proNGF to mature NGF and an increased NGF degradation in AD brains is observed (Bruno et al, 2009). Thus, the biological activities of proNGF *versus* NGF influence the balance between cell death and cell survival (Nykjaer et al, 2005; Hempstead, 2006), and an imbalance in this complex ligand/receptor system has been correlatively linked to AD neurodegeneration, and direct proof in vivo has been obtained recently (Capsoni and Cattaneo, 2006; Tiveron et al, 2013).

The NGF binding properties of anti-NGF mAbαD11, expressed in the brain of AD11 mice, provided a first clue (Covaceuszach et al, 2008) towards a mechanistic and not correlative link between proNGF/NGF imbalance and neurodegeneration. The anti-NGF mAbαD11, expressed in AD11 brains, binds NGF with a three orders of magnitude higher affinity than that for proNGF, with binding to NGF being virtually irreversible. The preferential binding of mAbαD11 to mature NGF, with respect to proNGF, would determine, under limiting concentrations in the mouse brain, an experimentally induced functional imbalance between NGF and proNGF by irreversibly "sequestering" mature NGF while leaving proNGF free to act in the functional "absence" of mature NGF. proNGF would activate the proneurodegeneration, proamyloidogenic pathways, interacting with sortilin and p75NTR receptors (Capsoni et al, 2006). A first test of this mechanism (Capsoni et al, 2010) comes from studies in which AD11 mice have been crossed to p75NTR knock-out mice (p75NTR –/–). The resulting offspring (AD12 mice) shows a complete reversion of the Aβ phenotype, demonstrating that amyloidogenesis in the AD11 model involves proNGF/p75 signaling. Accordingly, the main determinant of neurodegeneration in anti-NGF mice would be the selective neutralization of NGF versus proNGF by an antibody in the brain: "too little NGF, too much proNGF" (Cattaneo et al, 2008). In a more recent study, transgenic mice expressing a furin-uncleavable form of proNGF in the postnatal brain were derived. As a result, proNGF mice express not only high levels of proNGF, but also higher levels than normal of mature NGF. Surprisingly, these mice develop a striking cholinergic deficit phenotype, accompanied by formation of Amyloid beta oligomers and severe learning and memory behavioral deficits (Tiveron et al, 2013).

This provides the missing link for a proNGF/NGF centered vicious cycle, integrating data on Amyloid beta inducing dysmetabolism of proNGF (Bruno and Cuello, 2006; Bruno et al, 2009) and on proNGF increase in AD brains (Fahnestock et al, 2001).

NERVE GROWTH FACTOR AND ALZHEIMER'S DISEASE: THE "NEW" STORY

The experimental studies on NGF deficit induced neurodegeneration in transgenic mice, as well as the mechanistic studies on the anti amyloidogenic actions of NGF/TrkA signalling in primary neuronal

cultures demonstrated a novel causal link between neurotrophic signaling deficits and Alzheimer's neurodegeneration. Given these results, a new NGF hypothesis could proposed, with neurotrophic deficits of various types representing an upstream driver of core AD triad pathology (Cattaneo et al, 2008; Cattaneo and Calissano, 2012). Thus, AD neurodegeneration would arise from alterations of the homeostatic equilibrium of the NGF system, leading through a series of interconnected loops to the activation of local and global neurodegeneration processes, ultimately determining the central core of AD hallmarks.

These neurotrophic deficits would be "located" upstream of the "amyloid cascade", as currently described, but would be part of a negative feedback loop that involves several feedback steps from the downstream process itself (e.g., links between APP, Tau, and axonal transport). Also, the cellular targets for NGF/proNGF actions, in this negative loop, could be more widespread than envisaged so far.

Essential elements of this mechanism, besides the "classical" NGF/TrkA/p75NTR system, are the proNGF/sortilin pathway and immune and inflammatory effectors acting on astrocytes, microglia and synapses, progressively broadening the cellular basis of the pathology.

In any event, the "new" NGF hypothesis for LOAD has significant therapeutical implications. Any therapy aimed at reestablishing the homeostatis of the NGF system appears to have a clear and strong rationale, as being truly able to interfere directly with a neurodegeneration mechanism involved in the disease process.

NERVE GROWTH FACTOR BASED THERAPIES FOR ALZHEIMER'S DISEASE: TAKING PAIN OUT OF NGF

In this framework, the first therapeutic choice would be to use NGF itself as a drug. Clinical application of NGF requires solving two major problems: effective CNS delivery and limitation of adverse effects (most notably, pain). It is indeed a challenge to deliver NGF into the brain in a safe and efficient manner. First, NGF does not readily cross the blood brain barrier. A second major issue is represented by the pro-nociceptive actions of NGF. Thus, NGF is a key pain mediator, controlling both the neural and the inflammatory components of pain (Pezet and McMahon, 2006). The capacity of NGF to cause pain has been demonstrated in humans in the course of pilot clinical trials in AD patients (Eriksdotter Jonhagen et al, 1998), as well as during clinical trials undertaken to explore the potential use of NGF in peripheral polyneuropathies (Grimm et al, 2007).

This has severely limited, in previous clinical trials, the dosage administrable to patients, jeopardizing the efficacy of the treatment (Apfel, 2002).

The clinical application of NGF in AD is therefore limited by a double constraint, of achieving a pharmacologically adequate concentration in target brain areas while preventing its adverse pain effects. For this reason, clinical trials evaluating NGF for AD have used invasive approaches, such as neurosurgery for the implant of autologous fibroblasts, engineered to secrete NGF, directly in the brain (Tuszynski et al, 2005), the direct stereotactic delivery into the brain of adeno-associated viral vectors encoding human NGF, or the chronic neurosurgical implant into the brain of biopolymer capsules filled with NGF-producing cells.

These invasive clinical approaches of NGF gene/cell therapy provide an independent validation of the therapeutic potential of NGF in AD, and their outcome will provide insights into the safety, efficacy, and liabilities of NGF therapies. However, the approach is highly impractical for its extension to large numbers of AD patients. Therefore, a safe route for an effective, non-invasive delivery of NGF to the brain is required.

We have shown that the intranasal route allows a non-invasive, safe, and pharmacologically effective delivery of NGF to the brain (Capsoni et al, 2002b; De Rosa et al, 2005; Capsoni et al, 2009). NGF intranasal delivery represents an effective compromise to meet the required therapeutic window for NGF, leading to NGF build-up in target brain areas while minimizing its bio-distribution to non-targeted districts where it induces pain (CSF and bloodstream) (See Table 1).

To facilitate optimal dosing, we designed a modified human NGF (hNGFP61S), "tagged" with a single distinctive residue, that can be easily traced against endogenous NGF and has a

potency and bioactivity identical to that of hNGF (Covaceuszach et al, 2009). hNGFP61S constitutes a backbone whereby additional desirable functions, such as antinociceptive properties, could be further engineered into the therapeutic NGF molecule.

Is it possible to take pain out of NGF, engineering an NGF mutein having neurotrophic properties identical to hNGF but lacking its nociceptive pain-inducing activity? To answer this question, we were inspired by genetic data on rare human syndromes determining congenital insensitivity to pain. Two rare forms of human congenital insensitivity to pain are due to mutations in genes related to NGF signaling: hereditary sensory and autonomic neuropathy, type IV (HSAN IV), is due to mutations in the gene for TrkA (Indo et al, 1996; Indo, 2001), while HSAN type V is associated with a mutation (R100W) in the NGF gene (Einarsdottir et al, 2004). Both HSAN IV and V are characterized by profound loss of pain sensitivity and perception, accompanied, in HSAN IV, by severe mental retardation and learning problems. On the other hand, HSAN V patients show no mental retardation and have most neurological functions intact. The HSAN V mutation NGFR100W separates, from a clinical point of view, the neurodevelopmental effects of NGF from those involved in the activation of peripheral pain pathways, after development.

This mutation could form the rational basis for the design of a "painless" NGF variant that, while displaying a full neurotrophic activity, shows a reduced nociceptive activity (Covaceuszach et al, 2010; Capsoni et al, 2011). Indeed, receptor binding measurements demonstrated that while the affinity of NGF R100 mutants for the TrkA receptor was substantially unchanged, the binding for p75NTR was 100-fold lower (Covaceuszach et al, 2010) (see Table 2). The neurotrophic potency of hNGF R100 mutants, in a number of different cellular bioassays, was indistinguishable from that of wild type hNGF. Interestingly, mutants hNGFR100, and hNGFP61/R100, were equally effective as hNGF and hNGFP61S in activating, through TrkA, downstream Shc and Akt pathways but failed to activate PLC-1γ. This selective TrkA signaling failure is noteworthy since the PLC-1γ pathway has been implicated in TrkA-mediated sensitization of sensory nociceptors (Chuang et al, 2001; Prescott and Julius, 2003). Thus, from a pharmacological point of view, painless NGF acts as a biased agonist, selectively

To study the effects of the mutation in the NGF gene on nociceptive responses *in vivo*, hNGFR100 and hNGFP61/R100 were administered to mice by intraplantar injection in the hind paw and mechanical allodynia was measured (Capsoni et al, 2011). A significant time- and dose-dependent allodynic effect was induced by hNGF, reaching a maximum effect 5 hours after the injection. On the contrary hNGFR100 and hNGFP61/R100 failed to show any allodynic effect (Capsoni et al, 2011), confirming that the R100 mutation in NGF makes a molecule equally neurotrophic, but less nociceptive (see Figure 1). The lack of pronociceptive effect of mutant hNGF was also confirmed when thermal hyperalgesia was evaluated (Capsoni et al, 2011). In a dose response study, a 10 fold greater concentration of painless hNGFR100 is needed, to sensitize the mice to the same extent as wild type hNGF does. This implies that a 10 fold greater concentration of painless NGF can be used, than has been used in past clinical trials for NGF, before the pain inducing threshold is reached.

In vivo studies in neurodegeneration models have confirmed the therapeutic potential of these painless NGF molecules, by showing that intranasally delivered NGF in different neurodegeneration mouse models (including AD11 mice and APPxPS1 transgenic mice) is very effective in rescuing several aspects of the neurodegenerative phenotype (Capsoni et al, 2012). It is noteworthy that the therapeutic efficacy of painless NGF is observed at many different levels of the complex neurodegenerative phenotypes of transgenic AD models, including both neuropathological endpoints, such as cholinergic deficit, hyperphosphorylated tau pathology and Abeta related pathology, as well as behavioural learning and memory deficits.

Current efforts are aimed at completing the preclinical activities required to advance the painless NGF molecule into clinical testing in humans. Among these, optimization of the production process, to increase the yields of the expression system for recombinant painless NGF, represent a high priority. Current yields are in the order of 10-15 mg/l of bacterial culture, and further scaling up is ongoing.

