DEVELOPMENT OF ANALYTICAL TOOLS
FOR THE QUANTIFICATION OF
MANF AND CDNFG
IN DISEASE AND THERAPY

Emilia Galli

ACADEMIC DISSERTATION

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ABSTRACT

Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) are evolutionarily conserved proteins that are classified as the fourth family of neurotrophic factors. They are expressed ubiquitously in the body and show protective effects on neurons and other cell types. Genetic ablation of MANF in mice resulted in a severe type 1 diabetes-like phenotype characterised by postnatal reduction of pancreatic insulin-producing β-cells. Growing evidence points out a pivotal role of MANF, and the less-studied CDNF, in cellular endoplasmic reticulum (ER) stress. Chronic ER stress is linked to the pathology of various diseases, including Parkinson’s disease, Alzheimer’s disease, and diabetes. Thus, the proteins might play a role in the pathology of these diseases and, on the other hand, they carry potential for disease modifying therapy.

Although the effects of MANF and CDNF administration have been studied in various animal disease models, such as neurodegenerative diseases, ischemia, and diabetes, it is largely unknown whether their endogenous levels and expression patterns are changed in relation to the pathology of diseases in animal models and humans. Quantification of MANF and CDNF in pathological samples can give insights into their association with disease development and therapeutic potential. Furthermore, any changes in MANF or CDNF levels would open up the possibility to use them as biomarkers for disease prediction, diagnostics, or therapy response.

This thesis describes the development and validation of enzyme-linked immunosorbent assays (ELISAs) for the specific and sensitive quantification of MANF and CDNF in biological samples including serum, tissues, and in vitro cell samples. Due to the clear diabetic phenotype observed in MANF-deficient mice, we were interested to study the circulating MANF levels in type 1 diabetes patients. The aim was to find out whether MANF expression and/or secretion is differently regulated before and at the onset of clinical symptoms of the disease, thus reflecting whether MANF could be related to human type 1 diabetes. The observed MANF levels were unchanged at the pre-clinical state of the disease, suggesting that analysis of circulating MANF cannot be utilized in the disease prediction. However, MANF was significantly higher in a subgroup of children with recent onset diabetes compared to controls. We studied the circulating MANF concentration against functional β-cell mass and inflammation, but the exact mechanism behind the observed high MANF levels remained unclear.

Multiple studies have demonstrated neuroprotective and neurorestorative effects of MANF and CDNF. Delivering proteins to the central nervous system is challenging, since in many cases they are unable to penetrate the blood brain barrier and their tissue diffusion is poor. Consequently, the therapeutic proteins have to be delivered close to the target site within the central nervous system. In this thesis, the effects of intracranial
gene therapy of human CDNF were studied in animal models of Parkinson’s and Alzheimer’s disease. Transgene expression depends on multiple variables, such as transduction efficiency, stability, and virus titer. Thus, the quantification of expressed protein is important in the interpretation of its effects. The species-selectivity of the developed ELISAs was utilized when analysing human CDNF levels in rodent brain.

Another possibility for long-term protein administration to the brain is to deliver encapsulated cells secreting the therapeutic protein to the target site. This method relies heavily on stable protein secretion. The developed ELISAs were used for the quantification of secreted CDNF from cells. Despite the successful production of several genetically engineered cell clones with high CDNF expression, the secretion of CDNF was found to decrease substantially once the cells were confluent in cell culture plates or in polymeric microcapsules. Modifications to the CDNF coding sequence increased CDNF expression and secretion. Most importantly, removal of the putative ER-retention signal resulted in CDNF secretion from confluent cell clones. If the mutated CDNF proves to be biologically active it could be delivered to the central nervous system via encapsulated cells.

The developed sensitive, specific, and thoroughly validated ELISAs for the quantification of MANF and CDNF can be utilized in future studies aimed at understanding the regulation and functions of these proteins in health and disease. In addition, the ELISAs hold the potential for analysis of the proteins as biomarkers.


Useat tutkimukset ovat osoittaneet MANF:n ja CDNF:n hermosoluja


LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscript:


The publications and the manuscript are referred to in the text by their roman numerals. Supplementary, unpublished results are also presented. Reprints were made with the permissions of copyright holders.
Author's contribution:

I The author participated in designing the experiments, performed experiments (including ELISA development and validation, MANF measurement, cytokine measurement), analysed the data, and had a central role in writing of the manuscript.

II The author performed experiments with self-designed CDNF ELISA, contributed in the respective data analysis and writing of the manuscript.

III The author performed and conducted experiments with self-designed CDNF ELISA, contributed in the respective data analysis, and writing of the manuscript.

IV The author participated in designing the experiments, performed all the experiments, analysed the data, and wrote the manuscript.

Unpublished results presented in the thesis were generated by the author.
ABBREVIATIONS

6-OHDA  6-hydroxydopamine
AAV  Adeno-associated virus
AD  Alzheimer’s disease
ARPE-19  Human retinal pigment epithelial cell line
BBB  Blood brain barrier
BMSC  Bone marrow-derived stem cells
C  Carboxy (terminal end of amino acid chain)
CDNF  Cerebral dopamine neurotrophic factor
CMV  Cytomegalovirus
CNS  Central nervous system
CSF  Cerebrospinal fluid
DTT  1,4-Dithiothreitol
eIF2α  Eukaryotic initiation factor 2α
ELISA  Enzyme-linked immunosorbent assay
EndoC-βH1  Human β-cell line
ER  Endoplasmic reticulum
ERAD  ER-associated degradation
ERSE  ER stress response element
GDNF  Glial cell line-derived neurotrophic factor
h  Human
HA  Heterophilic antibody
HAIA  Human anti-animal immunoglobulin antibody
hCG  Human chorionic gonadotropin
HEK  Human embryonic kidney cell line
HeLa  Human cervical cancer cell line
HRP  Horseradish peroxidase
Ig  Immunoglobulin
IIR  Immunoglobulin inhibiting reagent
KO  Knock out
LPS  Lipopolysaccharide
m  Mouse
mAb  Monoclonal antibody
MANF  Mesencephalic astrocyte-derived neurotrophic factor
mRNA  Messenger RNA
MS  Mass spectrometry
N  Amino (terminal end of amino acid chain)
NGF  Nerve growth factor
NOD  Non-obese diabetic (mouse strain)
NTF  Neurotrophic factor
pAb  Polyclonal antibody
PCR  Polymerase chain reaction
PD  Parkinson’s disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Human neuroblastoma cell line</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box-binding protein</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>U2OS</td>
<td>Human bone osteosarcoma epithelial cell line</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>vg</td>
<td>Virus genome</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
</tbody>
</table>
1. Introduction to MANF and CDNF proteins

Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) are evolutionary conserved proteins (Lindholm & Saarma 2010). The expression of both MANF and CDNF is found in vertebrates. Instead invertebrates, such as fruit fly (Palgi et al. 2009), pea aphid (Wang et al. 2015) and marine sponge (Sereno et al. 2017), express only one homolog with slightly closer resemblance to the vertebrate MANF. This is clearly different from other neurotrophic factors (NTFs), for which a clear invertebrate ortholog has not been identified. MANF was first discovered in the conditioned medium of an immortalised rat mesencephalic astrocyte cell line and was found to have protective effects on primary dopaminergic cell cultures \textit{in vitro} (Petrova et al. 2003). The structural homolog CDNF, predicted by bioinformatics methods, was purified and characterised soon thereafter (Lindholm et al. 2007). Both CDNF (Lindholm et al. 2007) and MANF (Voutilainen et al. 2009) have neuroprotective and restorative effects on dopamine neurons in a toxin model of Parkinson’s disease in rats.

