A Review of MANF Literature: Implications for the Development Program of Amarantus Bioscience Holdings Inc.

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EXECUTIVE SUMMARY

- The discovery of mesencephalic, astrocyte-derived neurotrophic factor (MANF) as the first member of a new class of neurotrophic growth factors was published in 2003 by a team led by the scientific founder of Amarantus Bioscience Holdings Inc. (AMBS).

- MANF was originally found to selectively increase survival and to cause a marked degree of sprouting of mesencephalic dopaminergic neurons in culture.

- Subsequent work showed MANF is expressed in response to endoplasmic reticulum (ER) stress, secreted via the ER-Golgi pathway and active as a soluble autocrine / paracrine growth factor.

- Exogenous MANF displays anti-apoptotic activity against ER stress and serum-starvation and a neurotrophic effect as manifested by enhancement of survival and differentiation of dopaminergic neurons.

- In addition to these neurotrophic effects, there is evidence that MANF enhances GABA mediated post synaptic inhibitory currents in dopamine neurons of the substantia nigra. This effect may contribute to the protective action of MANF on dopamine neurons.

- The mechanism of MANF expression and secretion is well described but the MANF receptor and its down-stream signaling pathways are unknown. The biochemical and molecular characterization of MANF effects after its release offers an exciting area of original research.

- MANF is expressed in many human tissues and by virtue of its autocrine / paracrine growth factor function may display anti-apoptotic activities in a broad range of neuronal and non-neuronal target tissues. This offers opportunities in an expanded set of indications; orphan indications are particularly attractive for scientific, developmental and commercial reasons.

- MANF's activity in rodent models of Parkinson's disease (PD) is well established in peer-reviewed publications and further development of MANF in PD is clearly warranted.

- A key success factor for MANF's development in PD is its delivery to the right target in the human brain. Therefore, the ongoing evaluation and selection of the delivery device should be completed with high priority.

- A non-human primate pharmacokinetics study should be performed to establish delivery parameters such as dose, dose regimen, flow-rate, MANF tissue distribution and MANF intracellular transport.

- Profiling MANF in a non-human primate PD model is essential for proof of concept in this indication and to define design parameters for the first human clinical study.
- The GDNF development path with its translational data from rodents to humans serves as a guide for MANF development activities.
- Drafting of a target product profile (TPP) for PD should be initiated to focus the development plan and to facilitate FDA interactions.
- Mechanistic studies on the molecular biology and biochemistry of MANF have been largely conducted in academic laboratories, and therefore at little expense to AMBS. We believe the time is now ripe for AMBS to adopt a proactive role in generating original data through strategic collaborations and in-house efforts, so as to dominate the intellectual property and science in this emerging field.
- Given the known and unique biochemical and cellular properties of MANF, it is highly justified to expand investigations of MANF beyond PD to other clinical indications, including orphan diseases.

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1 Introduction

Mesencephalic, astrocyte-derived neurotrophic factor (MANF) (Petrova et al., 2003) and cerebral dopamine neurotrophic factor (CDNF) (Lindholm et al., 2007) form a distinct family of evolutionary conserved trophic factors with a unique domain organization. MANF was initially purified from conditioned media from an immortalized ventral mesencephalic astrocytic cell line (Petrova et al., 2003) and its sequence determined by a combination of proteomics and bioinformatics technologies. The second member of this protein family, CDNF, was discovered by a bioinformatics analysis of a sequence data base using the MANF sequence as template. Together, these two proteins form a family of growth factors with functions in the central nervous system and an emerging realization of their importance in peripheral tissues. The following sections will discuss the structures of these proteins, their cellular and *in vivo* activities and implications to their use as therapeutic entities in an increasing range of potential indications.

2 MANF and CDNF protein structures and implications to function

2.1 MANF and CDNF protein sequences

Human MANF and CDNF are expressed in a pre-form of 179 and 187 amino acid residues, respectively. Both proteins are N-terminally processed and a signal sequence of 21 and 26 amino acids, respectively, is removed, yielding the mature, secreted and active MANF (Petrova et al., 2003) and CDNF (Lindholm et al., 2007) of 158 and 161 amino acid residues, respectively. MANF and CDNF proteins function as secreted neurotrophic factors but some of their activities may occur while they are resident in the endoplasmic reticulum (ER) lumen. The former activities (i.e., those shown for the secreted form) are relevant to MANF and CDNF protein therapeutics development while the latter activities (i.e., those shown for the intracellular resident form) could be achieved best with a small molecule mimetic, inducer or enhancer of MANF and/or CDNF.

The mature human MANF protein starts with the amino acid sequence L_{22}RPGD… and ends with …TDL_{179}. The mature human CDNF protein starts with the amino acid sequence Q_{27}EAG… and ends with …TEL_{187}. The full-length human CDNF and MANF sequences are 52% identical and the mature forms are 58% identical. A sequence alignment in which notable structural elements are indicated is given in Figure 1.

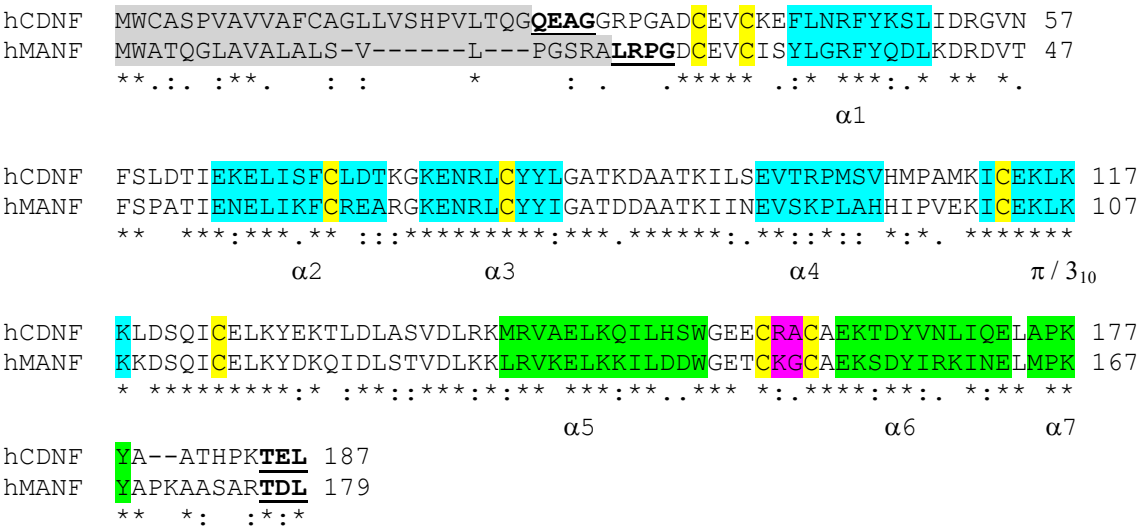

Figure 1: Human CDNF and human MANF sequences: Alignment of full-length sequences. Signal sequences in grey. Start and end of mature sequences in bold / underlined. Secondary structural elements (i.e., alpha helices, π helix, 3_{10} helix) in N-terminal saposin-like domain (cyan) and C-terminal SAP-like domain (green) are indicated according to Hoseki et al. 2010. "CXXC" motif (magenta). The eight conserved cysteines are indicated (yellow).

2.2 Three-dimensional structures of MANF and CDNF proteins

The three-dimensional structure of full-length MANF was determined by NMR (Hoseki et al., 2010; Hellman et al., 2010; Hellman et al., 2011) and by X-ray crystallography (Parkash et al., 2009). The structure of CDNF could be determined for the N-terminal domain only (Parkash et al., 2009) and it was hypothesized that the C-terminal domain might be unstructured. The nuclear magnetic resonance assignments for the N-terminal domain of CDNF will enable the determination of a solution structure for CDNF (Latgé et al., 2013). The following discussion will focus on the MANF structure since there is more structural information available for this protein. However, the CDNF structure very closely resembles that of MANF. The NMR-structure of mature MANF identified two distinct domains joined by a linker. A solution dynamics study determined that the two domains tumble independently and that the linker is highly flexible (Hoseki et al., 2010).

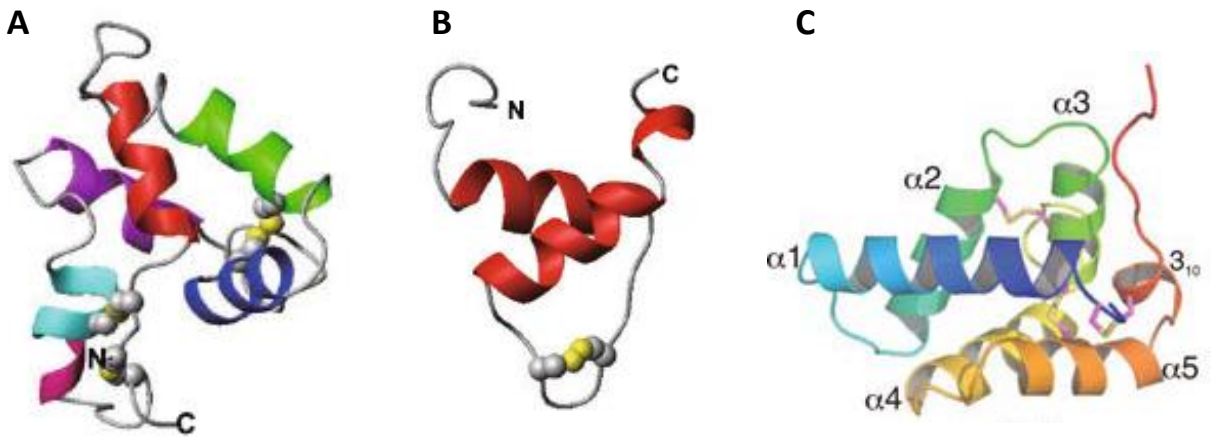

Figure 2: Three-dimensional structures of MANF and CDNF. Secondary structures (helices) are shown as ribbons. A: MANF N-terminal domain structure determined by NMR. Location of disulfide bonds indicated by CPK. B: MANF C-terminal domain determined by NMR. Location of CXXC motif indicated by CPK. C: CDNF N-terminal domain determined by X-ray crystallography.