In conclusion, these studies demonstrate that by

exploiting a mutation from a human rare genetic condition, that leads to congenital insensitivity to pain, the therapeutic window for the therapeutic uses of NGF in clinical conditions in man has been significantly broadened. The painless hNGFP61/R100 traceable NGF molecule represents a candidate drug that has the potential for being developed not only into a disease-modifying non invasive therapy for Alzheimer's Disease, but also for other indications where the systemic use of NGF in man has shown significant pharmacological potential, but also difficulties, linked to its nociceptive properties, such as diabetic polyneuropathy.

AKNOWLEDGMENTS

We dedicate this paper to Rita Levi Montalcini, immensely grateful to her for her life, her example and her mentorship.

REFERENCES

Apfel SC (2002) Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold? *Int Rev Neurobiol* **50**: 393–413.

Berardi N, Braschi C, Capsoni S, Cattaneo A, Maffei L (2007) Environmental enrichment delays the onset of memory deficits and reduces neuropathological hallmarks in a mouse model of Alzheimer-like neurodegeneration. *J Alzheimers Dis* **11**: 359–370.

Bruno MA, Cuello AC (2006) Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. *Proc Natl Acad Sci USA* **103**: 6735–6740.

Bruno MA, Leon WC, Fragoso G, Mushynski WE, Almazan G, Cuello AC (2009) Amyloid β–induced NGF dysmetabolism in Alzheimer disease. *J Neuropathol Exp Neurol* **68**: 857–869.

Capsoni S, Cattaneo A (2006) On the molecular basis linking nerve growth factor (NGF) to Alzheimer's disease. *Cell Mol Neurobiol* **26**: 619–633.

Capsoni S, Covaceuszach S, Ugolini G, Spirito F, Vignone D, Stefanini B, Amato G, Cattaneo, A (2009) Delivery of NGF to the brain: intranasal versus ocular administration in anti-NGF transgenic mice. *J Alzheimers Dis* **16**: 371–388.

Capsoni S, Covaceuszach S, Marinelli S, Ceci M et al and Cattaneo A (2011) Taking pain out of NGF: a "painless" NGF mutant, linked to hereditary sensory autonomic neuropathy type V, with full neurotrophic activity. *PLoS One* **6**(2):e17321.

Capsoni S, Giannotta S, Cattaneo A (2002a) Beta-amyloid plaques in a model for sporadic Alzheimer's disease based on transgenic anti-nerve growth factor antibodies. *Mol Cell Neurosci* **21**: 15–28.

Capsoni S, Giannotta S, Cattaneo A (2002b) Nerve growth factor and galantamine ameliorate early signs of neurodegeneration in anti-nerve growth factor mice. *Proc Natl Acad Sci USA* **99**: 12432–12437.

Capsoni S, Marinelli S, Ceci M, Vignone D, Amato G, Malerba F, Paoletti F, Meli G, Viegi A, Pavone F, Cattaneo A. (2012) Intranasal "painless" human Nerve Growth Factor [corrected] slows amyloid neurodegeneration and prevents memory deficits in App X PS1 mice. *PLoS One*; **7**(5):e37555.

Capsoni S, Tiveron C, Vignone D, Amato G, Cattaneo A (2010) Dissecting the involvement of tropomyosin-related kinase A and p75 neurotrophin receptor signaling in NGF deficit-induced neurodegeneration. *Proc Natl Acad Sci U S A*; **107**(27):12299-304.

Capsoni S, Ugolini G, Comparini A, Ruberti F, Berardi N, Cattaneo A (2000) Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice. *Proc Natl Acad Sci USA* **97**: 6826–6831.

Cattaneo A, Calissano P. Nerve growth factor and Alzheimer's disease: new facts for an old hypothesis. (2012) *Mol Neurobiol*; **46**(3):588-604.

Cattaneo A, Capsoni S, Paoletti F (2008) Towards non invasive nerve growth factor therapies for Alzheimer's disease. *J Alzheimers Dis* **15**: 255–283.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* **411**: 957–962.

Coulson EJ, May LM, Sykes AM, Hamlin AS (2009) The Role of the p75 Neurotrophin Receptor in Cholinergic Dysfunction in Alzheimer's disease. *Neuroscientist*; **15**(4): 317-323.

Counts SE, Nadeem M, Wuu J, Ginsberg SD, Saragovi HU, Mufson EJ (2004) Reduction of cortical TrkA but not p75(NTR) protein in early-stage Alzheimer's disease. *Ann Neurol* **56**: 520–531.

Covaceuszach S, Capsoni S, Ugolini G, Spirito F, Vignone D, Cattaneo A (2009) Development of a non-invasive NGF based therapy for Alzheimer's disease. *Curr Alzheimer Res* **6**: 158–170.

Covaceuszach S, Capsoni S, Marinelli S, Pavone

F, Ceci M, Ugolini G, Vignone D, Amato G, Paoletti F, Lamba D, Cattaneo A (2010) In vitro receptor binding properties of a "painless" NGF mutein linked to hereditary sensory autonomic neuropathy type V. *Biochem Biophys Res Commun* **391**: 824–829.

Covaceuszach S, Cassetta A, Konarev PV, Gonfloni S, Rudolph R, Svergun DI, Lamba D, Cattaneo A (2008) Dissecting NGF interactions with TrkA and p75 receptors by structural and functional studies of an anti-NGF neutralizing antibody. *J Mol Biol* **381**: 881–896.

De Rosa R, Garcia AA, Braschi C, Capsoni S, Maffei L, Berardi N, Cattaneo A (2005) Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice. *Proc Natl Acad Sci USA* **102**: 3811–3816.

Einarsdottir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, Holmgren G, Holmberg D, Holmberg M (2004) A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Hum Mol Genet* **13**: 799–805.

Eriksdotter Jonhagen M, Nordberg A, Amberla K, Backman L, Ebendal T, Meyerson B, Olson L, et al. (1998) Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. *Dement Geriatr Cogn Disord* **9**: 246–257.

Fahnestock M, Michalski B, Xu B, Coughlin MD (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. *Mol Cell Neurosci* **18**: 210–220.

Fahnestock M, Scott SA, Jetté N, Weingartner JA, Crutcher KA. (1996) Nerve growth factor mRNA and protein levels measured in the same tissue from normal and Alzheimer's disease parietal cortex. *Brain Res Mol Brain Res*; **42**(1):175-8.

Fahnestock M, Yu G, Coughlin MD (2004) ProNGF: a neurotrophic or an apoptotic molecule? *Prog Brain Res* **146**: 101–110.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**: 704–706.

Grimm MO, Grimm HS, Hartmann T (2007) Amyloid beta as a regulator of lipiod homeostasis. *Trends Mol Med*. **13**: 337-344.

Hempstead BL (2006) Dissecting the diverse actions of pro- and mature neurotrophins. *Curr Alzheimer Res* **3**: 19–24.

Holtzman DM, Li Y, Chen K, Gage FH, Epstein CJ, Mobley WC (1993) Nerve growth factor reverses neuronal atrophy in a Down syndrome model of age-related neurodegeneration. *Neurology*; **43**(12):2668-73.

Indo Y (2001) Molecular basis of congenital insensitivity to pain with anhidrosis (CIPA): mutations and polymorphisms in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. *Hum Mutat* **18**: 462–471.

Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, Mitsubuchi H, Tonoki H, Awaya Y, Matsuda I (1996) Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat Genet* **13**: 485–488.

Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. *Science* **294**: 1945–1948.

Levi-Montalcini R, Hamburger V (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J Exp Zool*. Mar; **116**(2):321-61.

Li Y, Holtzman DM, Kromer LF, Kaplan DR et al and Mobley WC. (1995) Regulation of TrkA and ChAT expression in developing rat basal forebrain: evidence that both exogenous and endogenous NGF regulate differentiation of cholinergic neurons. *J Neurosci*.; **15**(4):2888-905.

Longo FM, Massa SM (2005) Neurotrophin receptor-based strategies for Alzheimer's disease. *Curr Alzheimer Res*; **2**(2): 167-169. Review.

Matrone C, Barbagallo AP, La Rosa LR, Florenzano F et al and D'Adamio L. (2011) APP is phosphorylated by TrkA and regulates NGF/TrkA signaling. *J Neurosci*; **31**(33):11756-61.

Matrone C, Di Luzio A, Meli G, D'Aguanno S, Severini C, Ciotti MT, Cattaneo A, Calissano P (2008) Activation of the amyloidogenic route by NGF deprivation induces apoptotic death in PC12 cells. *J Alzheimers Dis* **13**: 81–96.

Matrone C, Marolda R, Ciafrè S, Ciotti MT, Mercanti D, Calissano P (2009) Tyrosine kinase nerve growth factor receptor switches from prosurvival to proapoptotic activity via Abeta-mediated phosphorylation. *Proc Natl Acad Sci USA* **106**:11358–11363.

Mobley WC, Rutkowski JL, Tennekoon GI, Gemski J, Buchanan K, Johnston MV. (1986) Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Brain Res.*; **387**(1):53-62.

Mufson EJ, Kroin JS, Sendera TJ, Sobreviela T. (1999) Distribution and retrograde transport of trophic factors in

the central nervous system: functional implications for the treatment of neurodegenerative diseases. *Prog Neurobiol*; **57**(4):451-84. Review.

Mufson EJ, Ma SY, Cochran EJ, Bennett DA et al and Kordower JH. (2000). Loss of nucleus basalis neurons containing trkA immunoreactivity in individuals with mild cognitive impairment and early Alzheimer's disease. *J Comp Neurol*; **427**(1):19-30.

Nykjaer A, Willnow TE, Petersen CM (2005) p75NTR—live or let die. *Curr Opin Neurobiol* **15**: 49–57.

Origlia N, Capsoni S, Domenici L, Cattaneo A (2006) Time window in

cholinomimetic ability to rescue long-term potentiation in neurodegenerating antinerve growth factor mice. *J Alzheimers Dis* **9**: 59–68.

Pezet S, McMahon SB (2006) Neurotrophins: mediators and modulators of pain. *Annu Rev Neurosci* **29**: 507–538.

Prescott ED, Julius DA (2003) Modular PIP2 binding site as a determinant of capsaicin receptor sensitivity. *Science* **300**: 1284–1288.

Ruberti F, Capsoni S, Comparini A, Di Daniel E et al and Cattaneo A (2000) Phenotypic knockout of nerve growth factor in adult transgenic mice reveals severe deficits in basal forebrain cholinergic neurons, cell death in the spleen, and skeletal muscle dystrophy. *J Neurosci*; **20**: 2589-2601.