Due to the documented trophic effect on neurons, MANF and CDNF are classified as the fourth family of NTFs (Lindholm & Saarma 2010), others being the 1) neurotrophins (Huang & Reichardt 2001), 2) glial cell line-derived neurotrophic factor (GDNF) family ligands (Airaksinen & Saarma 2001), and 3) neuropoietic cytokines (Bauer et al. 2007). Classically, NTFs are considered secreted proteins, which by binding to plasma membrane receptors activate intracellular signalling cascades and thereby promote neuron migration, differentiation, survival, regeneration, neurite outgrowth, and formation of synapses. Although MANF was initially reported to protect cultured dopaminergic neurons selectively in favour of GABAergic or serotonergic neurons (Petrova et al. 2003), it was later shown to have general cytoprotective actions (Apostolou et al. 2008). The finding of MANF ortholog in a marine sponge, \textit{Suberites domuncula}, lacking the conventional nervous system (Sereno et al. 2017), strengthens the idea that MANF and CDNF have additional actions in organisms beyond neurotrophic.

1.1. Protein structure

Consistent with the classical model of secreted NTFs, both MANF and CDNF contain an amino(N)-terminal signal sequence, which targets them to the secretory route via the endoplasmic reticulum (ER) (Lindholm, Saarma 2010). After cleavage of the signal peptide, the resulting mature proteins have a molecular weight of approximately 18 kDa. However, in contrast to classical NTFs, the carboxy(C)-terminal end of both MANF and CDNF contain a tetrapeptide sequence with affinity to KDEL receptors, which are involved in protein retrieval from the Golgi apparatus to the ER (Raykhel et al. 2007).
The 3D-structures of MANF and CDNF have been studied by X-ray crystallography (Parkash et al. 2009) and by nuclear magnetic resonance spectroscopy in aqueous solution (Hoseki et al. 2010, Hellman et al. 2011, Latge et al. 2015). These studies revealed high structural similarity between the homologs. The 3D-structures of MANF and CDNF were found to contain two separate domains connected with a short linear sequence. The separate N-terminal domain was observed in a preparation of recombinant CDNF produced in insect cells (Parkash et al. 2009), implying that the domains can be cleaved off from each other. The approximately 12 kDa N-terminal domain resembles saposin-like proteins, which are involved in lipid-binding (Parkash et al. 2009). Indeed, MANF has been demonstrated to bind to sulfatide lipids (Bai et al. 2018). The authors demonstrated that a single amino acid mutation at the end of the N-terminal domain significantly reduced MANF binding to the lipid. However, as the effect of the mutation to the 3D-structure of MANF was not addressed, the lipid-interacting domain cannot be exclusively defined. Binding of CDNF to a lipid has not been reported so far.

Interestingly, the 6 kDa C-terminal domain of MANF and CDNF shows homology to the SAP-like domain in Ku70, an inhibitor of proapoptotic Bax (Hellman et al. 2011, Latge et al. 2015). Interaction with Ku70 keeps cytosolic Bax inactive (Hada & Kwok 2014). When C-terminal MANF was microinjected to the cytoplasm of cultured neurons, it protected the cells against Bax-induced apoptosis to the same extent as Ku70 (Hellman et al. 2011). However, interaction between the C-terminal domain of MANF or CDNF with Bax has not been demonstrated. Also, as endogenous MANF and CDNF are largely retained in the ER, the biological relevance of the possible cytoplasmic interaction remains to be studied. The SAP-like domain of MANF and CDNF might be involved in DNA-binding (Hellman et al. 2011). The SAP-like domain of MANF was reported to interact with the DNA-binding subunit of NF-κB p65, but whether the supposed interaction occurred directly between the two molecules, or indirectly via binding of DNA, was not addressed (Chen et al. 2015).

The conserved structure of MANF and CDNF includes eight cysteine residues with similar spacing, forming four intramolecular disulphide bonds (Parkash et al. 2009). Three of the bonds are in the N-terminal and one in the C-terminal domain, where the interacting cysteines (C) are only two amino acids apart (Parkash et al. 2009, Hoseki et al. 2010, Hellman et al. 2011). Similar CXXC-sequences are found in reductases and disulphide isomerases, which catalyse the formation of intramolecular disulphide bonds in other proteins (van Anken & Braakman 2005), suggesting a role for MANF and CDNF in protein folding in the ER. Despite attempts, no oxidoreductase activity has been found for MANF (Mizobuchi et al. 2007, Hartley et al. 2013, Mätlik et al. 2015). However, the C-terminal CXXC-motif is important to MANF’s intra- and extracellular cytoprotective activity, as has been demonstrated by mutations disrupting the disulphide bond (Lindström et al. 2013, Mätlik et al. 2015).

MANF and CDNF appear as monomers in neutral and slightly acidic
(pH≥6) environment (Mizobuchi et al. 2007, Parkash et al. 2009, Hoseki et al. 2010, Hellman et al. 2011, Latge et al. 2015). At pH 4.6, the N-terminal CDNF domain crystallised as a dimer (Parkash et al. 2009). Complexes of higher molecular weight MANF were observed mouse urine samples analysed on western blot (WB) (Kim et al. 2016). The molecular weight of the most prominent band was around 37 kDa, implying that MANF could dimerise in urine. Alternatively, the higher molecular weight band could represent MANF in a complex with some other molecule. However, it remained unclear whether MANF was actually in a complex in the urine or whether the bands occurred due to aggregation at sample processing.

1.2. Intracellular localization and secretion

Intracellular MANF (Apostolou et al. 2008, Tadimalla et al. 2008, Henderson et al. 2013) and CDNF (Sun et al. 2011, Fernández et al. 2014) reside predominantly in the ER. Two suggested interacting partners of MANF and CDNF reside in the ER: 1) protein chaperone GRP78 and 2) KDEL receptors. Overexpression of both GRP78 (Oh-Hashi et al. 2012, Norisada et al. 2016) and KDEL receptors (Henderson et al. 2013, Norisada et al. 2016) in cell cultures resulted in greater intracellular retention of MANF and CDNF. In contrast, the removal of the C-terminal tetrapeptide of MANF (Glembotski et al. 2012, Oh-Hashi et al. 2012, Henderson et al. 2013, Mätlik et al. 2015) and mouse CDNF (Norisada et al. 2016), supposedly recognised by the KDEL receptors, resulted in mislocalization of the protein from the ER to the Golgi apparatus and increased secretion, indicating decreased ER-retention. The interaction of MANF and GRP78 has been studied by cross-linking and immunoprecipitation studies (Glembotski et al. 2012).