The N-terminal domain (N-domain) of MANF encompassing residues $L_{22} – L_{126}$ is entirely helical, with four α-helices and a rare structural element, a π helix, immediately followed by a 3_{10} helix (Hoseki et al. 2010). Most π-helices are involved in binding enzyme substrates or ligand molecules. The N-domain contains three disulfide bonds. A cluster of positively charged residues in the π and 3_{10} helices are conserved among MANF homologues and may indicate functionally important residues. A weak but significant structural similarity to the N-domain was found with saposin-like proteins (i.e., Saposin D). Saposin D has the same topology of secondary structural elements and the arrangement of disulfide bonds is identical. Saposins are required for the degradation of plasma membrane derived glycosphingolipids in the lysosome. However, the charged surface of MANF suggests that interacting molecules and the biological function may differ considerably between MANF and saposins. It is at present unclear whether MANF or CDNF interact with lipids or membranes. Nevertheless, this mechanism could be important for the function of these neurotrophic factors (Lindholm, 2009).

The C-terminal domain (C-domain) of MANF encompasses residues $T_{131} – L_{179}$ and is well defined in the NMR solution structure. This domain is also entirely helical and contains one disulfide bond between conserved cysteines in the CXXC motif between α-helices 5 and 6. The CXXC motif is a consensus sequence of proteins of the thiol-protein oxidoreductase superfamily, other members of which include thioredoxins, glutaredoxins, and peroxiredoxins. Common to this enzyme superfamily is that all members are involved in disulfide mediated redox reactions and glutathione metabolism in which the CXXC domain takes center stage. However, no enzymatic oxidoreductase activity has been detected for MANF so far (Mizobuchi et al, 2007,

data not shown). The presence of this prominent structural motif is intriguing and warrants further investigation in particular with respect to its role in the ER stress response. A further hypothesis on the function of the C-domain has been generated using structural alignment. Hellman et al., (2011) determined that the MANF C-domain is structurally similar to SAP-domains (SAF-A/B, Acinus, PLAS) and most similar to the SAP-domain of Ku70. Ku70 is a cytoplasmic protein with anti-apoptotic activity. Ku70 is associated with Bax, keeping the latter in an inactive conformation. Once Bax dissociates from Ku70 the mitochondrial cell death pathway is activated. Based on structural considerations it is thus conceivable that MANF displays functions related to apoptosis. Interaction studies of MANF with Bax or other proteins involved in apoptosis would shed light on the molecular mechanism of MANF in apoptosis. Whether a Ku70-derived peptide (VPMLK) can arrest Bax-mediated apoptosis remains controversial but MANF carries a similar sequence (VKELK) on α-helix 5 that could serve as a starting point for a small molecule mimetic of MANF. The Michael J. Fox Foundation funded a grant to Arumae, Saarma and Tuominen on the development of MANF-derived peptides for treatment of PD (Rapid Response Innovation Awards 2011). The mode-of-action of the MANF mimetic peptide is stated as prevention of cellular death by intracellular action. In an interim progress report of November 2012, the group announced that a tetrapeptide was active in a 6-OHDA model of Parkinson's disease (PD), although no details were given.

Structure/function analysis studies for MANF or CDNF have not been performed in a systematic manner. This is likely the case because there is no receptor known that mediates the effects of these growth factors and thus no detailed molecular mechanism for their intracellular action has been described. Nevertheless, Parkash et al. (2009) speculated on functionally important residues in MANF and CDNF based on a comparison of residues that are identical in MANF orthologues but different in CDNF. Of these, E100 and K109, located in the π and 3_{10} helical regions, were considered to be the most important. The method employed by Parkash et al., is likely to identify residues involved in selective activity of one growth factor versus another of the same family but might miss functionally important residues common in both growth factors. As a reference, in the neurotrophin family, the key arginine residue (i.e., R103 in NT-3) for receptor interaction is fully conserved across all orthologues of the nerve growth factor family. Therefore, further studies to explore the importance of common residues between MANF and CDNF on biological activity are justified.

The structural arrangement of domains in MANF and CDNF is unique within the family of growth factors. Both proteins have two independent domains that may perform different functions or mediate different aspects of a function. Moreover, extracellular and intracellular activities have been described for MANF and CDNF which is unusual for a secreted growth factor. However, this combined intracellular and extracellular activity is reminiscent of macrophage migration inhibitory factor (MIF) that combines cytokine and enzymatic activities in one relatively small protein. Interestingly, MIF also contains a CXXC motif and exhibited low redox catalytic activity *in vitro* (compared to thioredoxin and glutaredoxins) and modulated

cellular redox stress responses by elevating the intracellular glutathione (GSH) pool (Reviewed in Savaskan et al., 2012).

Since MANF and CDNF will be administered as exogenous proteins their therapy relevant activities will be mediated by extracellular events including receptor binding and potentially endocytosis / retrograde transport. On the other hand intracellular activities of MANF / CDNF may be of relevance for small molecule mimetics, enhancers or inducers of MANF / CDNF expression. Such compounds could affect both intracellular and extracellular MANF / CDNF pathways. The two PD drugs selegiline (Monoamine oxidase B inhibitor) and SCH58261 (adenosine A(2A) antagonist) did not influence CDNF expression levels in the mouse brain (Gyárfás et al., 2010), but valproic acid induced the expression of MANF, CDNF and glial cell-line derived neurotrophic factor (GDNF) (Almutawaa et al., 2012).

3 Cellular activities of MANF and CDNF

MANF was purified from conditioned media from an immortalized ventral mesencephalic astrocytic cell line (Petrova et al., 2003). Successive chromatographic protein purification steps were combined with a sensitive and robust bioassay that measured survival of dopaminergic neurons (Petrova et al., 2004) to identify active, neurotrophic factor containing fractions. An isolated, active protein was subjected to proteolytic digestion and N-terminal sequences of two peptidic fragments were determined. These peptides were then used in BLAST searches of the NCBI protein sequence database which led to a match with human Arginine-Rich Protein (ARP) also known as human Arginine-Rich, Mutated in Early stage Tumors (ARMET) (Shridhar et al., 1996). The full-length sequence for MANF, including the most likely position of the Met start codon, was identified by further bioinformatics analyses and vectors for mammalian and bacterial MANF expression were constructed. CDNF was discovered by bioinformatics analysis using the MANF sequence and a database search for related sequences. Full-length CDNF was cloned into expression vectors and characterized *in vivo* (Lindholm et al., 2007).

3.1 Tissue distribution of MANF and CDNF

MANF expression is widespread in the nervous system and in non-neuronal tissues (Lindholm et al., 2008). mRNA levels in human brain tissues was widespread and highest in cerebral frontal cortex, optic nerve, cerebellum, dentate nucleus and pons. High levels were also detected in medulla, cerebellum white matter, cerebral pedunculi, colliculi, corpus callosum and hippocampus. Low levels of mRNA were detected in many additional brain tissues, including substantia nigra. MANF protein expression in the substantia nigra was only partially co-localized with tyrosine hydroxylase (TH). MANF is thus expressed in potential target tissues relevant to the treatment of PD. In non-neuronal tissues, the highest mRNA levels were found in lung, stomach and testis and high levels in many other tissues including brain, heart and liver. At the protein level, in mouse, the highest levels occurred in liver, salivary gland and testis, but interestingly not in lung. An independent study (Mizobuchi et al., 2007) found the highest mouse MANF mRNA levels in muscle, testis and stomach.

Highest mRNA expression for CDNF was detected in heart, skeletal muscle and testis (Lindholm et al., 2007). Brain mRNA levels were low but detectable. Within the brain, levels of CDNF mRNA were detected (ranked from highest to lowest) in midbrain / pons / medulla > cerebellum > striatum > thalamus > hippocampus / cortex. At the protein level, CDNF was detected (ranked from highest to lowest) in heart > testis > skeletal muscle > liver / salivary gland > lung > kidney / brain. In the substantia nigra, CDNF was detected in solitary cells that did not express TH. CDNF mRNA and protein expression was also found in cultured midbrain astrocytes (Rocha et al., 2012).

Given that MANF and CDNF are considered to be autocrine / paracrine trophic factors, the tissue distribution of these growth factors will likely coincide with the target tissue of exogenously

applied therapeutic MANF and CDNF proteins. MANF and CDNF have overlapping yet distinct expression profiles. Hence, these differentiated expression profiles may aid in identifying the most promising indications for each of these two growth factors. MANF mRNA expression in the brain was relatively high while CDNF mRNA is low in brain tissues. On the other hand, CDNF mRNA and protein expression is high in skeletal muscle while the levels of MANF are unclear as two studies reported high and low levels in this tissue, respectively (Mizobuchi et al., 2007; Lindholm et al., 2008).