Salehi A, Delcroix JD, Mobley WC (2003) Traffic at the intersection of neurotrophic factor signaling and neurodegeneration. *Trends Neurosci* **26**: 73–80.

Schindowski K, Belarbi K, Buee L (2008) Neurotrophic factors in Alzheimer's disease: role of axonal transport. *Genes Brain Behav* **7**: 43–56.

Sola E, Capsoni S, Rosato-Siri M, Cattaneo A, Cherubini E (2006) Failure of nicotine-dependent enhancement of synaptic efficacy at Schaffer-collateral CA1 synapses of AD11 anti-nerve growth factor transgenic mice. *Eur J Neurosci* **24**: 1252–1264.

Tiveron C, Fasulo L, Capsoni S, Malerba F et al and Cattaneo A. (2013) ProNGF\NGF imbalance triggers learning and memory deficits, neurodegeneration and spontaneous epileptic-like discharges in transgenic mice. *Cell Death Differ*. **20**(8):1017-30.

Tuszynski MH, Thal L, Pay M, Salmon DP et al and Conner J (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med* **11**: 551–555.

NEED FOR NEW GUIDELINES FOR ALZHEIMER'S DISEASE CLINICAL TRIALS

G. NISTICO'[1], K. BROICH[2], and H. HAMPEL[3]

[1]Rita Levi-Montalcini Institute, EBRI Foundation, Rome; [2]Federal Institute of Drugs and Medical Devices, Bonn, Germany; [3]Department of Psychiatry, University of Frankfurt, Germany

Alzheimer's Disease (AD) is an irreversible and progressive neurodegenerative disease which affects approximately 5.1 million of people in USA (Hebert et al, 2003) and about 7.3 million in Europe. Patients affected by AD are unable to interact with their environment or care for themselves. Functional deficits appear in all the domains of cognition and behavior including memory loss, language difficulties, poor executive functions, behavioral and psychiatric disorders, e.g. delusion and depression, psychomotor agitation and decline in functional status, i.e. activities of daily living (ADLs) and self-care (See Herrup, 2012 and Burns & Illiffe, 2009). The first symptom of AD is often memory impairment. As the disease progresses, memory continues to decline and other functions like language capability and decision making become more difficult. Personality and behavior changes may also occur. An individual with AD may no longer recognize his family and friends and depends on other people for assistance in the most basic ADLs. A "National Plan to Address Alzheimer's Disease" has been recently published by the US Department of Health and Human Services of FDA (February 2013) as a consequence of US President Barack Obama signature into law of the National Alzheimer's Project Act (NAPA; see http://napa.alz.org/), on January 04, 2011. This law required the development of a national plan in order to make faster research toward prevention and treatment of AD, and to improve care, services, and support to AD people, their families, and caregivers.

Pathognomonic anatomo-pathological features of the AD brain are the senile plaques (SPs) and neurofibrillary tangles (NFTs). The main plaque constituent is represented by macromolecular aggregates of a short peptide termed beta-amyloid (Aβ) (Glenner and Wong, 1984). Notably, the predominant forms of the peptides are insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments. The former is the most abundant whereas the latter, highly hydrophobic, forms oligomers and fibrils accumulating in the extracellular plaques (Andreasen and Zetterberg, 2008).

The NFTs consist of paired helical filaments (PHFs) whose main constituent is a hyperphosphorilated form of the microtubule-associated protein tau (Grundke-Iqbal et al, 1986; Delacourte e Defossez, 1986). Therefore, the accumulation of abnormal intra- and extra-cellular protein deposits represents the main defect in protein processing and consequent alteration of normal neuronal functioning which ultimately results in neuronal death.

The most accredited pathogenetic hypothesis is the so-called "amyloid cascade". According to this hypothesis, the familial forms of AD (fAD), exhibit a genetic predisposition to an aberrant process of the Amyloid Precursor Protein (*APP*) gene leading to the accumulation of Aβ, in particular the $A\beta_{1-42}$ peptide which is the most amyloidogenic form.

Clinically, fAD accounts for only 1-5% of cases of AD and is caused by mutations in one of three definite genes, namely *APP*, presenilin-1 (*PSEN1*), and presenilin-2 (*PSEN2*) (Bateman et al., 2011). Moreover, fAD is usually referred to as early onset AD (EOAD) with an average onset of 50 years of age and some cases showing symptoms as early as

Correspondence address:
Prof. Giuseppe Nisticò
General Director
Rita Levi-Montalcini Institute – EBRI Foundation
Via del Fosso di Fiorano, 64-65 -00143 Rome
nistico@ebri.it

2279-5855 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

the mid-30's (Bertram et al., 2010).

In sporadic forms of AD (sAD) – without apparent familial aggregation (i.e., non-Mendelian inheritance) – the disease begins with a slow, age-related accumulation of extracellular Aβ. It is acknowledged that the majority of AD cases (>95%) are apparently non-Mendelian forms with age at onset of 65 years or above. As a result, such cases are referred to as late-onset AD (LOAD) (Bertram et al., 2010).

Mouse models of hereditary AD have demonstrated that a variety of transgenes produce heavy Aβ plaque deposition in the cortex. However, mice carrying an amyloid accumulation for three-quarters or more of their life show no significant neurodegeneration or behavioral problems, but only spatial orientation deficiencies and long-term memory deficits.

However, whatever are the mechanisms underlying cell death in various areas of the brain in AD, i.e. amyloid cascade hypothesis, tau hyperphosphorylation, mitochondrial dysfunction, and abnormal generation of reactive oxygen species (ROS) since clinical trials with several classes of drugs have begun too late in the disease process to be effective (see Herrup, 2012 and Schneider, 2012), no drugs for the treatment of mild cognitive impairment (MCI) and for the prevention of AD have been approved so far. With reference to the treatment of AD, no other drugs have been approved for marketing authorization since memantine in Europe and in USA in 2002 and 2003, respectively (Schneider, 2012).

The limited efficacy as well as the existence of adverse effects characterizing the available drugs has led to substantial controversy about the use and usefulness of current treatment options (see Schneider, 2012). The development of new pharmacological treatment for AD is driven by the enormous need, a large market perspective, and the high profit potential. Currently, drug development programs are aimed at new targets since the last clinical trials have been unsuccessful.

The regulatory criteria for marketing of symptomatic and disease modifying therapies for AD require the demonstration of efficacy in the cognitive domain accompanied by improvement in functional or global measure of the patients, i.e., ADLs and often evidence of overall clinical improvement or less overall decline, accompanied by adequate evidence of safety (Schneider, 2008). The criteria issued by the Food Drug Administration (FDA), as draft guideline, and by the European Medicine Agency (EMA), as formal guideline, have both tended to facilitate drug development by providing a road map of the types of clinical trials required, imposing development programs by indicating how drugs for a complex disorder can be developed. The guidelines encourage similar programs for very different drugs (see Broich et al, 2012 and EMA Guidelines). Unfortunately, despite numerous recent clinical trials, only few drugs with modest therapeutic effects are available and we should ask ourselves the reasons underlying such a failure.

Despite a partial restoration in synaptic plasticity and long-term potentiation (LTP) alterations in experimental models of AD was shown to occur after nerve growth factor (NGF), (LaRosa et al, 2013) , brain-derived neurotrophic factor (BDNF) (Lu et al, 2013) and drug treatment (γ-secretase modulators (Balducci et al, 2011), it is highly probable that the goal of treating AD when the symptomatology is overt is an almost impossible mission since, in this circumstance, millions of neurons are already killed both in the neocortex and in the hippocampal system. Therefore, only a treatment employing neural stem cells transplant or NGF could potentially repair the disrupted synaptic communication between neurons and eventually ameliorate the cognitive and behavioral disorders. However, the restoration of the brain areas destroyed in AD by means of implantation of neural stem cell is thought to be an hard task considering the complex architecture of neuronal networks and circuits in the affected areas. In particular, the different nature of neuronal pathways, the tens of neurotransmitters, the many other endings impinging on neurotransmitter nerve terminals as well as the mosaics of different pre- and post-synaptic receptors render the goal of stem-cells to repair or perfectly reproduce a functioning synaptic communication as remote goal.

The major risks of maintaining the present regulatory guidelines is represented on one side by the impossibility to reduce or cure the terrible symptoms of the patients and the suffering of their families, relatives, and friends. On the other side,

should the present guideline persist, another risk is represented by the discouragement of pharmaceutical industry or research consortia to carry out research and new developmental projects since so far they have in vain invested enormous resources and funds .

Obviously it seems logical to change the strategy to treat AD and recently the Food and Drug Administration has proposed and distributed for comments a draft guidance for applicants (February 2013) allowing alternative targets of intervention pointed in early stages of AD. Recently, an international task force of investigators from academia, industry, nonprofit foundations, and regulatory agencies met in Monte Carlo, Monaco, on October 31, 2012, to review lessons learned from the recent bapinezuzumab and solanezumab trials, and to incorporate insights gained from these failed trials into future clinical studies. Although there is a broad consensus that AD should be treated during its early stages, the concept of secondary prevention has evolved to be described more accurately as treatment of preclinical, presymptomatic, or early AD. There continues to be a strong emphasis on biomarkers and a need for new biomarkers; however, there has also been a realization, based on completed trials, that the most reliable indicator of clinical efficacy across the entire spectrum of disease from asymptomatic to AD dementia is likely a measure of cognition (Vellas, Carrillo, Sampaio, Brashear et al, 2013). Therefore, the new guidance for FDA suggests potential approaches to clinical trial design and execution that allow for regulatory flexibility and innovation (Kozauer and Katz, 2013).

Notably, the FDA guideline covers the selection of patients for trials in early stages of AD. In this regard, there is a consensus within the Alzheimer research community that clinical diagnosis of early cognitive impairment might be coupled with specific appropriate biomarkers of the disease.

The currently evolving status of multimodal core biomarker development and validation that encompass neurochemical/neurogenetic as well as structural/functional/metabolic neuroimaging studies has been extensively assessed and revised (Blennow et al., 2013; Hampel and Lista, 2013; Hampel et al., 2012; Hampel and Lista, 2012; Ewers et al., 2011; Hampel et al., 2008) providing also the converging perspectives of industry stakeholders and regulatory bodies on the AD biomarker discovery/development area (Hampel at al., 2010; Blennow et al., 2010). Notably, criteria have been established and are being validated by various working groups. In particular, the recent advances in biomarkers of AD, which provide *in vivo* information about the pathophysiologic processes associated with AD, have encouraged the proposal of new diagnostic criteria by the International Working Group (IWG) for New Research Criteria for the Diagnosis of AD (Dubois et al., 2007; 2010). According to the IWG/Dubois criteria, the diagnosis of AD was reconceptualized as a "clinical-biological" entity with a specific clinical phenotype and confirmatory *in vivo* pathophysiologic evidence of AD (Sarazin et al., 2012; Cummings et al., 2013).