Despite the predominant ER localization, both MANF and CDNF can also be secreted, as shown in vitro in overexpression (Lindholm et al. 2007, Apostolou et al. 2008, Lindholm et al. 2008, Sun et al. 2011). Their secretion is assumed to occur via Golgi apparatus, as the addition of Brefeldin A, a Golgi disruptor, inhibited MANF and CDNF secretion (Apostolou et al. 2008, Oh-Hashi et al. 2012). The proteins are suggested to be transported from the ER to the Golgi by the COPII-mediated pathway, as cotransfection of mutant Sar1, a key regulator of the assembly of COPII vesicles, reduced the amounts of MANF and CDNF in culture medium significantly (Oh-Hashi et al. 2012, Norisada et al. 2016).

2. MANF and CDNF functions in vitro

2.1. Effects on cells

Both extracellularly and intracellularly applied MANF and CDNF have cytoprotective actions in vitro (Table 1). It is largely unknown at this time how
Table 1. Examples on documented cytoprotective effects of MANF and CDNF applied intra- or extracellularly in vitro. The molar concentrations were calculated from the weight concentrations reported in the publications. Molecular weights of 18.1 and 18.3 kDa for MANF and CDNF, respectively, were used in the calculations. h = human, m = mouse, r = rat, TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling, 6-OHDA = 6-hydroxydopamine, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LDH = lactate dehydrogenase, NGF = nerve growth factor, TBP = TATA box-binding protein.

<table>
<thead>
<tr>
<th>Intracellularly applied</th>
<th>Protein</th>
<th>Type</th>
<th>Model (species)</th>
<th>Specified action (evaluated by)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMANF</td>
<td>Plasmid transfection</td>
<td>U2OS cell line (human)</td>
<td>Protection against cell death induced by glucose deprivation and tunicamycin (visual inspection of cell cultures)</td>
<td>(Apostolou et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>mMANF</td>
<td>Adenoviral transduction</td>
<td>Primary cardiac myocytes (rat)</td>
<td>Protection against death induced by serum-starvation and ischemia (activated caspase-3, live/dead-staining)</td>
<td>(Tadimalla et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>hMANF</td>
<td>Plasmid transfection, protein microinjection</td>
<td>Primary symaptetic neurons (mouse)</td>
<td>Protection against cell death induced by etoposide, staurosponine, and NGF-deprivation (number of cells)</td>
<td>(Hellman et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>mMANF</td>
<td>Lentiviral transfection</td>
<td>PC12 cell line (rat)</td>
<td>Protection against toxicity induced by mutant TBP (NGF-induced neurite outgrowth, MTS metabolic activity assay)</td>
<td>(Yang et al. 2014a)</td>
<td></td>
</tr>
<tr>
<td>hMANF</td>
<td>Plasmid transfection</td>
<td>SH-SY5Y cell line (human)</td>
<td>Protection against 6-OHDA (MTT metabolic activity assay)</td>
<td>(Hao et al. 2017)</td>
<td></td>
</tr>
<tr>
<td>hCDNF</td>
<td>Lentiviral transduction</td>
<td>Primary astrocytes (rat)</td>
<td>Protection against tunicamycin (medium LDH)</td>
<td>(Cheng et al. 2013a)</td>
<td></td>
</tr>
<tr>
<td>rCDNF</td>
<td>Plasmid transfection</td>
<td>H9C2 cell line (rat)</td>
<td>Protection against tunicamycin (metabolic activity assay, TUNEL-staining)</td>
<td>(Liu et al. 2017)</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Type (source)</td>
<td>Max effect</td>
<td>Model (species)</td>
<td>Specified action (evaluated by)</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
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</tr>
<tr>
<td>mMNF</td>
<td>Recombinant (bacterial)</td>
<td>26.4 nM</td>
<td>Primary cardiac myocytes (rat)</td>
<td>Protection against death induced by serum-starvation and ischemia (activated caspase-3, live/dead-staining)</td>
<td>(Tadimalla et al. 2008)</td>
</tr>
<tr>
<td>hMANF</td>
<td>Recombinant (bacterial)</td>
<td>27.6 nM</td>
<td>Primary cortical neurons (rat)</td>
<td>Promoted cell proliferation, protection against death induced by tunicamycin (TUNEL-staining)</td>
<td>(Yu et al. 2010)</td>
</tr>
<tr>
<td>hMANF</td>
<td>Recombinant (mammalian)</td>
<td>13.8 nM</td>
<td>SH-SY5Y cell line (human)</td>
<td>Protection against 6-OHDA (MTT metabolic activity assay)</td>
<td>(Hao et al. 2017)</td>
</tr>
<tr>
<td>hMANF</td>
<td>Recombinant (bacterial)</td>
<td>2.8 nM</td>
<td>Primary retinal ganglion cells (rat)</td>
<td>Protection against hypoxia induced by cobalt chloride (metabolic activity assay, activated caspase-3, TUNEL-staining)</td>
<td>(Gao et al. 2017)</td>
</tr>
<tr>
<td>hCDNF</td>
<td>Recombinant (bacterial)</td>
<td>10 000 nM</td>
<td>Primary neurons (mouse), differentiated N2s cell line (mouse)</td>
<td>Protection against toxic α-synuclein oligomers and 6-OHDA (live/dead-staining, MTT assay, medium LHD)</td>
<td>(Latge et al. 2015)</td>
</tr>
<tr>
<td>hCDNF</td>
<td>Recombinant (mammalian)</td>
<td>1 000 nM</td>
<td>PC12 cell line (rat)</td>
<td>Protection against methamphetamine (MTT assay)</td>
<td>(Wang et al. 2017)</td>
</tr>
</tbody>
</table>
MANF and CDNF exert their cytoprotective actions, although a role in ER stress is widely accepted (see chapter 2.2. Relation to cellular ER stress).