3.2 Expression of MANF and endoplasmic reticulum stress

MANF (and likely CDNF) display activities linked to their secreted, extracellular form and potentially a distinct set of activities while they reside within the cell. This distinction has important implications for the design of therapeutics based on the MANF (and CDNF) proteins. Recombinant forms of MANF (or CDNF) will display activities observed for the secreted, extracellular form of MANF while small molecule mimetics, inducers or enhancers will be able to perform intracellular MANF activities. Extracellular and intracellular activities may be performed in different cell types. MANF was initially described as a paracrine neurotrophic factor for dopaminergic neurons expressed in astrocytes (Petrova et al., 2003). However, MANF expression has also been reported in neurons (Yu et al., 2010; Shen et al., 2012). MANF intracellular effects may thus manifest themselves in both astrocytes and neurons. If MANF acts as an autocrine neurotrophic factor, then MANF receptor mediated effects could regulate intracellular pathways in the same cells that express MANF in response to pathological stimuli.

The endoplasmic reticulum (ER) is the key site of protein synthesis. ER quality control mechanisms monitor protein folding and prevent the transport and secretion of immature proteins. Misfolded proteins are discarded by ER-associated degradation. When ER stress, caused for instance by oxidative stress, overwhelms the capacity of the quality control system, unfolded or misfolded proteins accumulate in the ER. ER stress sensor proteins, PERK, IRE1 and ATF6 activate an intracellular signal transduction pathway called the unfolded protein response (UPR). The UPR increases the expression of several target genes to restore ER homeostasis. The functions of UPR target genes vary broadly and include protein folding helpers (i.e., chaperones) and proteins involved in glycosylation, oxidative stress response, protein trafficking, lipid biosynthesis and ER-associated degradation. Aspects of ER stress and the UPR have been linked to the development of several neurodegenerative disorders (Lindholm et al., 2006). In the context of PD, it is noteworthy that a prominent feature of this disease is the presence of intraneuronal cytoplasmic inclusion bodies, known as Lewy bodies. Studies of families with rare autosomal recessive PD identified several genes coding for mutated proteins that could be causative for PD. Among them, aggregated alpha-synuclein is found in Lewy bodies. In the transgenic mouse line A53TαS aggregated alpha-synuclein was associated with abnormal UPR that could promote neuronal death (Colla et al., 2012). It is thus conceivable that

a growth factor such as MANF whose expression is induced by ER stress and the UPR could counteract degenerative mechanisms caused by protein aggregation.

MANF was initially isolated from a cell-line that constitutively expressed and secreted MANF (Petrova et al., 2003) but regulation of expression and secretion of MANF, and to a lesser degree CDNF, was mostly studied in the context of the cellular stress response. MANF/ARMET was identified as an UPR target gene (Lee at el., 2003). In a subsequent microarray study of genes induced by the UPR, MANF was one of twelve regulated proteins (Apostolou et al., 2008). The MANF promoter contains an ER stress response element, ERSE-II, that is activated by known ER stressors like tunicamycin and thapsigargin (Apostolou et al., 2008; Tadimalla et al., 2008). Induction of MANF expression by ER stressors was demonstrated in several independent studies (NIH3T3 cells / tunicamycin, thapsigargin, Mizobuchi et al., 2007; U2OS, 293, SHST5Y cells / tunicamycin, thapsigargin, Apostolou et al., 2008; Primary cultured neurons / tunicamycin, Yu et al., 2010; Cardiac myocytes, HeLa cells / tunicamycin, thapsigargin, DTT, Glembotski et al., 2012; Neuro2a cells / thapsigargin, Oh-hashi et al., 2012).

Ischemia is a well-established pathophysiological mechanism leading to ER stress. Correspondingly, there is evidence from several independent studies that MANF expression is induced by ischemic conditions, including ischemia of the heart (Tadimalla et al., 2008) and the brain (Global ischemia, Lindholm et al., 2008; Focal ischemia, Yu et al., 2010, Shen et al., 2012).

Epileptic seizures may induce ER stress. Accordingly, animals exposed to two hours of electrically induced status epilepticus displayed a transient increase of MANF in a rather restricted area of the hippocampus (i.e., dentate granule cell layer), the thalamic reticular nucleus and several cortical regions (Lindholm et al., 2008). Chronic administration of valproic acid increased MANF and CDNF mRNA in the hippocampus (Niles et al., 2012).

Increased expression of MANF (ARMET) was also observed in oocytes after *in vitro* maturation (IVM) and MANF remained elevated in the brains of the resulting newborn mice (Wang et al., 2011). It is conceivable that the IVM procedure induces stress on oocytes resulting in an upregulation of MANF, however, the underlying mechanism of these observations has not been studied. Fibroblasts from long-lived Snell dwarf mice display a higher sensitivity to the lethal effects of ER stress than cells from normal mice (Sadighi Akha et al., 2011) and they express lower levels of MANF (ARMET), further confirming a potential role of MANF in counteracting ER stress. Finally, ER stress in chondrocytes induced by matrilin-3 mutations (Nundlall et al., 2010) or in an *in vivo* model of Schmid-type metaphyseal chondrodysplasia (Cameron et al., 2011) induced the expression of MANF (ARMET). These latter studies have not investigated the role of MANF in the development of the specific pathology of epiphyseal dysplasia and Schmid-type metaphyseal chondrodysplasia.

It is thus well established that ER stress induces MANF expression in different cell types. MANF may thus be cell protective and/or regenerative in a variety of pathological situations. Hence, this offers numerous exciting opportunities for development of MANF in indications beyond PD. Moreover, the fact that the MANF promoter contains an ER-stress response element could be exploited for the discovery of therapeutics (e.g. small molecule compounds) inducing expression of MANF that could mimic the effects of intracellular MANF.

3.3 Mechanisms of MANF/CDNF secretion

Upon translocation into the ER, the signal sequence of pro-MANF is removed to yield mature MANF. The protein is ER-resident on the luminal side of the ER (Apostolou et al., 2008) as it co-localizes with protein disulfide isomerase (PDI) (Mizobuchi et al., 2007). MANF is then secreted from the ER to the Golgi and correspondingly Brefeldin A, a Golgi disrupter, completely inhibits MANF secretion (Apostolou et al., 2008; Oh-hashi et al., 2012). It is unclear whether glycosylation of MANF is required for this process as one study found an effect by tunicamycin (Petrova et al., 2003) while another did not (Oh-hashi et al., 2012).This ER-Golgi transport is COPII-dependent (Oh-hashi et al., 2012). Therefore, MANF is secreted via the ER-Golgi pathway.

ER stress conditions can be created by thapsigargin which depletes the ER (and the sarcoplasmic reticulum) of Ca^{2+}. Thapsigargin has a rapid effect on Ca^{2+} depletion (within 30') and increases the levels of MANF in conditioned medium of HeLa cells and cardiomyocytes within the same time-frame (Glembotski et al., 2012). Overexpression of GRP78 (i.e., chaperone protein BiP) retains MANF intracellularly (Oh-hashi et al., 2012; Glembotski et al., 2012). GRP78 and MANF form a complex when overexpressed in HeLa cells or cardiac myocytes that is Ca^{2+} concentration dependent. Reducing Ca^{2+} by thapsigargin yields less MANF / GRP78 complex (Glembotski et al., 2012). The direct association of endogenous MANF and GRP78 was demonstrated in cardiac myocytes using zero distance photo cross-linking technology (Glembotski et al., 2012).

MANF carries a sequence element RTDL at the C-terminus which is related to the known ER retention signal KDEL. A MANF variant lacking the RTDL sequence is spontaneously secreted while the wild type and a mutant form carrying the KDEL sequence at the C-terminus are retained in the cell to some extent. Overexpression of GRP78 retained essentially all of the MANF protein of these three variants inside the cell (Glembotski et al., 2012). The GRP78 interaction is not directly dependent on the RTDL / KDEL sequence since a MANF mutant lacking this element was equally retained in the ER by GRP78. This was independently shown by Oh-hashi et al. (2012). MANF ER retention and secretion may be modulated by the KDEL receptor (KDELR) family by a mechanism involving the MANF C-terminal sequence RTDL (Henderson et al., 2012). Removal of RTDL increases MANF levels in cell supernatants possibly due to reduced ER / Golgi retention by KDELR. KDELR recombinant expression in SH-SY5Y cells reduces secreted MANF but not MANF lacking RTDL. MANF and KDELR were detected

at the cell surface but due to the qualitative detection method employed, the degree of KDELR cell surface display is unknown. An association between MANF and KDELR was not demonstrated with a direct method but peptides derived from MANF and KDELR inhibited MANF cell surface binding.

In summary, MANF and GRP78 form a complex in the ER lumen. This complex formation prevents secretion of MANF. The stability of the complex is Ca^{2+} concentration dependent. ER stress decreases the Ca^{2+} concentration in the ER, MANF dissociates from GRP78, is secreted and performs its functions as a paracrine or autocrine neurotrophic factor. In addition to GRP78 mediated retention, there exists a second ER retention mechanism for MANF based on the conserved sequence motif RTDL / KDEL. How these two mechanisms interact is unknown. Moreover, evidence of specific intracellular activity of the MANF / GRP78 or the MANF / KDELR complexes are absent and it is thus unknown whether these complexes fulfill any function beyond retaining MANF within the ER.