More recently, the National Institute of Aging-Alzheimer's Association (NIA-AA) workgroups published new diagnostic guidelines for AD (McKhann et al., 2011; Albert et al., 2001; Sperling et al., 2011) that also incorporate biological and imaging markers to establish an earlier diagnosis of AD (Sarazin et al., 2012).

In both IWG/Dubois and NIA-AA diagnostic criteria, a discussion of preclinical stages of AD is offered, focused on the existence of a pathophysiologic process that precedes the clinical manifestations of the disease (Sarazin et al., 2012) and data on specificity, sensitivity and predictive values are awaited.

Biomarkers discussed include brain β-amyloid load, as measured by positron-emission tomography (PET) and cerebro-spinal fluid (CSF) levels of β-amyloid and tau proteins (Hampel and Carrillo, 2012). However, validation of these biomarkers is still lacking although of the 19,104 published papers, 142 longitudinal studies related to the biomarkers of interest were identified which included subjects who had objective cognitive impairment but no dementia at baseline (see Noel-Storr et al, 2013).

As far as CSF biomarkers are concerned in a recent paper it was reported that the overall variability of data coming from a total of 84 laboratories remains too high to allow the assignment of universal biomarker cut-off values for the specific intended use (Mattsson et al, 2013) underpinning the need for better collaboration and standardization in such conditions.

A specific suggestion by the FDA guidance for clinical trials focusing on patients in whom overt dementia seems imminent is the use of a single scale that combines assessment of both cognition and function such as the score on the Clinical Dementia Rating Sum of Boxes (CDR-SB) (Kozauer and Katz, 2013). For patients, whose disease is at an even earlier clinical stage, we agree that despite biomarkers validation tests are not defined yet, it seems possible to approve a drug through an accelerated procedure pathway on the basis of assessment of only cognitive symptoms. The accelerated approval mechanisms will allow drugs that address an high unmet medical need to be approved on the basis of a surrogate end point or an intermediate clinical endpoint (i.e. a sensitive cognitive measure). This approach would be a "conditional" approval which implies that the applicant is obliged to carry out long-term longitudinal clinical studies after marketing authorization to confirm clinical efficacy and safety. Only after the approval and long-term treatment it would be possible to properly follow the amelioration of cognitive and behavioral disorders as well as the slowing of the progression of neurodegenerative lesions as shown by neuroimaging techniques (Hampel and Carrillo, 2012). In order to improve the diagnostic accuracy and possibilities for follow-up in patients with MCI, new validated neuropsychological test batteries, functional and global impairment measures are needed, which are sensitive to change. e.g. Richard et al (2013) have recently proposed a new memory test, the Net Reclassification Improvement (NRI) which, followed by MRI and CSF analysis, might be an attractive and easy to interpret measure for clinicians. EMA has established a qualification procedure for such novel methodologies for clinical trials and stakeholders are encouraged to use the procedure (Isaac M et al. 2011; Manolis E et al. 2011)

The focus of new drug development to be shifted to earlier stages of AD has been recently raised by one of us (G.N.) at EMA Management Board (March 2013) and remains a challenging scientific question. It is desirable that both EMA and FDA consult each other to decide the best design for the new clinical trials in AD prevention and treatment at the various stages of the disease , which should enrich the AD therapeutic armamentarium to alleviate clinical symptoms and/or to prevent progressive devastating effects.

ACKNOWLEDGEMENTS

The ideas expressed in this review are personal ideas of the single authors and do not reflect the view-point of the Agencies (AIFA, BfArM, EMA) where they pay their own activity.

H.H. was supported by grants of the Katharina-Hardt-Foundation, Bad Homburg vor der Höhe, Germany.

REFERENCES

Albert MS, DeKosky ST, Dickson D et al (2011) The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*; **7**:270-9.

Andreasen N, Zetterberg H (2008) Amyloid-related biomarkers for Alzheimer's disease. *Curr Med Chem* **15**:766-771.

Balducci C, Mehdawy B, Mare L, Giuliani A, Lorenzini L, Sivilia S, Giardino L, Calzà L, Lanzillotta A, Sarnico I, Pizzi M, Usiello A, Viscomi AR, Ottonello S, Villetti G, Imbimbo BP, Nisticò G, Forloni G, Nisticò R. The gamma-secretase modulator CHF5074 restores memory and hippocampal synaptic plasticity in plaque-free Tg2576 mice. *J Alzheimers Dis.* 2011;**24**(4):799-816.

Bateman RJ, Aisen PS, De Strooper B et al (2011) Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease. *Alzheimers Res Ther* **3**:1.

Bertram L, Lill CM, Tanzi RE (2010) The genetics of Alzheimer disease: back to the future. *Neuron* **68**:270–81.

Blennow K, Hampel H, Zetterberg H (2013) Biomarkers in β-Amyloid Immunotherapy Trials in Alzheimer's Disease. *Neuropsychopharmacology*. doi: 10.1038/npp.2013.154.

Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nat Rev Neurol*, **6**:131-44.

Broich K, Schlosser-Weber G, Weiergraber M, Hampel H. (2012) Regulatory requirement on clinical trials of Alzheimer's disease. In: Alzheimer's disease-Modernizing Concept, Biological Diagnosis and Therapy. Hampel H. & Carrillo M.C. Eds. Adv Biol Psychiatry, Basel, Karger, 2012, vol.28, pp 168-178.

Burns A, Illiffe S (2009) Alzheimer's diases. *Br Med J*; **338**, 158b.

Cummings JL, Dubois B, Molinuevo JL, Scheltens P (2013) International Work Group criteria for the diagnosis of Alzheimer disease. *Med Clin North Am*, **97**:363-8.

Delacourte A, Defossez A (1986) Alzheimer's disease: tau proteins, the promoting factor of microtubule assembly are major component of paired helical filaments. *J Neurol Sci*, **76**: 173-186.

Dubois B, Feldman HH, Jacova C et al (2007) Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. *Lancet Neurol*,**6**:734–46.

Dubois B, Feldman HH, Jacova C et al (2010) Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol*, **9**:1118-27.

EMA CHMP: Guideline on Alzheimer's disease: http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guidelines/2009/09/WC500003562.pdf.

Ewers M, Sperling RA, Klunk WE, Weiner MW, Hampel H (2011) Neuroimaging markers for the prediction and early diagnosis of Alzheimer's disease dementia. *Trends Neurosci*, **34**:430-42.

FDA Guidance for Industry. Alzheimer's disease: developing drugs for the treatment of early stage disease. Washington Center for Drug Evaluation and Research, February 2013 (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM338287.pdf).

Glenner G.G., Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebro-vascular amyloid protein. *Biochem biophys Res Commun*, **120**: 885-890.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM (1986) *J Biol Chem*, **261**:6084-6089.

Hampel H, Carrillo MC (2012) (Eds) Alzheimer's disease- Moderning Concept, Biological diagnosis and Therapy, Karger Publ. Co., Basel, 2012.

Hampel H, Lista S (2013) Use of biomarkers and imaging to assess pathophysiology, mechanisms of action and target engagement. *J Nutr Health Aging*, **17**:54-63.

Hampel H, Lista S (2012) Alzheimer disease: From inherited to sporadic AD-crossing the biomarker bridge. Nat Rev Neurol, 8:598-600.

Hampel H, Lista S, Khachaturian ZS (2012) Development of biomarkers to chart all Alzheimer's disease stages: the royal road to cutting the therapeutic Gordian Knot. Alzheimers Dement, 8:312-36.

Hampel H, Frank R, Broich K et al (2010) Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. Nat Rev Drug Discov 9:560-74.

Hampel H, Bürger K, Teipel SJ, Bokde AL, Zetterberg H, Blennow K (2008) Core candidate neurochemical and imaging biomarkers of Alzheimer's disease. Alzheimers Dement, 4:38-48.

Hebert LE, Scherr PA, Bienias JL et al (2003) Alzheimer's disease in the US population: prevalence estimates using the 2000 census. Arch Neurol, 60: 1119-1122.

Herrup K. (2012) Current conceptual views of Alzheimer's disease. In: Alzheimer's disease-Modernizing Concept, Biological Diagnosis and Therapy. Hampel H. & Carrillo M.C. Eds. Adv Biol Psychiatry, Basel, Karger, 2012, vol.28, pp.30-48.

Isaac M, Vamvakas S, Abadie E, Jonsson B, Gispen C, Pani L. Qualification opinion of novel methodologies in the predementia stage of Alzheimer's disease: cerebro-spinal-fluid related biomarkers for drugs affecting amyloid burden--regulatory considerations by European Medicines Agency focusing in improving benefit/risk in regulatory trials. *Eur Neuropsychopharmacol*. 2011 Nov;21(11):781-8

Kozauer N, Katz R (2013, March 13) Regulatory innovation and drug development for early-stage of Alzheimer's disease. New Engl J Medicine, 1-3.

La Rosa LR, Matrone C, Ferraina C, Panico MB, Piccirilli S, Di Certo MG, Strimpakos G, Mercuri NB, Calissano P, D'Amelio M, Nisticò R. Age-related changes of hippocampal synaptic plasticity in APP-null mice are restored by NGF through p75NTR. J Alzheimers Dis. 2013;33(1):265-72.

Lu B, Nagappan G, Guan X, Nathan PJ, Wren P. BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. Nat Rev Neurosci. 2013 Jun;14(6):401-16.

Manolis E, Vamvakas S, Isaac M. New pathway for qualification of novel methodologies in the European Medicines Agency. Proteomics Clin Appl. 2011 Jun;5(5-6):248-55. doi: 10.1002/prca.201000130. Epub 2011 Apr 28

Mattsson N, Andreasson U, Persson S, Carrillo MC et al and Käser SA (2013) CSF biomarker variability in Alzheimer's Association quality control program. *Alzheimer's & Dementia*; **9**, 251-261.

McKhann GM, Knopman DS, Chertkow H et al (2011) The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement, 7:263–9.

Noel-Storr A.H., Flicker L., Ritchie CW, Nguyen GH

et al and McShane R (2013) Systematic review of the body of evidence for the use of biomarkers in the diagnosis of dementia. *Alzheimer's & Dementia*; **9**, e96-e105.

Richard E, Schmand BA, Eikelenboom P, Van Gool WA (2013) MRI and cerebrospinal fluid biomarkers for predicting progression to Alzheimer's disease in patients with mild cognitive impairment: a diagnostic accuracy study. Br Med J Open; 3, e002541. doi:10.1136/bmjopen-2012-002541; pp. 1-8.