However, the cytoprotective effects of MANF and CDNF seem to be more diverse than that. Intracellular MANF protected cultured mouse sympathetic neurons against insults that mediate cell death by a Bax-dependent manner: deprivation of nerve growth factor (NGF), or treatment with etoposide or staurosporine (Hellman et al. 2011). When human embryonic kidney (HEK) cells, expressing toll-like receptors, were transfected with sponge MANF and challenged by bacterial lipopolysaccharide (LPS), a known inducer of ER stress, inflammation and apoptosis, Bax protein levels remained almost undetectable on WB, whereas, in the non-transfected cells Bax levels increased along with increasing LPS concentration (Sereno et al. 2017). In addition, lower levels of activated caspase-3 and increased viability were observed in cells expressing MANF transgene. The result suggests that MANF might regulate Bax levels. However, the relation of MANF to Bax accumulation and oligomerisation at mitochondrial membrane and release of cytochrome c, which are crucial steps in the Bax-mediated apoptosis (Westphal et al. 2014), were not addressed.

In addition to the possible involvement in ER stress and Bax-mediated apoptosis, both MANF and CDNF have shown anti-inflammatory effects on cultured cells in vitro. Pretreatment with recombinant MANF (Zhao et al. 2013, Chen et al. 2015, Zhu et al. 2016) or CDNF (Zhao et al. 2014), or overexpression of CDNF by lentiviral infection (Cheng et al. 2013a) suppressed inflammation in cultured cells by reducing the expression and secretion of pro-inflammatory cytokines as a response to various inflammation-inducing insults, such as LPS. MANF has been suggested to suppress the NF-κB pathway, a key regulator of inflammation, to attenuate inflammatory responses in diverse cells (Chen et al. 2015, Zhu et al. 2016, Hakonen et al. 2018). There are also implications that extracellular MANF can activate Akt-signal transduction pathways in 6-hydroxydopamine (6-OHDA)-injured human neuroblastoma cells (SH-SY5Y), thereby promoting cell survival (Hao et al. 2017, Zhang et al. 2017).

MANF has been shown to have a role in cell proliferation, which might be cell type specific. Apostolou and colleagues (2008) first reported that MANF had an inhibitory effect on cell proliferation, as knocking down MANF expression by small interfering RNA induced cell proliferation and overexpression of MANF decreased the proliferation rate of two human cancer cell lines (HeLa and U2OS) in vitro. On the contrary, extracellularly administered recombinant MANF increased primary mouse and human β-cell proliferation in vitro (Lindahl et al. 2014, Hakonen et al. 2018), and neural stem cells isolated from mice lacking MANF showed a normal proliferation rate in vitro (Tseng et al. 2017). Instead, MANF seemed to be crucial for the differentiation and neurite outgrowth of mouse neural stem cells. In addition, MANF was shown to affect the migration of neural progenitor cells, as cells lacking or overexpressing MANF showed reduced or increased migration, respectively, from explants in vitro (Tseng et al. 2018).
2.2. Relation to cellular ER stress

Accumulating data imply that MANF is an ER stress response factor with cytoprotective activity. The cellular organelle ER is the site for the folding and post-translational modification of secreted and transmembrane proteins, as well as the site for lipid biosynthesis (van Anken & Braakman 2005). Maturation of proteins requires a range of chaperones and enzymes involved in for example protein folding, disulphide bond formation, and glycosylation. Efficient protein folding requires a special environment, such as high Ca\textsuperscript{2+}-concentration and oxidising conditions. An oxidising environment is crucial for disulphide bond formation, whereas, high Ca\textsuperscript{2+} concentration is required for proper functioning of calcium-dependent chaperones. The ER also functions as an intracellular Ca\textsuperscript{2+} store. The high ER Ca\textsuperscript{2+} concentration is maintained by the sarco/ER Ca\textsuperscript{2+}-ATPase.

Chaperones function in the quality control of secretory proteins. For example, GRP78 binds to exposed hydrophobic patches on misfolded proteins, retaining them in the ER (van Anken & Braakman 2005). When a protein fails to fold correctly, it is removed to the cytosol and marked by ubiquitin, which destines it to proteasomal degradation, a process known as ER-associated degradation (ERAD) (Smith et al. 2011). In case of an imbalance between protein folding capacity and protein load in the ER, the accumulating misfolded or unfolded proteins result in ER stress (Walter & Ron 2011).

Cellular ER stress can be caused by mutations that lead to misfolding of nascent proteins, by changes in the ER folding capacity due to reduced chaperone, ATP, or Ca\textsuperscript{2+} concentrations, or by changes in the redox status due to e.g. chemical interference, hypoxia, or nutrient deprivation (Hetz & Papa 2018). Cells try to restore the protein-folding homeostasis and adapt to the new protein folding demand by activating unfolded protein response (UPR). The signalling pathways activated in UPR aim at 1) increasing ER size, 2) increasing the translation of proteins involved in folding and ERAD, and 3) decreasing overall protein translation. In mammalian cells, the main relays of UPR are three ER-transmembrane proteins: IRE1, PERK, and ATF6 (Table 2) (Walter & Ron 2011). In case of long-term non-resolved ER stress with no help from the adaptive responses, UPR aims toward cell apoptosis.

**MANF** messenger (m)RNA and protein expression increase when cells are challenged with chemical ER stress activators, including thapsigargin (sarco/ER Ca\textsuperscript{2+}-ATPase inhibitor), tunicamycin (N-glycosylation inhibitor), 1,4-Dithiothreitol (DTT, reducing agent), and lactacystin (proteasome inhibitor). This has been shown in multiple independent *in vitro* studies with several cell lines (Mizobuchi et al. 2007, Apostolou et al. 2008, Glembotski et al. 2012, Oh-Hashi et al. 2012) and primary cells including mouse pancreatic β-cells (Mizobuchi et al. 2007), rat cardiac myocytes (Tadimalla et al. 2008, Glembotski et al. 2012), rat neurons (Yu et al. 2010), and rat glial cells (Shen et al. 2012). Ischemia is a known inducer of ER stress (Doroudgar et al. 2009), and hypoxia-related increase in MANF expression has been reported in mouse embryonic fibroblast cultures (Romero-Ramirez et al. 2004) and cardiac
Table 2. ER-transmembrane proteins involved in the initiation of mammalian UPR as reviewed by Walter & Ron (2011) and Hetz & Papa (2018). Accumulation of incorrectly folded proteins in the ER leads to activation of IRE1 and PERK by oligomerisation and trans-autophosphorylation and ATF6 by translocation to the Golgi apparatus, where it gets cleaved and obtains its active form. The information of the cross talk between UPR and MANF is added as reported in the literature. IRE1 = Inositol-requiring enzyme 1, PERK = PKR-like ER kinase, ATF6 = Activating transcription factor 6, XBP1 = X-box binding protein 1, sXBP1 = spliced XBP1, TRAF2 = Tumour necrosis factor receptor-associated factor 2, JNK = c-Jun N-terminal kinase, NF-κB = Nuclear factor-κB, RIDD = Regulated IRE1-dependent decay, eIF2α = Eukaryotic initiation factor 2α subunit, ATF4 = Activating transcription factor 4, ERAD = ER-associated degradation, CHOP = transcription factor C/EBP homologous protein, ERSE = ER stress response element.