The information on CDNF expression and secretion is much sparser. In PC-12 cells, CDNF is secreted via constitutive and regulated pathways and follows the ER-Golgi-secretory granule route (Sun et al., 2011). CDNF is also secreted when expressed in HEK293 cells and secretion is completely inhibited by Brefeldin A but not by tunicamycin (Apostolou et al., 2008). Given the structural similarity of CDNF and MANF it is conceivable that CDNF secretion is similarly regulated as MANF's but there is currently no evidence for this.

3.4 Activities mediated by MANF overexpression or mRNA knock-down

MANF is expressed in response to ER stress and the UPR. The question arises whether MANF could have activities that manifest themselves intracellularly that would not require secretion of MANF and the subsequent activation of a receptor-mediated signaling pathway. Three studies used molecular biology techniques to increase or decrease the expression levels of MANF intracellularly by overexpression or knock-down, respectively (Apostolou et al., 2008; Tadimalla et al., 2008; Hellman et al., 2011).

Knock-down of MANF expression by siRNA rendered HeLa cells more sensitive to cell death induced by ER stress. Moreover, overexpression of MANF in U2OS cells protected cells from ER-stress induced cell death (Apostolou et al., 2008). Knock-down of MANF expression by micro-RNA increased cell death of cardiomyocytes after simulated ischemia / reperfusion while overexpression of MANF protected these cells from serum-deprivation induced caspase-3 activation and ischemia-induced cell death (Tadimalla et al., 2008). Hence, the MANF protein protects cells from stress induced by ischemia, serum-deprivation and more specifically, ER stress.

However, the design of these experiments prevents a conclusion on whether this protective effect is mediated by intracellular MANF or by a secreted MANF-induced receptor signaling pathway.

Both siRNA-mediated knockdown and overexpression of MANF will affect ER-resident MANF as well as secreted MANF. The observed effects could thus be explained by extracellular MANF binding to a receptor or by ER-resident MANF performing an intracellular function. This distinction is critical to understanding the potential therapeutic uses of exogenously administrated MANF.

Overexpression or microinjection of MANF and the C-terminal domain of MANF prevented apoptotic cell death of sympathetic neurons induced by NGF-deprivation (i.e., physiological apoptotic stimulus), DNA double-strand breaks (i.e., etoposide inhibition of topoisomerase II) and staurosporine (i.e., broad-spectrum kinase inhibitor) (Hellman et al., 2011). Intracellularly expressed or microinjected full-length MANF was modestly active in the NGF-deprivation assay (15%-25% protection) but provided some protection (35%) from etoposide- and staurosporin-induced cell death. The C-terminal fragment displayed stronger activity in all three assays and the protective effect against apoptotic stimuli reached approximately 50%. It needs to be noted, however, that the C-terminal fragment is an artificial product that does not exist as such in cells and given the modest activities observed with the full-length construct these data need to be interpreted with caution.

Exogenous recombinant MANF had no anti-apoptotic effect against NGF-deprivation of sympathetic neurons (Hellman et al., 2011). Moreover, MANF did not bind to these cells nor was it taken up. Hence, the observed anti-apoptotic effects of overexpressed or microinjected MANF were attributed to a strictly intracellular mechanism not involving secreted MANF or a receptor mediated signaling pathway. However, anti-apoptotic activities by exogenous MANF were described elsewhere and these data are summarized in the following section.

3.5 MANF cellular activities after exogenous administration of recombinant protein

The experimental systems most relevant to the therapeutic use of MANF and/or CDNF proteins are those in which exogenous MANF or CDNF is administered to cells and effects on cell survival and differentiation are observed. There have been several studies published for MANF and a coherent profile of activities is emerging. On the other hand, little information is available on CDNF cellular activities. Moreover, nothing is known about potential MANF or CDNF receptors and receptor mediated intracellular signaling and therefore, this area of research offers significant opportunities to further the understanding of MANF and CDNF biology.

Recombinant CDNF (100 ng/ml, 5.5 nM, assuming a molecular weight of 18.3 kD) did not display survival promoting activities in primary cultures of P1 mouse sympathetic neurons, E14 rat motorneurons, or E14 and E15 mouse dorsal root ganglion sensory neurons (Lindholm et al., 2007). CDNF enhanced the survival of dopaminergic neurons determined by TH immunostaining (Vilponen et al., 2008). CDNF (0.2 to 1.5 μM concentration range) did not protect LUHMES cells or primary embryonic neurons from 6-OHDA-induced toxicity and HT-

22 cells from glutamate-induced toxicity (Barkholz, 2012). Moreover, media collected from mesencephalic astrocytes containing CDNF and other growth factors attenuated Zymosan A-induced microglia activation but this effect was not dependent on the CDNF present in the conditioned medium (Rocha et al., 2012). Hence, activity of CDNF in cellular systems has not been unequivocally demonstrated even though CDNF's *in vivo* activity is well established (Lindholm et al., 2007).

Recombinant MANF selectively increased the survival of dopaminergic (i.e., TH^+) neurons (Petrova et al., 2003) in mixed neuronal cultures containing 20% dopaminergic, 50% GABAergic, 5% serotonergic, 8% cholinergic, 5% bi-potential $vimentin^+$ cells, 0% astrocytes and 12% uncharacterized cells (Petrova et al. 2004). A neurotrophic effect (100% of control) was observed with 50 ng/ml (2.76 nM, assuming a molecular weight of 18.1 kD) E.coli expressed MANF and a partial effect (66% of control) with 2.5 ng/ml (0.14 nM) HEK293 expressed MANF. No effects were seen on GABAergic and serotonergic neurons. The mammalian expressed MANF also caused a marked degree of sprouting of dopaminergic neurons but not of GABAergic or serotonergic neurons. Enhancement of dopaminergic neurons survival by MANF was confirmed independently (Vilponen et al., 2008).

Recombinant MANF (E.coli expressed) decreased caspase-3 activation in a dose-dependent manner (Half-maximal effect at 6.6 nM) in cardiomyocytes that were serum-starved (Tadimalla et al., 2008). Moreover, recombinant MANF strongly (>50%) protected cardiomyocytes against simulated ischemia and simulated ischemia / reperfusion injury ([MANF] = 26 nM).

Recombinant MANF/ARMET (27.62 nM, assuming a molecular weight of 18.1 kD; E.coli expressed, exact construct unknown) fully protected primary mixed (cortex, hippocampus) neuronal cultures exposed to tunicamycin using quantification of terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL)-positive cells as a marker of apoptosis (Yu et al., 2010).

The effects of MANF on gamma-aminobutyric acid (GABA)-receptor mediated inhibitory postsynaptic currents (IPSCs) were studied in dopaminergic neurons of the substantia nigra (Zhou et al., 2006). MANF (0.25 nM) increased the frequency of spontaneous and miniature IPSCs without changing the mean amplitudes in mechanically dissociated cells. In enzymatically dissociated neurons, MANF had no effect on currents induced by exogenous GABA. The absence of an effect in enzymatically dissociated neurons could be indicative of the need for a cell surface receptor to mediate MANF effects.

In summary, recombinant MANF displayed protective effects on dopaminergic neurons, mixed cortical / hippocampal neurons and cardiomyocytes. Exogenously administered MANF protected against serum-starvation and ER-stress but also displayed a neurotrophic effect on neuronal survival and differentiation. Mechanistically, MANF administration was anti-apoptotic as evidenced by decreased caspase-3 activation and a reduced number of $TUNEL^+$ cells. MANF

effects were observed in a concentration range of 2.5 to 25 nM for E.coli expressed material and at 0.125 to 0.25 nM for the mammalian expressed protein. This concentration range is well within the ligand concentrations observed for cell-surface receptor mediated events and it seems thus likely that MANF (and CDNF) exert their neuroprotective and anti-apoptotic activities via binding to cell-surface receptors and activation of downstream signaling pathways. The spectrum of activities demonstrated for MANF offers numerous opportunities for cell protective and / or regenerative action in indications with physiopathology involving apoptosis and cellular stress.

3.6 MANF and CDNF single nucleotide polymorphisms

The gene for human MANF was formerly known as human ARP/ARMET (Shridhar et al., 1996). Interest in ARP/ARMET arose because its sequence contained a point mutation (ATG_{50} to AGG; deletion of ATG_{50}) that was thought to be enriched in sporadic renal cell carcinoma (Shridhar et al., 1996a) and pancreatic (Shridhar et al., 1997), lung, breast, prostate and head and neck cancer (Shridhar et al., 1996b). However, subsequent studies could not confirm a preferential occurrence of ARP/ARMET mutations in cancer (Evron et al., 1997; Tanaka et al., 2000; Piepoli et al., 2006) and it was concluded that variations in the ARP/ARMET trinucleotide repeat region represent normal polymorphisms rather than tumor-specific mutations (Evron et al., 1997). In order to investigate potential associations of single nucleotide polymorphisms (SNP) in the MANF and CDNF coding sequences with disease, a search of the NCBI database was performed and results are shown in Table 1.