Sarazin M, de Souza LC, Lehéricy S, Dubois B (2012) Clinical and research diagnostic criteria for Alzheimer's disease. Neuroimaging Clin N Am, 22:23-32.

Schneider LS (2008) Issues in design and conduct of clinical trials for cognitive-enhancing drugs. In: McArthur RA & Borsini F (Eds.): Animal and translational Models for CNS Drug Discovery. San Dieco Academic Press, pp. 21-76.

Schneider LS (2012) Pharmacological treatment of Alzheimer's disease. In: Alzheimer's disease-Modernizing Concept, Biological Diagnosis and Therapy. Hampel H. & Carrillo M.C. Eds. Adv Biol Psychiatry, Basel, Karger, 2012, vol.28, pp122-167.

Sperling RA, Aisen PS, Beckett LA, et al (2011) Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement, 7:280–92.

Vellas B, Carrillo MC, Sampaio C, Brashear Hr et al and Aisen P (2013) The signing drug trials for Alzheimer's disease: What we have learned from the release of the phase III antibody trials: a report from the EU/US/CTAD Task Force. *Alzheimer's & Dementia*; **9**, 438-444.

MESENCEPHALIC CELL CULTURES FROM CYP2E1 KNOCKOUT MICE: A STUDY ON MPP+ TOXICITY

C. PARDINI, C. VIAGGI, C. GERACE, G.U. CORSINI, F. VAGLINI

Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Italy

The relevance of the P450 2E1 isozyme (CYP2E1) in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in C57bl mice has been reported previously. Recently, we generated mesencephalic cell cultures from Cyp2e1(–/–) and wild-type 129S1/SvImJ (Cyp2e1(+/+)) embryos. In the present study we demonstrated that Cyp2e1(–/–) primary cultures proved to be a valuable in vitro model of 1-methyl-4-phenylpyridinium ion (MPP+) toxicity and metabolic study. After 24 h of MPP+ exposure, Cyp2e1(–/–) mesencephalic cells were less sensitive to the toxic insult compared with Cyp2e1(+/+) cultures. The MPP+ kinetic study revealed long-term enhanced uptake of the toxin inside the neurons with a total retention that was double that of controls. The dose-response study of the long-term retention of MPP+ revealed that the difference is observed only at a low concentration of the toxin. Our data suggest that in Cyp2e1(–/–) cultures, once MPP+ is inside the cells, it enters preferentially into vesicles where its storage represents a sort of protection with respect to other toxic sites such as mitochondria.

Parkinson's disease (PD) is a neurodegenerative disorder characterised by the degeneration of dopaminergic (DA) neurons in the substantia nigra (SN). Despite extensive investigation, the aetiology of the sporadic disease is still unknown. Attention has recently focused on neurotoxins, both endogenous and environmental, which can rapidly and selectively damage mesencephalic DA neurons. The discovery that a contaminant found in an illicit substance of abuse, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was able to induce a severe syndrome of parkinsonism in humans, gave a considerable impulse to research on the pathophysiology of PD (Davis et al, 1979; Langston et al, 1983). Treatments with the neurotoxin MPTP and its active metabolite 1-methyl-4-phenylpyridinium ion (MPP+) have been used as experimental models for PD. DA neurons accumulate MPP+ through the high-affinity dopamine transporter (DAT) (Javitch & Snyder, 1984). Inside the neuron, MPP+ is taken up by vesicles and mitochondria, where it is accumulated, directly inhibiting complex I (Nicklas et al, 1985). Accordingly, MPP+ toxicity has been directly associated with failure of energy supplies in some in vitro models (Di Monte et al, 1986).

Compounds such as diethyldithiocarbamate (DDC), diallyl sulphide (DAS), and phenylethyl isothiocyanate (PIC) have been shown to potentiate MPTP toxicity and the consequent selective DA neuron degeneration (Corsini et al, 1985; Vaglini et al, 2004). These compounds share a common feature: they are all substrates/inhibitors of the cytochrome P450 2E1 isozyme (CYP2E1) (Vaglini et al, 2004). Recently, Cyp2e1 knockout mice were challenged with MPTP and, surprisingly, the animals showed a reduced sensitivity to the toxin (Viaggi et al, 2009). Cyp2e1 has been previously detected in the brain of rodents and humans, and also localised in mesencephalic DA neurons (Tindberg & Ingelman-Sundberg, 1996;

Key words: MPP+, Cyp2e1, mesencephalic cells, Parkinson's disease

Corresponding author: Francesca Vaglini
Dept. of Translational Research and New Technologies in Medicine and Surgery
Via Roma 55, 56126 Pisa.
Tel.+39 050 2218701;
fax: .+39 050 2218717
e-mail: francesca.vaglini@med.unipi.it

2279-5855 (2013)

Upadhya et al, 2000;Watts et al, 1998).

It is well known that primary cultures from mesencephalic cells obtained from dissected ventral mesencephalon of embryos at 13 days of gestation are a good in vitro model for DA neuron development. Although the number of DA neurons in such cultures is extremely limited (2–5%), toxicological and biochemical studies on these neurons have been extensively performed (Prochiantz et al, 1983).

The objective of this study was to show that primary cultures from Cyp2e1 knockout and wild-type 129S1/SvImJ (Cyp2e1(+/+)) embryos are a valuable in vitro model for toxic and metabolic studies by investigating MPP+ toxicity and its retention time.

MATERIALS AND METHODS

Materials

Chemicals: Eagle's minimum essential medium (MEM), Ham's nutrient mixture F-12, L-glutamine, and phosphate buffer saline (PBS) were all purchased from Sigma Aldrich (St. Louis, MO, USA), NU-serum V was purchased from Collaborative Research (BD Biosciences, Steroglass, Perugia, Italy), rabbit tyrosine hydroxylase (TH) antibody was purchased from Chemicon (Temecula, CA, USA), the Vectastain Elite ABC kit was purchased from Vector Laboratories (Burlingham, CA, USA), and [3H]MPP+ from NEN Research Products , CytoScint (ICN Research Products, Costa Mesa, CA, USA). All tissue culture supplies were provided by Falcon (Steroglass).

Knockout mice

As previously described (Viaggi et al, 2009), Cyp2e1 knockout mice 129/SV-Cyp2e1tm1Gonz (Cyp2e1(–/–) stock number: 002910) and their wild-type counterparts 129S1/SvImJ (Cyp2e1(+/+) stock number: 002448) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Cyp2e1 –/– status in adult animals was confirmed by the absence of CYP2E1 as determined by tail-tip DNA polymerase chain reaction (PCR) phenotyping. The animals were treated in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The experiments described in this article were formally approved by the Committee for Scientific Ethics of the University of Pisa.

Cell cultures

Timed pregnant Cyp2e1(–/–) and Cyp2e1(+/+) mice were generated in our animal room. Brain were obtained from 13-day-old mouse embryos and placed in cold, sterile, balanced salt solution (PBS). Briefly, the midbrain was dissected under microscopic observation and placed in a nutrient medium composed of F-12 and Eagle's MEM (ratio 1:1), supplemented with 2 mM glutamine, 10% Nu-serum V, and 33.3 mM glucose. The mesencephalon was mechanically dispersed, using borosilicate-coated fire-polished Pasteur pipettes, centrifuged at 800 x g for 3 min, and re-suspended in the culture medium. Cells were counted with a haemocytometer and plated at a density of 5 x 105 in multi-well plates (16-mm-diameter wells; Falcon). The surfaces of the multi-well plates were pre-coated with 15 μg/ml of poly-D-lysine (high molecular weight, i.e. > 300,000) in an aqueous solution for 1 h at 37 °C, and rinsed three times with sterile water before plating. The cultures were maintained in a humidified atmosphere of 5% CO2/95% air at 37 °C. Experiments were performed in the absence of glial cells (Vaglini et al, 2008).

Cell morphology was assessed by direct microscopic observation of the mesencephalic monolayer, using an optic-inverted phase-contrast microscope (Nikon TMS; Nikon).

Experimental procedure

Mesencephalic cells Cyp2e1(–/–) and Cyp2e1(+/+) were grown in 2 ml of culture medium. On day 9 after plating, cells were treated with different doses (0.5, 1, and 5 μM) of MPP+. After 24 h, cells were functionally tested for [3H]dopamine uptake. In addition, a separate set of cells were fixed in paraformaldehyde (4% in PBS), and TH immunocytochemistry was performed.

In a separate set of experiments at 9 days in vitro (DIV), mesencephalic cell cultures were either exposed to [3H] MPP+ at 2 nM for 1, 2, 4, 6, and 24 h, after which the cells were scraped and evaluated for [3H]MPP+ content, or to [3H]MPP+ at 1, 10, 100, and 1,000 nM for 4 h, after which the cells were scraped and evaluated for [3H]MPP+ content.

Assay of [3H]dopamine uptake

Mesencephalic cells Cyp2e1(–/–) and Cyp2e1(+/+) were grown in 2 ml of culture medium. On day 9, cells were functionally tested for dopamine uptake. Mesencephalic cultures were rinsed with PBS with 1 mM CaCl2, 1 mM MgCl2, and 33.3 mM glucose, and incubated for 15 min with 50 nM [3H]dopamine (28.0 Ci/mmol). Uptake was stopped by removing the reaction mixture containing the radioligand, and rinsing the wells three times with ice-cold PBS. The cells were scraped into 0.5 ml of 0.2 N NaOH containing 0.2% Triton X-100. The pH was neutralised by adding 0.5 ml of 0.2 N HCl. An aliquot of 10 □l was taken for protein determination (Lowry et al, 1951). Blank values were obtained by incubating cells at 0 °C, a condition that blocks specific uptake in vitro. The radioactivity was

counted with 10 ml of CytoScint in a liquid scintillation β-counter (Wallac). Results are expressed as disintegrations per minute (dpm)/mg of protein, based on three experiments performed in triplicate wells.

TH immunocytochemistry

Cultures from Cyp2e1(–/–) and Cyp2e1(+/+) were washed twice in PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. After two washes in PBS, the cultures were incubated overnight at 4 °C with a rabbit TH antibody diluted 1:1,000 in PBS (containing 0.2 % Triton X-100). Cultures were then washed with PBS, and incubated with a biotinylated anti-rabbit immunoglobulin G, followed by incubation with an avidin-biotin conjugate of peroxidase (Vectastain). The peroxidase was visualised using diaminobenzidine and hydrogen peroxide.