<table>
<thead>
<tr>
<th></th>
<th>IRE1</th>
<th>PERK</th>
<th>ATF6</th>
</tr>
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<tbody>
<tr>
<td><strong>Downstream</strong></td>
<td>XBP1 mRNA splicing → sXBP1 protein</td>
<td>Binding TRAF2 → 1) JNK phosphorylation 2) NF-κB activation</td>
<td>Phosphorylation of eIF2α Activation by its cleavage in the Golgi apparatus</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td>Transcriptional activator (e.g. chaperones, ERAD-associated proteins †)</td>
<td>1) Apoptotic signal 2) Pro-inflammatory signal</td>
<td>Degradation of selected mRNA molecules General attenuation of protein translation, but induction in the translation of some proteins such as ATF4 and CHOP</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td>MANF expression †</td>
<td>NF-κB target gene expression ‡</td>
<td>MANF expression †</td>
</tr>
</tbody>
</table>
myocytes (Tadimalla et al. 2008). Further, MANF expression is increased in cells expressing mutant proteins, which are misfolded and retained in the ER causing ER stress (Mizobuchi et al. 2007, Hartley et al. 2013, Kim et al. 2016).

**MANF** promoter contains ER stress response elements (ERSE), which have been reported to be crucial for its transcriptional induction under ER stress in mouse and human cells (Lee et al. 2003, Mizobuchi et al. 2007, Oh-Hashi et al. 2013, Wang et al. 2018). The ERSE sequences upstream of **MANF** gene start codon are recognised by transcription factors ATF6 and XBP1, activated in UPR (Lee et al. 2003, Wang et al. 2018). **MANF** mRNA and protein levels were increased by 10- and 5-fold, respectively, in rat cardiac myocytes overexpressing activated forms of ATF6 and XBP1 (Tadimalla et al. 2008).

How MANF surpasses the general translational arrest mediated by PERK-induced phosphorylation of eukaryotic initiation factor 2α (eIF2α), is currently unknown. Induced translation of for example **ATF4** and **CHOP** at ER stress rely on leaky ribosome scanning of inhibitory open reading frames upstream of the main coding open reading frame in the presence of limited eIF2α forming the ternary complex required for translation initiation (Hinnebusch et al. 2016). However, in contrast to **ATF4** and **CHOP** which show preferential translation when eIF2α is phosphorylated (Lu et al. 2004, Palam et al. 2011), **MANF** seems to be translated well in normal conditions with ample non-phosphorylated eIF2α, implying a different regulation in translation. Increased translation of **GRP78** during ER stress has been proposed to occur through an eIF2α-independent mechanism involving a so-called internal ribosomal entry site (Macejak & Sarnow 1991), but such a site has not been reported in **MANF** (www.iresite.org).

Most proteins whose expression is induced in ER stress are retained within the cell. Interestingly, the secretion of MANF increases in ER stress. Although robust secretion of endogenous MANF from rat primary cardiac myocytes was shown after tunicamycin treatment (Tadimalla et al. 2008), two other studies have reported induction in the secretion of overexpressed MANF only with thapsigargin, not with tunicamycin or DTT (Glembotski et al. 2012, Henderson et al. 2013). Glembotski et al. (2012) showed that the secretion of N-terminally tagged MANF was partly regulated by its complexation with GRP78. The interaction was suggested to require Ca²⁺, as the amount of immunoprecipitated complex decreased in lower calcium concentrations. Further, Henderson and co-workers (2013, 2014) showed that MANF’s C-terminal sequence ((ASA)RTDL) had an independent role in the secretory response to thapsigargin. Although the mechanism behind ER Ca²⁺-depletion to induced secretion of C-terminal RTDL-sequence remained unclear, the authors discussed that it could be due to altered KDEL-receptor mediated retrieval process (Henderson et al. 2014). Perhaps increased secretion occurred due to translocation of KDEL receptors to plasma membrane by thapsigargin (Henderson et al. 2013), thus transporting and liberating MANF outside the cell pH-dependently (Capitani & Sallese 2009).
Taken together, these studies suggest that MANF secretion is regulated by at least two factors, GRP78 and KDEL receptors, both of which are sensitive to changes in ER calcium levels. If this is the case, the induction of MANF secretion in ER stress is not depended on its increased expression. Instead, decreased ER Ca^{2+} levels would cause liberation of an intracellular pool of MANF, suggesting additional ways for regulated protein secretion to the well-known Ca^{2+}-dependent secretion via cytoplasmic secretory granules (Burgess & Kelly 1987). The functions of secreted MANF in ER stress are intriguing. Does it act in an autocrine manner to exert activities beyond those it potentially has in the ER? Or does it act as a sensor or signalling molecule in paracrine mode to boost the resilience of neighbouring cells? Is it secreted to affect other cells, such as those of the immune system, activated in tissue injury?

Evidence for CDNF acting as an ER stress response protein is scarce due to limited published data. The CDNF promoter has not been reported to contain an ERSE sequence. Differently from MANF, CDNF expression did not change in the human bone osteosarcoma epithelial cell line (U2OS) under up to seven hours of tunicamycin (2.5 μg/ml) incubation (Apostolou et al. 2008). In contrast, CDNF mRNA and protein levels increased in rat myoblast cells (H9c2) with 24-hour tunicamycin (5-20 μg/ml) incubation (Liu et al. 2017). The discrepancy between results might arise from differences in the used tunicamycin concentration and incubation time, and possibly from differently responding cells. The secretion of CDNF in ER stress has not been addressed, but it might be lesser compared to MANF since a reporter construct with a C-terminal sequence KTEL, from human CDNF, was secreted less from the SH-SY5Y cell line in the presence of thapsigargin than a construct containing RTDL, from MANF (Henderson et al. 2014).

The functional importance of MANF in cellular ER stress has been demonstrated by knocking down of MANF expression with small interfering RNA in vitro (Apostolou et al. 2008, Cunha et al. 2017). Reduction in endogenous MANF in HeLa cell line induced UPR-related gene expression and rendered cells more vulnerable to thapsigargin and tunicamycin (Apostolou et al. 2008). Similarly, reduction of MANF increased rat (INS-1E) and human (EndoC-βH1) pancreatic β-like cell apoptosis under thapsigargin treatment (Cunha et al. 2017). On the contrary, overexpression of MANF in U2OS cells (Apostolou et al. 2008) or extracellularly applied MANF in rat primary neuronal culture (Yu et al. 2010) protected cells against ER stress-induced apoptosis in vitro. Although CDNF has not been consistently found to respond to ER stress, it has shown protective functions in cells under ER stress. Overexpression of CDNF rendered cultured cells less sensitive to tunicamycin-induced cell damage (Cheng et al. 2013a, Liu et al. 2017). Recombinant CDNF reduced markers of UPR, including GRP78, phosphorylated eIF2α, and JNK, in cultured neurons challenged with amyloid beta peptide (Zhou et al. 2016). Interestingly, the changes in UPR markers were obtained with one-hour preincubation of CDNF, implying a priming effect against later applied amyloid beta peptides, although, the effect on cell survival was not addressed.
2.3. Mode of action

Since MANF and CDNF can be secreted, they are assumed to act in an autocrine or paracrine manner as extracellular proteins. Indeed, extracellularly applied recombinant MANF and CDNF are cytoprotective (refer to Table 1 and chapter 3.3. Therapeutic functions). However, it is not known whether MANF and CDNF act on cells as extracellular ligands or whether they need to be internalized for their intracellular actions.