MANF SNP (NCBI)	Chr: bp (ENSEMBL)	Transcript number	Alleles	Type	AA position (Full-length MANF)	AA change
rs192358306	3:51423677	ENST00000528157	A/G	Missense variant	42	K/E
rs199721651	3:51423690	ENST00000528157	T/A	Missense variant	46	V/D
rs200345870	3:51423699	ENST00000528157	C/T	Missense variant	49	S/L
rs201048315	3:51426347	ENST00000528157	G/A	Missense variant	123	D/N
rs113724505	3:51423767	ENST00000528157	G/T	Splice donor variant		-
rs11538558	3:51426519	ENST00000528157	A/G	Stop lost	180	*/W
rs202189850	3:51423700	ENST00000528157	A/C	Synonymous variant	49	S
rs34659596	3:51426468	ENST00000528157	/A	Frameshift	163	Glu/ins
CDNF SNP (NCBI)	Chr: bp (ENSEMBL)	Transcript number	Alleles	Type	AA position (Full-length CDNF)	AA change
rs139150625	10:14867500	ENST00000465530	G/T	Missense variant	121	S/R
rs142592274	10:14867609	ENST00000465530	A/G	Missense variant	85	L/P
rs145772897	10:14867562	ENST00000465530	G/A	Missense variant	101	R/C
rs145939473	10:14879938	ENST00000465530	C/T	Missense variant	3	C/Y
rs146907260	10:14870169	ENST00000465530	T/C	Missense variant	73	D/G
rs147031877	10:14867592	ENST00000465530	C/T	Missense variant	91	A/T
rs148342214	10:14870203	ENST00000465530	T/G	Missense variant	62	T/P
rs148962227	10:14867578	ENST00000465530	G/C	Missense variant	95	I/M
rs149495183	10:14879930	ENST00000465530	G/A	Missense variant	6	P/S
rs150464447	10:14870239	ENST00000465530	A/G	Missense variant	50	S/P
rs201197981	10:14870206	ENST00000465530	C/A	Missense variant	61	D/Y
rs201935618	10:14867573	ENST00000465530	C/T	Missense variant	97	S/N
rs61740068	10:14862140	ENST00000465530	C/A	Missense variant	135	A/S
rs61738953	10:14862082	ENST00000465530	C/G	Missense variant	154	W/S
rs61746971	10:14862042	ENST00000465530	A/G	Synonymous variant	167	Y
rs61843027	10:14867557	ENST00000465530	T/G	Synonymous variant	102	P
rs147677694	10:14867593	ENST00000465530	G/A	Synonymous variant	90	D
rs141533461	10:14870210	ENST00000465530	C/T	Synonymous variant	59	S
rs137976027	10:14870261	ENST00000465530	T/C	Synonymous variant	42	E

Table 1: Single nucleotide polymorphisms (SNP) in the human MANF and CDNF genes. The "rs" designation of each SNP, the location on the human genome (Chr:bp), the transcript identifier, the alleles, the classification of the nucleotide change (Type), the position of the affected amino acid and the resulting residue changes are shown.

For the MANF coding sequence there were 8 validated SNPs documented at the time of the search. Four of these SNP resulted in single amino acid substitutions, one in a frame-shift after position E163 and one in the substitution of the stop codon by a tryptophan residue. Finally, one SNP represents a splice donor variant and may affect MANF protein expression. None of these SNPs were associated with a known disease as of this date. However, even in the absence of a disease association with these SNPs, it would be interesting to investigate the functional consequences of these mutations.

For the CDNF coding sequence there were 19 validated SNPs documented at the time of the search. Fourteen of these resulted in amino acid substitution located throughout the protein sequence, five SNPs were synonymous variants not affecting the translated protein sequence. Also for the CDNF SNPs none were associated with known diseases at this time. Moreover, in a study where subjects with early onset PD and healthy controls were compared, no significant associations between disease and SNPs were identified (Choi et al., 2011). Furthermore, no significant differences in allele, genotype, or haplotype frequencies of CDNF variants between cocaine dependent individuals and unaffected controls were detected (Lohoff et al., 2009).

4 MANF and CDNF *in vivo* studies

MANF and CDNF were investigated in genetic models to study their roles in development and in particular the development of the nervous system. Moreover, pharmacodynamic studies were performed in experimental models of PD, stroke and myocardial infarction. To date, the most extensive data is available for studies in models of PD while neuroprotective activity against ischemic injury was demonstrated in the stroke and the myocardial infarction studies. The latter addressed in particular the activity of MANF in reperfusion injury.

4.1 Characterization of MANF and CDNF in genetic model systems

MANF and CDNF activities were studied in genetic model systems of mice, fruitflies (*Drosophila melanogaster*) and zebrafish (*Danio rerio*). CDNF knock-out mice were generated but these mice died at an early embryonic stage (Lindholm, 2009). This indicates that CDNF is critically important in development and that there are no compensatory mechanisms for this protein. No MANF transgenic or knock-out animals have been described in the literature.

MANF biology has been studied in *Drosophila* (Palgi et al., 2009). An invertebrate neurotrophic factor in *Drosophila*, DmMANF, was identified as the *Drosophila* ortholog of human MANF. DmMANF was required for the development of the *Drosophila* nervous system and two lines with differing DmMANF genetic deletions were embryonic and larval lethal, respectively. Maternal and zygotic DmMANF null mutants led to a complete loss of dopaminergic neurites and a drastic reduction of dopamine levels. These events were followed by a degeneration of axonal bundles in the embryonic central nervous system with subsequent cell death. Genetic rescue experiments showed that human MANF can compensate for the loss of DmMANF. Human MANF and DmMANF are thus functional orthologs. Human CDNF could not compensate for the loss of DmMANF suggesting that MANF and CDNF have complementary, non-redundant functions in *Drosophila* development. These developmental studies with DmMANF were complemented with gene expression analyses of two differing DmMANF genetic deletion mutants (Palgi et al., 2012) using microarrays followed by functional annotation clustering. DmMANF deletion led to expression changes in about 40% of known ER / UPR genes supporting a role of MANF in this cellular stress response pathway. However, the study did not report on mechanistic follow-ups and given that stress genes were up-regulated while UPR genes were down-regulated it seems difficult to reach firm conclusions on the role of DmMANF in these pathways. Interesting findings with regards to MANF regulation of PD-relevant genes included the upregulation of TH and 3,4-dihydroxyphenylalanine decarboxylase as a result of DmMANF deletion. It is known that DmMANF deletion depletes dopamine (Palgi et al., 2009) and these gene regulatory changes may be the result of a compensatory mechanism. Overexpression of DmMANF in a different *Drosophila* line increased the expression of genes involved in oxidation reduction, hinting at the possibility that DmMANF could be involved in protecting dopaminergic neurons from oxidative stress.

MANF is widely expressed during embryonic development and in adult organs of zebrafish (Chen et al., 2012). In adult organs, the most prominent expression was found in the liver but MANF could also be detected in brain, eyes and kidney. In the brain, MANF-positive cells were located close to tyrosine hydroxylase 1 (TH1) positive cells in preoptic, ventral and dorsal thalamic regions and only few MANF-containing cells were found to co-express TH1. Knockdown of MANF expression during development with antisense oligonucleotides resulted in no apparent phenotype. However, the level of dopamine was reduced by about 50% and the expression of the two TH genes, *th1* and *th2*, was reduced in some brain regions. The effects on the dopamine level and the *th1* / *th2* expression changes could be rescued by expression of zebrafish MANF mRNA. MANF is thus involved in the development of the dopaminergic system in zebrafish. Since developmental processes are sometimes re-activated in response to neuronal injury it is conceivable that MANF could have regenerative activity in PD.

Spinocerebellar ataxia 17 (SCA17) belongs to a family of nine inherited neurodegenerative diseases characterized by selective neuronal degeneration in specific brain regions. SCA17 is caused by a poly-glutamine expansion of the transcription factor TBP leading to cerebellar Purkinje cell degeneration. A conditional knock-in mouse of mutated TBP decreased expression of MANF in the cerebellum and thus a lack of MANF may contribute to the region specific neuropathology in SCN17 (Yang et al., 2012).

4.2 Parkinson's disease (PD)

MANF was identified as a mesencephalic astrocyte-derived neurotrophic factor and, together with CDNF, forms a novel family of neurotrophic factors with distinct structures and functions as described in the previous sections. MANF, and to a lesser degree CDNF, display a spectrum of cellular activities that could translate to neuroprotective or restorative effects in PD. Consequently, both MANF and CDNF were tested in models of PD and the activities of these neurotrophic factors were described in four published studies (Lindholm et al., 2007; Voutilainen et al., 2009; Voutilainen et al., 2011; Airavaara et al. 2012). The study by Lindholm et al. (2007) tested the activities of CDNF and GDNF in the intrastriatal 6-hydroxy dopamine (6-OHDA) model administering the growth factors to the striatum in a neuroprotection and a neuroregeneration protocol, respectively. In the neuroprotection protocol, the growth factors were applied shortly (i.e., 6 hours) before the striatal 6-OHDA administration while in the neuroregeneration protocol the growth factors were applied long after (i.e., 4 weeks) the 6-OHDA administration. Voutilainen et al. (2009) followed essentially the same protocol as Lindholm et al. (2007) to compare the activities of MANF and GDNF. In contrast, the study by Voutilainen et al. (2011) tested the activities of CDNF, MANF and GDNF in the 6-OHDA model in a protocol in which the growth factors were applied by an osmotic mini-pump starting two weeks after 6-OHDA for two weeks with a two-site intrastriatal infusion. All studies measured effects of growth factor treatment on amphetamine-induced rotational behavior,

survival of TH^+ cells in the substantia nigra and TH^+ fiber density in the striatum. The results of these studies are summarized in Table 2.