Total TH+ cell numbers were counted in 10 randomly selected fields (1.13 mm2/field) at 20x magnification with a Nikon inverted microscope, and cell morphology was assessed by microscopic observation of the immunostained mesencephalic monolayer. Micrographs were taken with a Nikon D40x digital camera mounted on a phototube.

Assay of [3H]MPP+ uptake

Mesencephalic cells Cyp2e1(–/–) and Cyp2e1(+/+) were grown in 2 ml of culture medium. On day 9, cells were functionally tested for MPP+ uptake. Mesencephalic cultures were rinsed with PBS with 1 mM CaCl2, 1 mM MgCl2, and 33.3 mM glucose, and incubated for different times (1, 2, 4, 6, and 24 h) with 2 nM [3H]MPP+ (81.0 Ci/mmol). In the dose-dependent uptake (described in section 4.5), different concentrations of [3H]-conjugated toxin were stopped after 4 h of incubation. Uptake was stopped by removing the reaction mixture containing the radioligand, and rinsing the wells three times with ice-cold PBS. The cells were scraped into 0.5 ml of 0.2 N NaOH containing 0.2% Triton X-100. The pH was neutralised by adding 0.5 ml of 0.2 N HCl. Blank values were obtained by incubating cells at 0 °C, a condition that blocks specific uptake in vitro. The radioactivity was counted with 10 ml of CytoScint in a liquid scintillation β-counter. Results are expressed as dpm/mg of protein, based on three experiments performed in triplicate wells.

Statistical evaluation

Data are presented as mean values β SEM. The statistical significance between individual data was assessed using ANOVA (Scheffé F test); the null hypothesis was rejected when p was lower than 0.05.

RESULTS

In 2007 we have demonstrated that ventral mesencephalon cell cultures from Cyp2e1 knockout mice were similar to those from wild-type animals. With phase-contrast microscopy, the apparent images of the two types of cell culture overlapped with regard to number of surviving cells and their shape. (Pardini et al, 2008). In order to confirm our preliminary observation about the possibility that mesencephalic CYP 2e1 -/- cells were less sensitive to MPP+ toxicity in respect to wild type, we have performed new experiments.

Fig. 1 shows the dose-dependent reduction of [3H] dopamine uptake observed after 24 h in the DIV 9 cultures exposed to MPP+ at different concentrations (0.5, 1 and 5μM). The reduction curve is less steep in cell cultures from Cyp2e1(–/–), suggesting that DA neurons from knockout mice are less sensitive to the toxic effect of MPP+. The decrease in [3H] dopamine uptake is significantly less pronounced at all doses tested; treatment with 0.5, 1, and 5 μM of MPP+ caused a reduction of [3H]dopamine uptake in the wild-type cell cultures of 69.9% ± 1.3%, 58.3% ± 1.4%, and 41.6% ± 1.2%, respectively, whereas for knockout cell cultures the reduction was 85.3% ± 1.7%, 74.5% ± 1.0%, and 41.3% ± 1.1%, respectively.

Direct microscope observation showed the normal aspect of a mesencephalic culture containing polygonal-shaped neurons with their axon processes from both wild-type and knockout mice as shown by TH immunoreactivity (Fig. 2 A, B). Immunocytochemistry analysis on mesencephalic cells with an anti-TH antibody from Cyp2e1(+/+) mice showed that treatment with increasing doses of MPP+ resulted in TH+ neurons with progressively damaged fibres (Fig. 2 C, E). In particular, at the highest MPP+ dose (5 μM), the spared TH+ neurons had a roundish-shaped body and were without fibres (Fig. 2E). In contrast, TH+ neurons from knockout mesencephalic cultures (Fig. 2 D, F) showed longer processes. At 5 μM mainly, the TH immunoreactivity cell count indicated that Cyp2e1(–/–) cultures showed a relative higher number of DA neurons (Fig. 2F), suggesting that these cultures were more resistant to the toxic insult compared with those from Cyp2e1(+/+) mice.

The relative percentages of TH+ cells after neurotoxin treatment are shown in Fig.2.

MPP+ kinetics

Trace amount of [3H]MPP+ (2 nM) uptake into

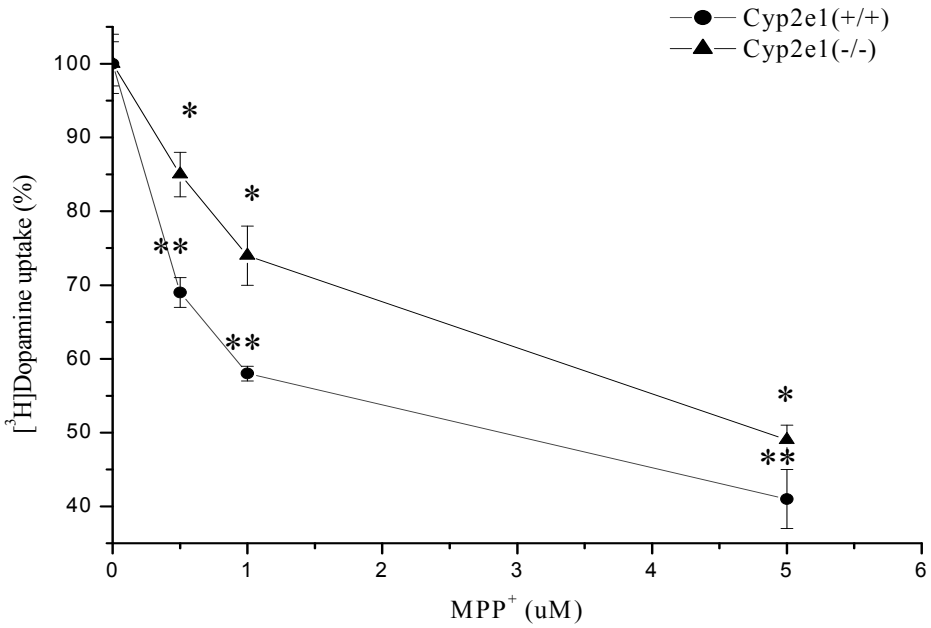


Fig. 1. *Effect of different concentrations of MPP+ on the survival of mouse mesencephalic cell cultures from Cyp2e1(+/+) and Cyp2e1(–/–), evaluated as [3H]dopamine uptake. After 9 DIV, mesencephalic cell cultures were exposed to MPP+ 0.5, 1, or 5 μM. [3H]dopamine uptake assay was performed 24 hours later. Values represent means ± SE. Statistical analysis consisted of ANOVA Scheffé F test. *p < 0.05 vs. control value (basal); **p < 0.05 vs. Cyp2e1(+/+) at the same MPP+ concentration.*

mesencephalic cultures from different strains of mice was performed to determine whether there were any strain differences to accumulate the toxin. At DIV 9, mesencephalic cells from wild-type and knockout mice were tested for long-term in vitro uptake of [3H] MPP+. In Cyp2e1(–/–) cultures, long-term exposure (1, 2, 4, 6, and 24 h) to a trace amount of [3H]MPP+ (2 nM) induced an enhanced (almost double) uptake of the toxin compared with Cyp2e1(+/+) cultures, with a maximum at 6 h of exposure (73.2 ± 3.3 and 41.03± 9.1 fmol/mg of protein respectively); uptake remained almost at a plateau until 24 h (71± 1.6 and 46.8 ± 2.1 fmol/mg of protein respectively) (Fig. 3).

Short-term uptake ([3H]MPP+ exposure ≤ 15 min) did not show any significant differences between the two types of culture (data not shown).

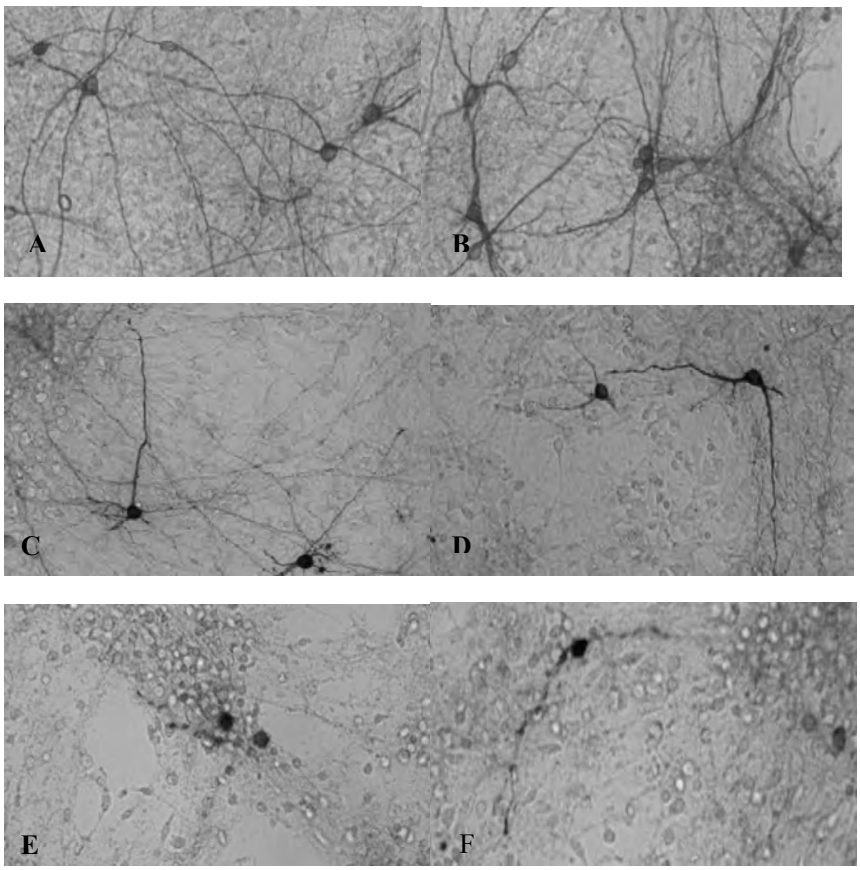
The dose-response curve, shown in Fig. 4, indicates that at increasing concentrations of MPP+ the difference between the two cultures, observed after 4 h of MPP+ exposure, tended to cancel out progressively, already reaching a plateau at 0.1 μM.

DISCUSSION

In the 80's numerous compounds were tested under acute treatment in order to modify MPTP toxicity; only a few enhancers of MPTP toxicity (DDC, ethanol and acetaldehyde) were found (Corsini, 1987). The 'enhancers' prolonged the striatal half-life of MPP+, the toxic metabolite of MPTP (Irwin et al, 1987; Zuddas et al, 1989), and this was interpreted as the causal factor underlying this enhancement. However, a more recent article demonstrated that striatal in vivo MPP+ levels do not necessarily correlate with MPTP toxicity in the same animal species (mouse) (Vaglini et al, 1996).