No conventional plasma membrane receptor has been described for either MANF or CDNF. Transmembrane KDEL receptors, which are normally located in the cell surface fraction of SH-SY5Y cells after treatment with thapsigargin in vitro (Henderson et al. 2013). Interestingly, there is an indication that MANF may bind plasma membrane-located KDEL receptors (Henderson et al. 2013). The cell surface fraction of MANF, labelled with cell-impermeable cross-linker, increased with overexpression of KDEL receptors, and deletion of C-terminal RTDL sequence abolished MANF from this fraction. The amount of MANF found in the cell surface fraction reduced dose-dependently with incubation of KDEL receptor binding peptides (YTSEKDEL and ASARTDL).

When located to the Golgi apparatus, the cytoplasmic domain of KDEL receptors can activate Src family kinases and p-38 MAP kinase upon ligand binding (Capitani & Sallese 2009). However, it is unknown whether the same can happen on the plasma membrane, and whether MANF binding can induce signalling. In addition to acting as putative transmembrane signalling receptors, KDEL receptors could act as co-receptors anchoring MANF and CDNF to the plasma membrane or even have a role in the internalization of them. However, the C-terminal tetrapeptide sequence was not crucial for the neuroprotective effect of extracellularly applied MANF in vivo in rats in an experimental model of cerebral ischemia (Mätlik et al. 2015), implying KDEL receptor independent actions of MANF in the protected cells.

Based on the characteristic N-terminal domain of MANF and CDNF, they could affect cells via interaction to plasma membrane lipids (Lindholm & Saarma 2010). Indeed, interaction of MANF with sulfatide lipids was found to enhance its internalization into HEK cells, as indicated by immunocytochemistry (Bai et al. 2018). Direct evidence of CDNF internalization in vivo was shown by immunoelectron microscopy analysis, which indicated CDNF localization in neuronal endosomes two hours after its extracellular administration to rat brain (Mätlik et al. 2017). No signal was found in cellular organelles, suggesting that either internalized CDNF was degraded by the lysosomal pathway, or reached cellular organelles in too low quantities to be observed by the method used. Thus, the destination of the internalized protein is yet to be studied: whether it is degraded or is delivered, for example, to the ER to exert its actions.

Similarly, in the case of intracellularly expressed MANF and CDNF, it is unknown whether their activities are mediated directly intracellularly or only after secretion. However, there are strong indications of intracellular actions
of MANF at least in neurons. Extracellularly applied MANF did not protect cultured mouse sympathetic neurons against NGF deprivation, in contrast to treatment with intracellularly delivered recombinant protein or transfection plasmid, which increased the cell survival (Hellman et al. 2011). As radiolabelled recombinant human MANF did not show binding on the plasma membrane of cultured mouse sympathetic neurons, the result suggests that these cells do not respond to extracellular MANF at least under the conditions used. ER localization of intracellular MANF was suggested to be crucial for its neuroprotective effect, as the transfection of neuronal populations of two different types with RTDL-deleted MANF only protected the ones where MANF localised to the ER, despite the mutation (Mätlik et al. 2015).

Alternatively, MANF and CDNF might act both intra- and extracellularly and induce different actions depending on their location. This notion is supported by a recent publication from Hao and colleagues (2017), who report that the SH-SY5Y cell line was protected against 6-OHDA toxin by different mechanisms depending on whether MANF was applied as an extracellular recombinant protein or by viral gene transduction. In the case of the extracellularly applied protein, they found that activation of PI3K/Akt/mTOR signalling pathway was involved in survival promotion and the protective effect of MANF was abolished with a PI3K-inhibitor. Instead, intracellularly overexpressed MANF was found to decrease the level of ER stress-related proteins, which was not the case with extracellularly applied MANF.

3. MANF and CDNF functions in vivo

3.1. Expression

Both MANF and CDNF are expressed ubiquitously in mouse and rat neuronal and peripheral tissues during embryonic development and in adulthood (Lindholm et al. 2007, Lindholm et al. 2008, Wang et al. 2014a). The expression of MANF and CDNF in human tissues has been confirmed as well (Lindholm et al. 2007, Lindholm et al. 2008), although less studied compared to mice. mRNA expression has been studied with reverse transcriptase polymerase chain reaction (PCR), Northern hybridisation and in situ hybridisation, and protein expression by immunohistochemistry and WB. At present, no quantitative data on MANF or CDNF protein levels in tissues is published.

In the mouse and rat brain, MANF is mostly localised in neurons and is enriched in the cerebral cortex, hippocampus, thalamus, hypothalamus, and cerebellar Purkinje cells (Lindholm et al. 2008, Wang et al. 2014a). CDNF has been detected in the cerebral cortex neurons, hippocampal neurons, cerebellar Purkinje cells, striatum, and substantia nigra (Lindholm et al. 2007).
Differently from MANF (Lindholm et al. 2008, Wang et al. 2014a), CDNF staining did not co-localise with a dopamine neuron marker, tyrosine hydroxylase (TH), in the substantia nigra (Lindholm et al. 2007).

Despite its wide expression in the brain, the highest MANF levels are found in professional secretory tissues of the peripheral body such as the endocrine pancreas, testis and salivary gland (Lindholm et al. 2008, Lindahl et al. 2014). Similarly to MANF, the expression of CDNF in the body is ubiquitous, although at overall lower levels compared to MANF (Lindholm et al. 2007). Interestingly, CDNF levels are highest in the skeletal muscle and heart, the tissues with the lowest MANF levels compared to other tissues. The differential expression implies specialised functions of the homologs. This view is strengthened with the observation of distinct phenotypes of MANF and CDNF knock out (KO)-mice (Lindahl et al. 2017).

3.2. Effects of knock out and overexpression

In order to reveal the functions of MANF and CDNF in a biological context, the effect of their removal has been studied in non-mammalian model organisms and in mice. In fruit fly, *Drosophila melanogaster*, genetic deletions in the *Manf/Cdnf* homolog (*DmManf*) were developmentally lethal (Palgi et al. 2009). Interestingly, human *MANF* (Palgi et al. 2009) and *CDNF* (Lindström et al. 2013) could rescue the *DmManf* mutants, implying evolutionarily conserved activity. In addition, the mutant flies could be rescued only with the expression of full-length *DmManf* and not with the separate N- or C-terminal domains, even if expressed simultaneously (Lindström et al. 2013). This implies that, despite the putative independent functions of the two distinct domains, the intact protein has an important role of its own.