Further, Airavaara et al. (2012) tested CDNF in an MPTP model of PD with a neuroprotection and a neuroregeneration protocol in which CDNF was applied by a single bilateral intrastriatal injection. The effects of CDNF treatment were assessed by measuring the horizontal and vertical travel activity, counting TH^+ neurites in the striatum and TH^+ neurons in the substantia nigra. CDNF (10 μg) administered 20h prior to MPTP significantly increased the horizontal and vertical activity two weeks after MPTP compared to placebo. CDNF-treated animals also had significantly higher numbers of TH^+ neurons in the subtstantia nigra and TH^+ neurites in the striatum. When CDNF (10 μg) was applied in the neuroregeneration protocol (i.e., one week after MPTP) a significant increase in the horizontal and vertical activities two weeks after MPTP were observed compared to placebo. CDNF treated animals also had significantly higher numbers of TH^+ neurons in the subtstantia nigra and TH^+ neurites in the striatum.

Table 2: Profiling of MANF, CDNF and GDNF in the 6-OHDA model of Parkinson's disease.

Protein	Pre or post 6-OHDA	Doses (ug) Intrastriatal	Amphetamine-induced rotations (reduction vs. vehicle estimated from publications)	TH^+ cells in substantia nigra (protection est. from publications)	TH^+ fibers in striatum (protection est. from publications)	Reference
CDNF	6h pre Protection	1, 3, 10 Single injection	75% @ 10 (2w post) 50% @ 10 (4w post) Dose response	100% @ 10 (4w post) Dose response	50% @ 10 (4w post)	Lindholm et al. 2007
GDNF		10 Single injection	75% @ 10 (2w post) 50% @ 10 (4w post)	100% @ 10 (4w post)	40% @ 10 (4w post)	
CDNF	4w post Rescue	1, 3, 10 Single injection	Time-dependent decrease to w12 65% reduction @ 10 (12w post)	50% @ 10 (12w post)	ND	
GDNF		10 Single injection	Time-dependent decrease to w12 70% reduction @ 10 (12w post)	50% @ 10 (12w post)	ND	
MANF	6h pre Protection	3, 10, 30 Single injection	80% @ 10 (2w post) 90% @ 10 (4w post) U-shaped dose-response	70% @ 10 (4w post)	10% @ 10 (4w post) (n.s.)	Voutilainen et al. 2009
GDNF		10 Single injection	30% @ 10 (2w post) 60% @ 10 (4w post)	70% @ 10 (4w post)	5% @ 10 (4w post) (n.s.)	
MANF	4w post Rescue	3, 10, 30 Single injection	Time-dependent decrease to w12 50% reduction @ 10 (cumulative)	25% @ 10 (12w post) (n.s.)	ND	
GDNF		10 Single injection	Time-dependent decrease to w12 25% reduction @ 10 (cumulative)	25% @ 10 (12w post) (n.s.)	ND	
CDNF	2w post Rescue	21, 42, 63 (total) 2w infusion	70% @ 42 (cumulative rotations) U-shaped dose response	0% at 21 (12w post) 50% at 42 (12w post) 45% at 63 (12w post)	50% at 21 (12w post) 75% at 42 (12w post) 55% at 63 (12w post)	Voutilainen et al. 2011
GDNF		42 (total) 2w infusion	50% @ 42 (n.s.)	45% at 42 (12w post) (n.s.)	50% at 42 (12w post) (n.s.)	
MANF		21, 42, 63 (total) 2w infusion	No effect (Vehicle was too low GDNF had no effect)	No effect (Vehicle was too high, GDNF had no effect)	No effect (GDNF had no effect)	

Two of the 6-OHDA studies followed very similar designs (Lindholm et al. 2007; Voutilainen et al. 2009) and included striatal administration of growth factors in neuroprotection and neuroregeneration protocols. CDNF, MANF and GDNF displayed neuroprotective and neurorestorative activities, albeit to varying degrees. Effects on amphetamine-induced rotations by pre-treatment of growth factors were observable 2 weeks and 4 weeks after intrastriatal 6-OHDA administration. Hence, these factors displayed a neuroprotective activity that manifested itself relatively short-term. On the other hand a single post-treatment with growth factors (4 weeks after 6-OHDA) reached the same low level of amphetamine-induced rotations at week 12 as was observed with pre treatment at week 2. Hence, these growth factors were neurorestorative and activated a relatively slow mechanism. Nigral TH^+ cell protection was far more effective with a pre-treatment regimen than through neuroregeneration. The number of TH^+ cells in the substantia nigra never recovered to the level before 6-OHDA administration but they could be almost fully protected by pre-treatment with growth factors.

Comparison of the activities of CDNF and MANF is limited by an absence of direct head-to-head comparisons in the published studies. However, two of the studies (Lindholm et al. 2007; Voutilainen et al. 2009) were almost identically designed and used the common control GDNF. CDNF and GDNF display similar activities on both amphetamine-induced rotational behavior and TH^+ cell protection. MANF was better in protection and restoration of amphetamine-induced rotational behavior than GDNF. However, both growth factors were almost equally potent in their ability to protect TH^+ cells in the substantia nigra and TH^+ fiber density in the striatum. Given that CDNF and MANF are closely related in structure it is not surprising that their activities are quite similar. However, the situation could be reminiscent of the neurotrophins where the interaction of each factor with its preferred receptor determines the spectrum of *in vivo* and *in vitro* activities. Identifying these receptors for CDNF and MANF and determining their tissue expression pattern will shed light on MANF's and CDNF's cellular targets and guide the choice of indications for each of these growth factors.

While single intrastriatal injections of MANF and CDNF resulted in substantial and significant neuroprotective and neuroregenerative effects, chronic infusions of MANF into the striatum (Voutilainen et al., 2011) yielded disappointing results with no difference to vehicle treated animals. However, it is noteworthy that in Vouitilainen et al. (2011) the vehicle control displayed unusually low cumulative rotation counts indicating rapid spontaneous recovery. Moreover, GDNF was not different from vehicle even though in a parallel experiment of the same study, GDNF showed a clear trend towards reduction of rotational behavior. Therefore, both the negative (vehicle) and positive (GDNF) controls failed in the part of the study in which MANF was assessed. Therefore, conclusions on MANF activity after chronic intrastriatal infusion should not be based on the Voutilainen et al. (2011) study.

Distribution studies of ^{125}I-labeled CDNF, MANF and GDNF after intrastriatal injection (Voutilainen et al. 2011; Voutilainen et al. 2009) revealed a slightly differentiated pattern of retrograde transport for each of the growth factors. ^{125}I -GDNF was detectable in frontal cortex, dorsal striatum and substantia nigra. ^{125}I -CDNF was detectable in frontal cortex, dorsal striatum, substantia nigra and hippocampus. ^{125}I -MANF was detectable in frontal cortex and dorsal striatum but not in hippocampus and substantia nigra. This differential transport might be a consequence of different receptor interactions for each of the growth factors.

An alternative approach to striatal injection / infusion of growth factors is based on the introduction of adeno-associated virus (AAV) based expression vectors into specific brain regions. An AAV-CDNF vector was delivered into the striatum of 6-OHDA treated rats and a robust restoration of motor behavior was observed (Bäck et al., 2013; Ren, 2012). Treatment with AAV2-CDNF resulted in a marked decrease in amphetamine-induced ipsilateral rotations but it provided only partial protection of TH^+ cells in the substantia nigra and TH^+ fibers in the striatum (Bäck et al., 2013). In contrast, Ren (2012) found that behavioral recovery was paralleled by protection of TH^+ neurons in the substantia nigra and TH^+ fibers in the striatum. An AAV-MANF vector was used in a study of neuroprotection in ischemia (Airavaara et al., 2010) and will be discussed in Section 3.2.1. of this report.

The totality of the results in published studies warrants further development of MANF for the treatment of Parkinson's Disease.

4.3 Acute neuroprotection

4.3.1 Stroke

The studies of MANF expression and application as a therapy in experimental stroke is linked to its involvement in the ER stress response. Two studies reported induction of MANF expression after middle cerebral artery occlusion (MCAO). MCAO-induced expression was exclusively neuronal (Yu et al., 2010) while in a more recent study by the same group MANF was highly up-regulated in astrocytes, microglia and oligodendrocytes, but only modestly in neurons (Shen et al., 2012). However, expression of MANF in non-ischemic brains was mostly neuronal (Shen et al., 2012). Transient global hypoxia-ischemia in piglets down-regulated MANF mRNA in the cortex, striatum, thalamus and hippocampus. Hypothermia treatment increased the MANF mRNA levels in the parietal cortex but not in the prefrontal cortex, striatum, thalamus and hippocampus (Olson, 2011).

Administration of MANF (1, 6, 12, 24 μg, three cortical injection sites) in a transient MCAO model in a pre-treatment regimen (20 min pre-MCAO) reduced infract size on day 2 (50%) and enhanced functional recovery on day 7 but not on day 14 compared to vehicle treated animals (Airavaara et al., 2009). Interestingly, also in this pharmacological model, a U-shaped dose

response curve was observed, with the 6 μg dose being the most effective. Extending the known anti-apoptotic effects of MANF in cellular systems to an *in vivo* situation, MANF significantly reduced TUNEL pixel density at the site proximal to MANF injection.

In the second study in experimental stroke, MANF was expressed in the cortex using an AAV-based vector and animals were subjected to transient MCAO (Airavaara et al., 2010). Infarct size was reduced by 40% and again early effects on functional recovery were observed. By day 14, there was no difference between the MANF and GFP (control) expressing animals.