More recently, similar to DDC, it was found that DAS or PIC also markedly enhanced MPTP toxicity, as measured by the dramatic decrease in striatal dopamine content after the treatments (Vaglini et al, 2004) . All the enhancers share the common feature of being substrates/inhibitors of CYP2E1.

CYP2E1 is an enzyme of the P450 family whose



Treatment	*Cyp2e1(+/+)*	*Cyp2e1(-/-)*
	%TH+ cells	
Control	100±0.5	98±0.7
MPP+ (1μM)	27±0.2	28±0.4*
MPP+ (5 μM)	20±0.3	26±0.5**

Fig. 2. *TH immunoreactivity in mesencephalic cell cultures from Cyp2e1(+/+) and Cyp2e1(–/–) mice after exposure to different MPP+ concentrations. A, B: control microphotograph of typical aspect of the culture; C, D: after 24 h of incubation of MPP+ at 1 μM; E, F: at 5 μM, of Cyp2e1(+/+) and Cyp2e1(–/–), respectively. Scale bar = 40 μm. The table shows the TH immunoreactivity neuronal counts expressed as a percentage, compared with control culture. The average number of TH+ cells in the control cultures was 8 cells/field. Values represent means ± SE. Statistical analysis consisted of ANOVA Scheffé F test. *n.s. with respect to 1 μM of MPP+ treated Cyp2e1(+/+)cells; ** $p < 0.05$ vs. 5 μM of MPP+ treated Cyp2e1(+/+)cells. n.s. = not significant; TH = tyrosine hydroxylase.*

occurrence at high concentration is typical in the liver. Its presence in extrahepatic organs such as kidney, lung, and brain has been reported (de Waziers et al, 1990; Hansson et al, 1990; Thomas et al, 1987). The enzyme has many endogenous and exogenous substrates, some of which are toxic and, generally,

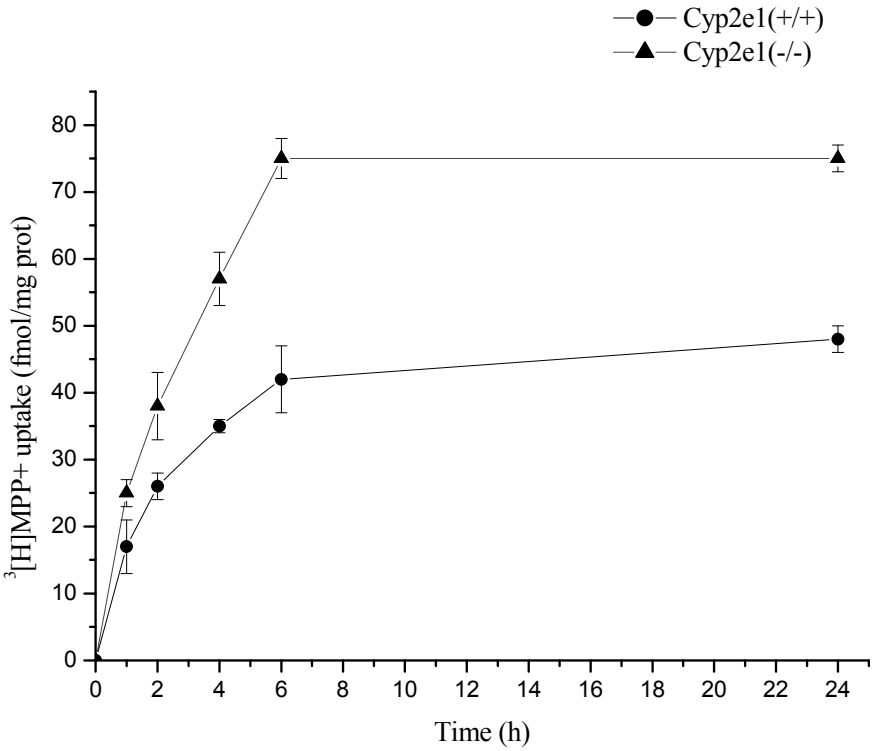


Fig. 3. *Time curve of the long-term in vitro [3H]MPP+ uptake in mesencephalic cell cultures from Cyp2e1(+/+) and Cyp2e1(–/–) mice. At 9 DIV, mesencephalic cell cultures were exposed to [3H]MPP+ at 2 nM for 1, 2, 4, 6, and 24 hours, after which the cells were scraped and evaluated for [3H]MPP+ content. *p < 0.05 with respect to Cyp2e1(+/+) values. DIV = days in vitro; n.s. = not significant.*

small non-polar molecules (Lieber, 1997). To date, many inhibitors and inducers of CYP2E1 have been found, and a complete list is available at the cytochrome P450 database (http://cpd.ibmh.msk.su) CYP2E1 was detected in neurons and glial cells of many nuclei including the SN of the rat brain (Hansson et al, 1990). Later, the enzyme was found in the rat basal ganglia, including the striatum, and it could be induced by ethanol or nicotine administration (Anandatheerthavarada et al, 1993; Sohda et al, 1993). Cyp2e1 mRNA expression was demonstrated in several areas of the human brain, including the SN (Farin & Omiecinski, 1993). In this same region, the enzyme was found in cells that morphologically resembled DA neurons (Riedl et al, 1996). Watts et al. (1998) showed definitively that inducible CYP2E1 existed in the same compartment as TH in the rat SN. In addition, localisation of the enzyme in monkey brain, as well as in prenatal and adult human brain was confirmed (Upadhya et al, 2000; Brzezinski et al, 1999; Joshi & Tyndale, 2006).

Recently, we investigated the response of Cyp2e1 knockout mice to the toxic insult by MPTP in a typical paradigm of an acute challenge, and we compared the results obtained in these animals with those observed in their wild-type counterparts. For that study, we reported that Cyp2e1(–/–) mice were less sensitive to the typical nigral damage produced by the toxin, which strongly indicated that CYP2E1 is involved in the MPTP mechanism of toxicity (Viaggi et al, 2009).

However, the reduced sensitivity to MPTP and not the absence of toxicity in Cyp2e1(–/–) mice may also be due in part to compensatory mechanisms taking place as a consequence of the missing protein. This phenomenon was observed very clearly by Gonzalez when his team generated these mice in order to study acetaminophen-induced liver toxicity (Lee et al, 1996). The drug causes liver and kidney necrosis

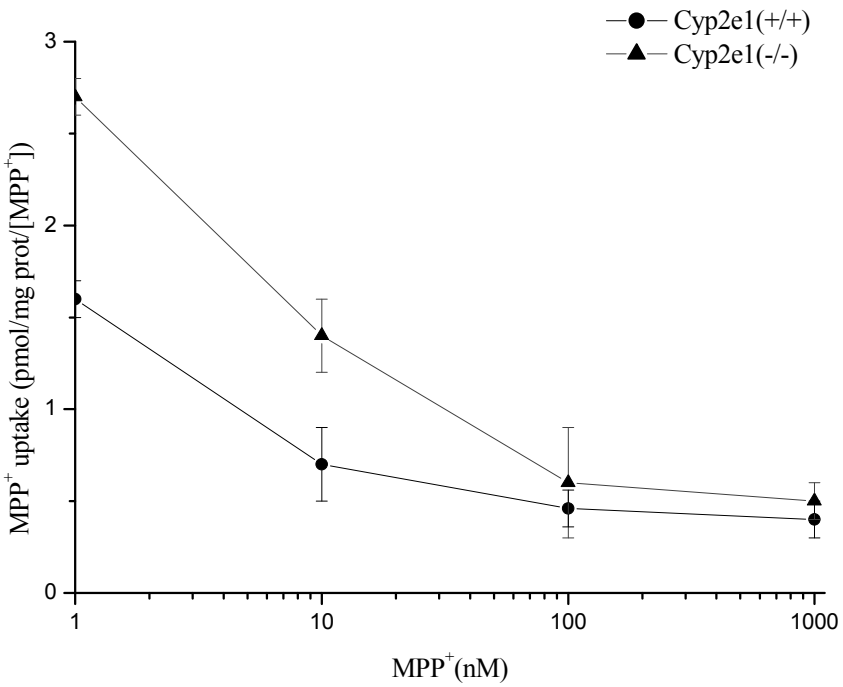


Fig. 4. *Dose-response curve of long-term uptake of [3H]MPP+ in mesencephalic cell cultures from Cyp2e1(+/+) and Cyp2e1(–/–) mice after 4 h of exposure. At 9 DIV, mesencephalic cell cultures were exposed to [3H]MPP+ at 1, 10, 100, and 1,000 nM for 4 hours, after which the cells were scraped and evaluated for [3H]MPP+ content. MPP+ is expressed as pmoles per mg of protein per MPP+ concentration. *p < 0.05 vs. Cyp2e1(+/+). DIV = days in vitro; n.s. = not significant*

when it is metabolised to an alkylating intermediate by the P450 system, and more specifically by CYP2E1 (Gonzales, 2007; Jollow et al, 1973; Mitchell et al, 1973).

To gain greater insight into the role of CYP2E1 in MPTP toxicity in mice, in this study we have prepared mesencephalic cell cultures from Cyp2e1(–/–)and wild type mice and we studied the effect of 24h MPP+ exposure. Here we found that Cyp2e1(–/–) cells are less sensitive to the MPP+ toxic insult as measured by [3H]dopamine uptake and microscopic observations after TH immunostaining, in a dose-response curve of MPP+ with a range of concentrations from 0.5 to 5 μM. Again, as observed in vivo, the difference from the wild-type control is not dramatic, although significant, suggesting further that CYP2E1 is involved in MPTP toxicity. In contrast, the difference we obtain in the long-term uptake of MPP+ in our cultures is dramatic. When MPP+ was measured inside the cells of the cultures, the total amount retained in the Cyp2e1(–/–) preparation was almost double that of the control culture. The rate of accumulation inside the cells was almost similar but the amount of MPP+ at 'plateau' at trace doses (2 nM) was significantly and dramatically enhanced.

The dose-response study of the long-term retention of MPP+ revealed that the difference is observed only at small amounts of the toxin. Increasing concentrations of MPP+ nullified the difference suggesting that the affinity of the CYP2E1 enzyme for the substrate is highly specific.

At least two important issues must be considered. Firstly, CYP2E1 is an enzyme that produces reactive oxygen species (ROS) in the liver and also the brain (Ronis et al, 1996; Karuzina & Archakov, 1994; Shahabi et al, 2008; Staudt et al, 1974). The production of ROS is elicited either by endogenous and exogenous substrates or also by inhibitors

(Shahabi et al, 2008; Montoliu et al, 1995; Tierney et al, 1992). Thus, the lack of the enzyme leads to a reduction of oxidative stress in the cell overall when it is challenged by a toxin.