The *DmManf* mutant larvae had a deficiency in nervous system development, particularly affecting the neurites of dopamine neurons, while motorneurons and serotonin neurons were intact (Palgi et al. 2009). These findings delineate the importance of MANF/CDNF in the maturation and/or survival of dopamine neurons in the developing fly. However, the effects of *DmManf* ablation on other organ systems were not addressed even if MANF was found to be expressed throughout the developing organism. A later microarray gene expression study of the developmentally lethal *DmManf* mutant embryos and larvae indicated major changes in gene expression related to metabolism and membrane transport (Palgi et al. 2012). The ERs in the mutant cells appeared dilated and microarray data pointed in the direction of activated UPR. Furthermore, the phosphorylation of eIF2α was more robust in mutants than in wild types, indicative of activated PERK pathway. Specific deletion of *DmManf* from glial cells by RNA interference was linked with appearance of a novel MANF-positive cell type in the pupal brain (Stratoulias & Heino 2014). A closer study indicated that the cells resembled potentially proinflammatory hemocytes, the invertebrate counterpart of microglia.
Knock-down of MANF protein expression by antisense morpholino oligonucleotides during the development of zebra fish, *Dario rerio*, did not result in any gross phenotype, although MANF is widely expressed during normal fish larval stages and in adult tissues (Chen et al. 2012). It is possible that CDNF could overcome some of the effects of MANF ablation in zebra fish. More specific analysis of the nervous system revealed deficits in a subpopulation of dopamine neurons characterised by reduction in the gene expression of dopamine cell markers: dopamine transporter and TH, an enzyme required in dopamine synthesis. Consequently, reduced dopamine levels were found. At the same time, no changes were observed in e.g., GABAergic or serotoninergic systems, implying a cell-type, and even subtype specific role of MANF in the development of zebra fish.

In mice, a strikingly different phenotype was observed compared to those found in the fly and fish. Deletion of mouse *Manf* gene led to progressive postnatal reduction of the pancreatic β-cell mass, resulting in a type 1 diabetes (T1D)-like state characterised by high blood glucose and low insulin levels (Lindahl et al. 2014). As pointed out by immunohistochemistry, there were no differences in pancreatic β-cell mass at embryonic state (day E18.5) compared to the wild type littermates. In contrast, the β-cell mass was decreased by 50% at postnatal day one, while almost total loss of β-cells was observed at 8 weeks of age. The loss in β-cell mass was found to occur by two mechanisms: increased β-cell death and decreased β-cell proliferation, assessed by TUNEL- and Ki67-stainings, respectively. A phenotype similar to *Manf*−/− was observed in mice with a specific deletion of MANF in the β-cells by Pdx1-Cre-recombinase (Lindahl et al. 2014), verifying that the phenotype was due to specific vulnerability of β-cells to the loss of MANF and not due to secondary effects.

All three branches of UPR regulators were activated in the pancreatic islets of *Manf*−/− mice, implying an overt ER stress (Lindahl et al. 2014). Increased expression of UPR-related genes and phosphorylation of eIF2α were observed throughout the life span of the KO mice, demonstrating constitutively activated UPR, which could be the reason for the observed increase in β-cell apoptosis. As MANF was found to stimulate β-cell proliferation *in vitro* and *in vivo* in mice, the lack of MANF could explain the observed decrease in β-cell proliferation in the *Manf*−/− mice.

ER stress was only assessed in the postnatal pancreatic islets, thus the ER stress status in other organ systems of *Manf*−/− mice remained unknown. In the pancreas, the loss of MANF affected specifically only β-cells and no other cell types, such as exocrine acinar cells, although MANF is equally expressed in these cell types as well (Lindahl et al. 2014). β-cells are especially prone to ER stress as their demand for insulin secretion is very high (Cnop et al. 2017). Further, insulin secretion needs to be adapted in response to changing blood glucose concentration, laying another level of demand for functional UPR. The importance of functional UPR is evident in *Perk*−/− mice, which develop a T1D-like phenotype due to postnatal apoptosis of β-cells (Harding et al. 2001, Zhang et al. 2002).
Since CDNF is expressed normally in the $Manf^{-/-}$ mice, including pancreas (personal data measured by mouse CDNF enzyme-linked immunosorbent assay (ELISA)), it is plausible that CDNF does not compensate for the role of MANF in the mouse pancreas. On the other hand, as the measured CDNF concentration in pancreas was over 10 000-fold less than that of MANF in wild type mice, compensation is unlikely. $Cdnf^{-/-}$ mice do not develop diabetes or show any other major phenotype in relation to viability, growth, fertility, or life-span (Lindahl et al. 2017). The in-depth analysis of the $Cdnf^{-/-}$ is yet to be published (Lindahl, Chalazonitis, Saarma et al., manuscript in preparation).

The effects of MANF overexpression have been studied in transgenic mice, where C-terminally tagged MANF was expressed under mouse prion promoter for specific expression in the central nervous system (CNS) (Yang et al. 2017). In these mice, MANF was found to be involved in the regulation of food intake and obesity. The transgenic mice gained more weight and adipose tissue than their wild type littermates beginning at four months of age. Overexpression of MANF was found to impair hypothalamic control of feeding by reducing insulin signalling. Specifically, MANF was linked with increased ER localization of PIP4k2b, a kinase that is involved in reduced Akt activation in the signalling cascade of insulin.

On the contrary, knock-down of MANF expression by targeted RNA-interference in the hypothalamus of mice resulted in decreased food intake and body weight compared to controls (Yang et al. 2017). Similarly, $Manf^{-/-}$ mice were reported to be smaller compared to their littermates and have less adipose tissue (Lindahl et al. 2014). The phenotype was not secondary to diabetes as it was not observed in the mice with conditional deletion of MANF in pancreas. However, whether the growth retardation was linked only to the hypothalamic feeding control, or additionally to some other mechanism, such as impairment in the hormonal control of growth, is currently unknown (Lindahl et al. 2017).

### 3.3. Therapeutic functions

In this paragraph I will review the therapeutic areas where current literature points out the most solid evidence for MANF and/or CDNF effects. These are diabetes, ischemia, inflammation, and neurodegenerative diseases, in which the highest efforts have been directed to Parkinson’s disease.