In summary, there are good reasons for investigating MANF for treatment of stroke and other indications involving ischemia based on MANF's unique mode-of-action counteracting cellular stress and apoptosis. The initial results obtained in two studies of experimental stroke provided a modest enhancement of functional recovery and encourage future studies of MANF in this indication. Application of MANF was performed as pre-treatment in both studies which does not represent the clinical situation in which patients will be treated likely several hours after stroke onset. Nevertheless, the current study design could be adapted to a neuroregeneration protocol and MANF expression or administration could be initiated after onset of experimental stroke or during the recovery phase to better reflect the situation likely encountered in clinical development of MANF.

4.3.2 Myocardial infarction

MANF expression was induced in cardiac myocytes and in other cell types in the hearts of mice subjected to *in vivo* myocardial infarction (Tadimalla et al., 2008). Knock-down of MANF expression by micro-RNA increased cell death of cardiomyocytes after simulated ischemia / reperfusion while overexpression of MANF protected these cells from ischemia-induced cell death and serum-deprivation induced caspase-3 activation. Recombinant MANF strongly (>50%) protected cardiomyocytes against ischemia / reperfusion injury and decreased caspase-3 activation in cardiomyocytes that were serum-starved (Tadimalla et al., 2008). MANF displayed cell protective activity when administered exogenously and therefore these effects may be mediated through cell surface receptors.

Administration of recombinant MANF (300 ng/h/g; total amount of subcutaneously administered MANF was estimated at about 300 μg per animal) by mini-pumps in a pre-treatment regimen in a model of myocardial infarction (30-min occlusion of the left ascending coronary artery, followed by 24 h of reperfusion) significantly reduced infarct size by 40% compared to saline treated animals (Glembotski et al., 2012).

Given its function and mechanism of expression in the heart, MANF was designated a "cardiomyokine", which are defined as heart-derived, secreted proteins that affect cardiovascular

function via autocrine, paracrine and/or endocrine mechanisms (Glembotski, 2011). MANF expression, as part of the response to ischemia-induced ER stress could thus function in an autocrine/paracrine manner to restore homeostasis in ischemic cardiomyocytes. MANF demonstrated potential for treatment of myocardial infarction and pathologies involving cardiomyocytes stress. Further studies with MANF in myocardial infarction models will evaluate whether MANF is also effective in a post-treatment regimen.

4.3.3 Traumatic brain injury

The events that occur after a traumatic brain injury (TBI) resemble the excitotoxic cascade observed after acute stroke. Release of the neurotransmitter glutamate activates glutamate receptors, leading to excessive entry of calcium into the neurons, destabilizing them and preventing normal energy metabolism from restoring homeostasis. Neuronal cell death due to excitotoxicity combines aspects of apoptosis and necrosis. MANF demonstrated potent anti-apoptotic activity in several cellular assays systems and it is thus conceivable that MANF could protect neurons from excitotoxic injury. To this end, Amarantus Bioscience, in collaboration with Banyan Biomarkers, conducted two studies of the effects of MANF (2.5 to 100 ng/ml / 0.14 nM – 5.5 nM) on N-methyl D-aspartate (NMDA)-induced toxicity on cortical neurons. MANF displayed neuroprotective activity against NMDA-induced cell death in both studies and the observed active concentration range (2.5 ng/ml (0.14 nM) to 10 ng/ml (0.55 nM)) is in agreement with MANF activities in other cellular assays. MANF is thus a potent neuroprotectant against NMDA-induced toxicity in cortical neurons. Having established the foundations for MANF as a potential therapy for TBI, *in vivo* studies will be conducted to determine whether the observed cellular effects translate to an improvement of motor function and cognitive ability.

5 Scientific points to consider

5.1 Intracellular versus extracellular activities of MANF and CDNF

It is of importance to distinguish MANF and CDNF activities that are performed by the ER-resident versus the secreted MANF / CDNF proteins, respectively. The most relevant activities for the development of a MANF recombinant protein product are those that were demonstrated with exogenously applied MANF or CDNF (summarized in section 2.3.). However, much interest in this growth factor family stems from the activities in the ER-stress response, UPR and inhibition of apoptosis. In this respect, it is encouraging that exogenous MANF counteracts tunicamycin-induced ER stress and apoptosis in primary neurons (Yu et al., 2010) and serum starvation-induced apoptosis in cardiomyocytes (Tadimalla et al., 2008). It will thus be of great interest to investigate the activities of exogenously applied MANF and CDNF to counteract ER-stress/UPR and to understand anti-apoptotic activities in greater mechanistic details, in particular on how they relate to disease mechanisms.

5.2 Neuroprotective and neuroregenerative activities of MANF and CDNF

Profiling of MANF and CDNF in models of PD revealed both neuroprotective and neuroregenerative activities *in vivo*. The treatment of chronic brain diseases (i.e., PD and AD) with a neuroprotectant is promising and due to the slow evolution of neuronal degeneration is not faced with the same time constraints as acute indications. A deceleration or arrest of disease progression would yield a tremendous benefit for PD patients. Moreover, a combination approach with a MANF therapeutic and an early diagnostic tool would allow the identification and treatment of patients during the prodromal phase of the disease and would offer an integral management of PD.

Neuroregeneration in acute or chronic diseases is of great interest and if successful will yield great benefits for patients. Moreover, neuroregenerative therapy could be initiated in symptomatic patients because its success may only in part depend on the presence of remaining functioning neurons. Neurorestorative therapy by MANF could induce regain of function in dopaminergic neurons that have been rendered dysfunctional or if significant cell loss has already occurred MANF might stimulate the regeneration of lost neurons. The cellular and molecular mechanisms underlying MANF neurorestorative activity are largely unknown and therefore, this area of research presents a rich opportunity to generate original scientific data with direct utility for the clinical development of MANF.

5.3 Search for new indications

The unique mechanism of MANF counteracting ER stress and apoptosis offers numerous opportunities for further development of MANF in several indications, including orphan indications. A systematic search for diseases with ER stress as an important element of

pathology could identify promising new indications with a focus on indications affecting tissues in which MANF (or CDNF) is expressed and will include non-CNS indications.

The current literature points towards a number of indications that have not implicated MANF directly in the disease process but could be further investigated. Spinocerebellar ataxia 17 (SCA17) is characterized by selective neuronal degeneration in the cerebellum and caused by a poly-glutamine expansion of the transcription factor TBP. A conditional knock-in mouse of mutated TBP decreased expression of MANF and thus a lack of MANF may contribute to the region specific neuropathology in SCN17 (Yang et al., 2012). Clarity on MANF skeletal muscle expression levels would be of interest to better understand the role of MANF in muscle physiology. Increased expression of MANF was observed in oocytes after IVM (Wang et al., 2011) possibly in response to stress on oocytes by the IVM procedure. Finally, ER stress in chondrocytes in models of chondrodysplasia (Nundlall et al., 2010; Cameron et al., 2011) induced the expression of MANF.

5.4 Peripheral versus CNS indications

The therapeutic forms of MANF and CDNF are recombinant proteins that will not readily cross the blood-brain-barrier. In order for MANF or CDNF to reach their targets in the CNS either a direct delivery to the target tissue in the brain or the design of modified proteins will be necessary. Direct delivery methods are complex but not unprecedented and a significant development effort will have to be made to achieve targeted delivery into the relevant tissue. Conceptual approaches exist to increase BBB crossing for recombinant proteins and such an approach would likely yield new composition of matter intellectual property.

MANF and CDNF are considered autocrine / paracrine growth factors. Therefore, the site of expression of these factors is likely close to the site of their action. The cells that express MANF and those that respond to secreted MANF are likely under similar physiological conditions and located in close proximity (paracrine) or are the same (autocrine). Based on these considerations, the tissue distributions for MANF and CDNF provide important clues as to their peripheral and CNS target tissues which in turn can be exploited to identify the most promising indications for these growth factors. In addition, expression of MANF is regulated by certain pathological mechanisms (i.e., ER stress, ischemia) which further expands the range of potential target tissues of this protein. A better understanding of the target tissues for MANF and CDNF, respectively, will also lead to a differentiation of these factors between themselves and GDNF, and to the definition of complementary development paths maximizing the potential for each of these factors.

5.5 The MANF and CDNF receptors

MANF and CDNF are likely to engage cell-surface receptors to mediate their anti-apoptotic and ER stress response-related activities. Despite the fact that these growth factors were cloned and

expressed several years ago no receptors have been identified so far. The lack of defined signaling pathways emanating from these growth factors limits the understanding on how they function. Therefore, a key research effort will include the identification and characterization of MANF and CDNF receptors. If successful, this could enable a small molecule growth factor mimetic program.

6 Parkinson's disease strategy

The previous sections of this document summarized published data on the MANF structure and its cellular and *in vivo* activities. In this section implications to the development of MANF for the treatment of PD will be discussed, gaps in the knowledge base identified and a series of issues to be addressed in progressing MANF to clinical trials for PD outlined.

6.1 MANF data summary

The available literature established MANF as a protein that is expressed in response to ER stress, secreted via the ER-Golgi pathway and active as a soluble growth factor. Exogenously applied MANF displays anti-apoptotic activity against ER stress and serum-starvation and a neurotrophic effect as manifested by enhancement of survival and differentiation of dopaminergic neurons. The fact that MANF is expressed in many human tissues in combination with its function as an autocrine / paracrine growth factor offers the possibility of MANF anti-apoptotic activities in the CNS and in non-neuronal target tissues. While the mechanism and regulation of MANF expression and secretion is well described it is currently unknown how MANF mediates its activities once it is released.