The second issue concerns the results we obtained previously in short-term experiments on ex vivo striatal MPP+ kinetics and dopamine levels (Viaggi et al, 2009). MPP+ clearance is markedly accelerated, indicating that MPP+ is eliminated from a pool with a high turnover rate such as that represented by vesicular storage and dopamine, which is displaced by MPP+, decreases at a faster rate in ex vivo measurements (Jones et al, 1998) as measured by the rise of 3-methoxytyramine (3-MT) (Bocchetta et al, 1985; Del Zompo et al, 1993). Our data from this study might therefore indicate that, in mice lacking CYP2e1, MPP+, once inside the cell enters preferentially into vesicles where its storage represents a sort of protection with respect to other toxic sites such as mitochondria (Kopin, 1992). Our preliminary results may indicate also that CYP2E1 participates in the fragile balance of mechanisms producing or removing highly reactive molecules in the MPTP-induced impairment of DA neurons. Relevance of all these findings in animal models is further strengthened by a recent study by Kaut et al. in humans, who found decreased methylation of the cytochrome CYP0 2E1 gene and increased expression of CYP 2E1 messenger RNA in PD patients' brains, suggesting that epigenetic variants of this cytochrome contribute to PD susceptibility (Kaut et al Wüllner, 2012) .

More recently Kaut et al (2012) found decreased methylation of the cytochrome CYP0 2E1 gene and increased expression of CYP 2E1 messenger RNA in PD patients' brains, suggesting that epigenetic variants of this cytochrome contribute to PD susceptibility.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs Natalia Jaksic for her assistance in the preparation of the manuscript, and Mrs Ornella Baroni and Lucia Aguirre for their help in taking care of the animals.

REFERENCES

Anandatheerthavarada HK, Shankar SK, Bhamre S, Boyd MR, Song BJ, Ravindranath V (1993) Induction of brain cytochrome P-450IIE1 by chronic ethanol treatment. Brain Res; 601: 279–285.

Bocchetta A, Piccardi MP, Del Zompo M, Pintus S, Corsini GU. (1985) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine: correspondence of its binding sites to monoamine oxidase in rat brain, and inhibition of dopamine oxidative deamination in vivo and in vitro. J Neurochem; 45: 673–676.

Brzezinski MR, Boutelet-Bochan H, Person RE, Fantel AG, Juchau MR (1999) Catalytic activity and quantitation of cytochrome P-450 2E1 in prenatal human brain. J. Pharmacol Exp Ther; 289: 1648–1653.

Corsini GU, Pintus S, Chiueh CC, Weiss JF, Kopin IJ. (1985) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by pretreatment with diethyldithiocarbamate. Eur J Pharmacol; 119:127–128.

Corsini GU, Zuddas A, Bonuccelli U, Schinelli S, Kopin IJ (1987) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by ethanol or acetaldehyde. Life Sci; 40: 827–832.

Davis CG, Williams AC, Markey SP, Ebert MH, Caine ED, Reichert CM, Kopin IJ (1979) Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. Psychiatry Res; 1: 249–254.

Del Zompo M, Piccardi MP, Ruiu S, Quartu M, Gessa GL, Vaccari A. (1993) Selective MPP+ uptake into synaptic dopamine vesicles: possible involvement in MPTP neurotoxicity. Br J Pharmacol; 109: 411–414.

de Waziers I, Cugnenc PH, Yang CS, Leroux JP, Beaune PH. (1990) Cytochrome P 450 isoenzymes, epoxide hydrolase and glutathione transferases in rat and human hepatic and extrahepatic tissues. J. Pharmacol Exp Ther; 253: 387–394.

Di Monte D, Jewell SA, Ekström G, Sandy MS, Smith MT (1986) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridine (MPP+) cause rapid ATP depletion in isolated hepatocytes. Biochem. Biophys Res Commun;137: 310–315.

Farin FM, Omiecinski CJ (1993) Regiospecific expression of cytochrome P-450s and microsomal epoxide hydrolase in human brain tissue. J Toxicol Environ Health; 40: 317–335.

Gonzalez FJ. (2007) The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. Drug Metab Dispos. 35: 1–8.

Hansson T, Tindberg N, Ingelman-Sundberg M, Köhler C. (1990) Regional distribution of ethanol-inducible cytochrome P450 IIE1 in the rat central nervous system.

Neuroscience; 34: 451–463.

Irwin I, Wu EY, DeLanney LE, Trevor A, Langston JW (1987) The effect of diethyldithiocarbamate on the biodisposition of MPTP: an explanation for enhanced neurotoxicity. Eur J Pharmacol; 141: 209–217.

Javitch JA, Snyder S (1984) Uptake of MPP(+) by dopamine neurons explains selectivity of parkinsonism-inducing neurotoxin, MPTP. Eur J Pharmacol; 106: 455–456.

Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR, Brodie BB (1973) Acetaminophen-induced hepatic necrosis. II. Role of covalent binding in vivo. J Pharmacol Exp Ther; 187: 195–202.

Jones SR, Gainetdinov RR, Wightman RM, Caron MG. (1998) Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. J Neurosci; 18: 1979–1986.

Joshi M, Tyndale RF. (2006) Regional and cellular distribution of CYP2E1 in monkey brain and its induction by chronic nicotine. Neuropharmacology; 50: 568–575.

Karuzina II, Archakov AI (1994) The oxidative inactivation of cytochrome P450 in monooxygenase reactions. Free Radic Biol Med; 16: 73–97.

Kaut O, Schmitt I, Wüllner U. (2012) Genome-scale methylation analysis of Parkinson's disease patients' brains reveals DNA hypomethylation and increased mRNA expression of cytochrome P450 2E1. Neurogenetics; 13: 87-91.

Kopin I. (1992) Mechanisms of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced destruction of dopaminergic neurons. In: Herken H, Hucho F. (Eds.), Handbook of Experimental Pharmacology. Vol. 102: Selective Neurotoxicity. Springer, Berlin. p. 333–356

Langston JW, Ballard P, Tetrud JW, Irwin I (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219: 979–980.

Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ. (1996) Role of CYP2E1 in the hepatotoxicity of acetaminophen. J Biol Chem; 271: 12063–12067.

Lieber CS (1997) Cytochrome P-4502E1: its physiological and pathological role. Physiol Rev; 77: 517–544.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem; 193: 265–275.

Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. (1973) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther; 187: 211–217.

Montoliu C. Sancho-Tello, M, Azorin I, Burgal M, Vallés S, Renau-Piqueras J, Guerri C. (1995) Ethanol increases cytochrome P4502E1 and induces oxidative stress in astrocytes. J Neurochem; 65: 2561–2570.

Nicklas WJ, Vyas I, Heikkila RE (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci; 36: 2503–2508.

Pardini C, Vaglini F, Viaggi C, Caramelli A, Corsini GU. (2008) Role of CYP2E1 in the mouse model of MPTP toxicity. Parkinsonism Relat Disord;14 Suppl 2:S119-23.

Prochiantz A, Daguet MC, Di Porzio U, Herbet A, Glowinski J (1983) In vitro studies on central dopaminergic neurons' development. Prog Brain Res; 58: 365–368.

Riedl AG, Watts PM, Edwards RJ, Boobis AR, Jenner P, Marsden CD (1996) Selective localisation of P450 enzymes and NADPH-P450 oxidoreductase in rat basal ganglia using anti-peptide antisera. Brain Res; 743: 324–328.

Ronis MJJ, Lindros KO, Ingelman-Sundberg M. The CYP2E subfamily. In: Ioannides, C., (Ed.), Cytochromes P450: Metabolic and Toxicological Aspects. CRC Press 1996. Boca Raton. p. 211–240.

Shahabi HN, Andersson DR, Nissbrandt H. (2008) Cytochrome P450 2E1 in the substantia nigra: relevance for dopaminergic neurotransmission and free radical production. Synapse. 62: 379–388.

Sohda T, Shimizu M, Kamimura S, Okumura M (1993) Immunohistochemical demonstration of ethanol-inducible P450 2E1 in rat brain. Alcohol Alcohol; Suppl. 1B: 69–75.

Staudt H, Lichtenberger F, Ullrich V. (1974) The role of NADH in uncoupled microsomal monoxygenations. Eur J Biochem; 46: 99–106.

Thomas PE, Bandiera S, Reik LM, Maines SL, Ryan DE, Levin W. (1987) Polyclonal and monoclonal antibodies as probes of rat hepatic cytochrome P-450 isozymes. Fed Proc; 46: 2563–2566.

Tierney DJ, Haas AL, Koop D.R. (1992) Degradation of cytochrome P450 2E1: selective loss after labilization of the enzyme. Arch Biochem Biophys; 293: 9–16.

Tindberg N, Ingelman-Sundberg M (1996) Expression, catalytic activity, and inducibility of cytochrome P450 2E1 (CYP2E1) in the rat central nervous system. J Neurochem; 67: 2066–2073.

Upadhya SC, Tirumalai PS, Boyd MR, Mori T, Ravindranath V. (2000) Cytochrome P4502E (CYP2E) in

brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. Arch Biochem Biophys; 373: 23–34.

Vaglini F, Fascetti F, Tedeschi D, Cavalletti M, Fornai F, Corsini GU. (1996) Striatal MPP+ levels do not necessarily correlate with striatal dopamine levels after MPTP treatment in mice. Neurodegeneration; 5: 129–136.

Vaglini F, Pardini C, Viaggi C, Bartoli, C, Dinucci D, Corsini GU (2004) Involvement of cytochrome P450 2E1 in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease. J Neurochem; 91: 285–298.

Vaglini F, Pardini C, Viaggi C, Caramelli A, Corsini GU. (2008) Apomorphine offers new insight into dopaminergic neuron vulnerability in mesencephalic cultures. Neuropharmacology; 55: 737–742.

Viaggi C, Vaglini F, Pardini C, Caramelli A, Corsini G.U. (2009) MPTP-induced model of Parkinson's disease in cytochrome P450 2E1 knockout mice. Neuropharmacology; 56: 1075–1081.

Watts PM, Riedl AG, Douek DC, Edwards RJ, Boobis AR, Jenner P, Marsden CD (1998) Co-localization of P450 enzymes in the rat substantia nigra with tyrosine hydroxylase. Neuroscience; 86: 511–519.

Zuddas A, Corsini GU, Schinelli, S, Barker, JL, Kopin, IJ, di Porzio U (1989) Acetaldehyde directly enhances MPP+ neurotoxicity and delays its elimination from the striatum. Brain Res; 501: 11–22.