MANF activities *in vivo* in models of PD are well established. The aggregate of this information shows that MANF displays neuroprotective and possibly neuroregenerative activities in the 6-OHDA model. These activities manifest themselves in a reduction in amphetamine-induced rotational behavior and protection of TH^+ cells in the substantia nigra and TH^+ fibers in the striatum.

MANF *in vivo* activity was assessed exclusively in rodents within a narrow dose-range (i.e., 3 – 30 μg per injection) using a delivery method (i.e., intrastriatal injection) that will not be applicable in human clinical studies. There is thus some uncertainty as to the minimally effective dose, the dose-effect relationship, the optimal frequency of application and total amount of MANF to be delivered, the site and method of delivery. Moreover, effective doses in the rodent model will have to be translated to appropriate doses and dosing schedules in non-human primates and humans. However, the *in vivo* rodent data does provide a useful basis for initiating development towards the development of MANF in human clinical studies. To this end, data from the translational chain from rodents to human subjects described for GDNF will be highly useful for the design of non-human primate PD studies for MANF. These studies in turn will serve as pre-clinical proof-of-concept for MANF in PD and as design support for the initial human clinical study.

6.2 Positioning of MANF

PD is a neurodegenerative disorder that is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta. The lack of dopamine causes the classical motor

symptoms of bradykinesia, rigidity and resting tremors. Current PD therapy is strictly symptomatic and employs dopamine replacement strategies. These include administration of L-DOPA, dopamine receptor agonists, monoamine oxidase B (MAOB) inhibitors and catechol O-methyltransferase inhibitors. Current active trials of new compounds and agents for treatment of PD symptoms with unmet needs attempt to address motor symptoms, dyskinesia, gait disorders, hallucinations / psychosis, depression / anxiety, autonomic failure and an additional few aim to achieve disease modification or neuroprotection (Meissner et al., 2011). The intended application of MANF falls into this latter category offering the possibility of a neuroprotective (i.e., halting disease progression) and a neuroregenerative (i.e., reverse of disease) treatment of PD. An important unmet medical need for PD remains disease modification (Datamonitor 2011) but the availability of a neuroprotective therapy would change the market landscape profoundly (Huynh, 2011). Independent on whether MANF is neuroprotective and/or neuroregenerative its demonstrated activities when translated to the human clinical situation would address key unmet medical needs. A neuroprotective MANF would likely be most effective in early PD patients that are still in the prodromal phase of the disease. This would however, necessitate the availability of a reliable assay predictive for PD disease progression. In the absence of such a test, an initial clinical study in PD would likely include subjects with a more advanced disease. Nevertheless, the continued progression of PD symptoms will make it possible to demonstrate neuroprotective activity even in patients with more advanced PD.

6.3 GDNF as a model for translational research in PD growth factor therapy

GDNF has been in development for treatment of PD and there is extensive data on pre-clinical rodent models, on a non-human primate model and on human clinical studies from three independent trials. GDNF (10 μg, single intrastriatal injection; 42 μg total in 2 week infusion) consistently displayed neuroprotective and neuroregenerative activities when used as a positive control compound in recent studies of CDNF and MANF in the 6-OHDA rodent model (Lindholm et al., 2007, Voutilainen et al., 2009, Voutilainen et al., 2011).

Chronic, controlled infusion of GDNF in advanced parkinsonian monkeys promoted structural and functional recovery (Grondin et al., 2002). Late-stage PD was modeled in rhesus monkeys by infusion of MPTP through the right carotid artery resulting in elimination of TH^+ cells in the right striatum but sparing of these cells in the left striatum. GDNF (5 μg/day or 15 μg/day) was infused into the lateral ventrical or the putamen using programmable pumps for three months. GDNF treatment resulted in an improvement from baseline in a non-human primate parkinsonian scale, modeled after the human Unified Parkinson's Disease Rating Scale (UPDRS), while vehicle treatment did not. The treatment with GDNF started three months after MPTP-induced injury to the nigrastriatal system and thus GDNF effects are primarily attributable to a restorative action. GDNF treatment also affected dopamine and DOPAC levels in the medial striatum but not in the intermediate or lateral striatum. Quantitative analysis of striatal dopamine fibers revealed that there was a significant increase of TH^+ fibers in the ventrical border of the right

striatum that gradually faded in a gradient from the ventrical to the lateral border of the putamen. The dopamine level and TH^+ fiber data may reflect the diffusion pattern of the growth factor. Interestingly, the effects of GDNF on nigral dopamine neurons were bilateral even though the growth factor was applied unilaterally.

GDNF was studied in three independent human clinical trials of differing designs. The first study enrolled five PD patients and administered GDNF via a continuous bi-lateral infusion into the putamen at a rate of 6 μl/h delivering 14.4 μg/putamen/day for 8 weeks. The study did not have a placebo group. An increase of GDNF dose levels after the first 8 weeks was attempted but had to be reversed to the original dose level. Results were reported at the one year (Gill et al., 2003) and two year (Patel et al., 2005) time-points. In the off-medication motor sub-score of the UPDRS an improvement from baseline of 39% (1 year) and 57% (2 years) was observed. In the activities of daily living sub-score an improvement of 61% and 63% was observed after 1 year and 2 years, respectively. The second study enrolled 10 patients with advanced PD and administered GDNF via continuous unilateral infusion into the putamen in a dose-escalation starting with 3 μg/day GDNF for two months, increasing to 10 μg/day for two months and ending with 30 μg/day for two months (Slevin et al., 2005). The study did not have a placebo group. The UPDRS total scores in the on and off states significantly improved by 34% and 33%, respectively, compared to baseline at the 6 month time-point. Even though administration was unilateral the improvements occurred bilaterally. The third study was a placebo controlled, randomized clinical trial sponsored by Amgen (Lang et al., 2006). A total of 34 PD patients were enrolled and were assigned GDNF or placebo in a 1:1 ratio. GDNF was administered by bi-lateral continuous infusion into the putamen at 15 μg/putamen/day for 6 months. The mean percentage changes in the off UPDRS score were -10% and -4.5% in the GDNF and placebo groups, respectively. This difference was not statistically significant and also all secondary endpoints did not differ between GDNF and placebo groups. The development of GDNF for the treatment of PD was discontinued for three reasons: (1) GDNF was not effective in the randomized placebo controlled study, (2) several subjects developed anti-GDNF antibodies and (3) a toxicology study in non-human primates revealed irreversible cerebellar changes (i.e., focal cerebellar lesions). Significant controversy ensued after the decision to discontinue the development of GDNF (Peck, 2005).

6.4 Lessons from the GDNF program applied to MANF development

In the following, the potential reasons for GDNF's lack of efficacy in a controlled trial despite its positive effects in pre-clinical rodent and non-human primates and in open-label human studies will be further analyzed with a view on lessons for the MANF development program.

GDNF infusion in rodents delivered a total of 42 μg in a two week period (Voutilainen et al., 2011). In the monkey study 70 μg and 210 μg, respectively, was delivered in any two week period. In the human studies, about 200 μg was administered in any two week time-period.

Hence, the dose was increased from the rodent to the non-human primate study but then stayed that same for the human clinical study. It is thus possible that the human study was under-dosed. It is unknown whether there were dose level limitations based on safety data.

There is no information available on the effect of MANF infusion in PD rodent models as this part of the study described in Voutilainen et al. (2011) likely failed. However, CDNF and MANF display their strongest effects when administered intrastriatally at the same dose (i.e., 10 μg). Therefore, the most active dose for CDNF in the infusion protocol (Vouitilainen et al., 2011) of 42 μg total CDNF for two weeks might be the same for MANF. Hence, a non-human primate study with MANF (or CDNF) should center on a delivery rate of about 200 μg/day with a higher and a lower dose in additional treatment groups. In a human study, a further increase of the dose should be considered and that needs to be reflected in the design of the monkey toxicology study.

The delivery device and protocol to be used in human and non-human primate studies is of critical importance. It was speculated that one reason for the failure of the placebo controlled trial was the use of a device that kept GDNF very localized versus the one used in previous human studies that promoted CED, resulting in an optimized tissue distribution. It is thus essential that the delivery protocol is optimized in a non-human primate study and appropriately adapted in the human clinical study.

MPTP or 6-OHDA treatment of rodents and non-human primates result in a phenotype that resembles human PD. However, the etiology of human PD is unknown and the cellular mechanisms leading to the death of dopaminergic neurons in the substantia nigra are possibly different from MPTP or 6-OHDA induced cell death. Consequently, dysfunction of dopaminergic neurons in rodents or non-human primates at the cellular physiology level will likely differ in a significant way. Therefore, if specific cellular mechanisms are required for the successful action of MANF (or other growth factors) then there might be significant limitations in the translation of findings observed in rodents and non-human primates to humans. In particular, a disruption in the retrograde transport pathway from the striatum to the nigral cell bodies is observed in patients with advanced-stage PD but not in non-human primates (Bartus et al., 2011). Hence, depending on the site of action for MANF and on whether retrograde transport of the MANF signaling complex is required there could be differences between non-human primates and humans. Therefore, the site of MANF administration is highly critical and concomitant administration to the putamen and the substantia nigra may be required in human subjects.

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