#### 3.3.1. MANF and CDNF in neurorestoration

As MANF was found to specifically affect dopaminergic neurons in mixed neuronal cell culture (Petrova et al. 2003), and the removal of MANF expression in fruit fly (Palgi et al. 2009) and zebra fish (Chen et al. 2012) was linked with problems in dopaminergic neuron maturation/development, the therapeutic effect of MANF and CDNF in Parkinson’s disease (PD) has been
under vigorous investigation. PD is a movement disorder characterised by selective and progressive loss of midbrain dopaminergic neurons (Poewe et al. 2017). The neuronal bodies of these cells are located in the substantia nigra pars compacta, and they innervate to the striatum via their axons. The pathologic hallmark of PD is widespread appearance of cytoplasmic inclusion bodies in neurons. These so-called Lewy bodies mainly consist of aggregated α-synuclein, which in normal state is present in neurons in soluble form.

The ability of human MANF and CDNF to protect dopamine neurons against subsequent administration of toxins, or to restore them after a toxin-induced injury, has been studied by intrastriatal administration of recombinant proteins in experimental animals including rats, mice, and marmoset monkeys (Lindholm et al. 2007, Voutilainen et al. 2009, Voutilainen et al. 2011, Airavaara et al. 2012, Garea-Rodríguez et al. 2016, Voutilainen et al. 2017). Effects of MANF and CDNF gene therapy has been studied in rats by transducing striatal (Bäck et al. 2013, Ren et al. 2013, Cordero-Llana et al. 2015, Hao et al. 2017) or nigral cells (Nadella et al. 2014, Cordero-Llana et al. 2015). In these animal models, the degeneration of dopamine neurons has been induced by 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP, that is converted into its toxic metabolite in the brain. Both toxins are taken up by dopamine neurons through dopamine transporters and induce relatively rapid cell death by mitochondrial dysfunction and oxidative stress (Blandini & Armentero 2012). The consequential loss of dopamine neurons can be assessed by behavioural tests and by post mortem immunohistochemical staining of dopamine neuron markers, such as TH.

In the PD animal models, administration of CDNF a few hours before or several weeks after the toxin has been linked with an increased number of TH-positive neurons in substantia nigra and increased TH-immunoreactivity in striatum compared to controls (Lindholm et al. 2007, Voutilainen et al. 2011, Airavaara et al. 2012, Ren et al. 2013). The results indicate protective and/or restorative effects of CDNF on dopamine neuron circuitry in the basal ganglia. More importantly, the function of dopamine neurons can be restored by CDNF treatment, as pointed out by higher dopamine transporter activity in the treated compared to the non-treated hemisphere observed by neuroimaging of live but anaesthetised rats (Ren et al. 2013) and monkeys (Garea-Rodríguez et al. 2016). As well, improved locomotor behaviour has been reported.

The mechanism of CDNF action on dopaminergic neurons is unclear. When administered to the striatum, the hypothesis is that CDNF acts as a classical target-derived neurotrophic factor to promote axon regeneration and stability of synaptic connections, thus keeping the cell bodies in the substantia nigra alive (Voutilainen et al. 2015). However, there are also implications that CDNF is transported from the striatum to cell soma in substantia nigra in naïve rat brain (Voutilainen et al. 2011, Mätlik et al. 2017), which suggests that it could act directly on dopaminergic cell bodies.

As compared to vehicle controls, striatal administration of extracellular CDNF in naïve rats increased Akt phosphorylation, suggesting activation of
the survival promoting PI3/Akt-signalling pathway (Voutilainen et al. 2017). Nigral transfection of CDNF was shown to suppress neuroinflammation in the rat 6-OHDA model (Nadella et al. 2014). The anti-inflammatory effect was reported to be mediated through astrocytes and microglia, i.e., not by direct effect on dopamine neurons. The 6-OHDA model is not known to induce Lewy bodies (Blesa & Przeborski 2014) but CDNF can potentially affect human disease by protecting neurons against toxic $\alpha$-synuclein oligomers, as observed in vitro (Latge et al. 2015).

Single intrastriatal injection of MANF has been linked with beneficial effects on dopamine neurons and improved behavioural outcome when given six hours before or four weeks after 6-OHDA in rats (Voutilainen et al. 2009). Although chronic infusion of MANF failed to reproduce the previously observed positive effects (Voutilainen et al. 2011), intrastratal transduction of MANF by adeno-associated viral serotype 9 (AAV9) vector in rats resulted in increased TH-staining in the substantia nigra and striatum and improved locomotor behaviour compared to controls (Hao et al. 2017). In this study the effects of gene therapy, implemented 10 days after 6-OHDA administration, were evident up to 16 weeks from viral injection, and long-term human MANF expression was observed in the neurons of transduced striatum and ipsilateral substantia nigra.

The mechanism of MANF action in PD models is less studied compared to CDNF. After three days of intrastratal protein infusion in rats, MANF diffusion was greater than that of CDNF (Voutilainen et al. 2011). However, MANF was distributed from the striatum preferentially to the cortex, not to the substantia nigra (Voutilainen et al. 2009). Differently from CDNF, the lentiviral delivery of MANF to the substantia nigra did not affect striatal TH-fibre density or improve the behavioural outcome caused by simultaneously administered 6-OHDA (Cordero-Llana et al. 2015). This result implies that MANF and CDNF act differently in the 6-OHDA model, although relative protein expressions were not reported. The observation of a synergistic effect of MANF and CDNF, when applied together in the same lentiviral construct, further strengthens the idea of differential actions on dopamine cells (Cordero-Llana et al. 2015). However, the used fusion construct included an extra C-terminal tail on CDNF, which could affect its biological characteristics, such as secretion, and thereby its therapeutic outcome.

Extracellular MANF increased GABA-mediated dopamine neuron inhibition in patch clamp studies of rat midbrain slices in vitro (Zhou et al. 2006), which could account for increased neuron survival in PD models. Intrastratal injection of MANF in naïve rats, has been reported to enhance stimulus-evoked dopaminergic neurotransmission and dopamine turnover (Renko et al. 2018), and thus, it could have an effect on animal behaviour. However, whether these observations have a role in the outcome of MANF-treated PD animal models, is unknown.

The restorative effects of MANF and CDNF have also been studied in rats with spinal cord neuron injury leading to paralysis of the hind limbs.
Administration of recombinant MANF into the brain ventricles or to the spinal cord improved motor performance and attenuated neuronal apoptosis which was linked to increased Akt-phosphorylation (Gao et al. 2018). Injection of CDNF-expressing bone marrow-derived stem cells (BMSCs) to the injury site was accompanied by better locomotor recovery, increased regeneration of neural fibres, increased myelination of the regenerated fibres, and decreased expression of pro-inflammatory cytokines, including PEGα, TNF-α and IL-1β, compared to the injection of control-BMSC (Zhao et al. 2016). As the CDNF-expressing BMSCs survived better in vivo compared to control-BMSCs, the observed regenerative and anti-inflammatory effect could be related to the BMSCs, rather than CDNF itself. However, lentiviral transduction CDNF to the injury site was reported to have an effect on axonal regeneration and myelination after sciatic nerve transection (Cheng et al. 2013b), implying a restorative effect of CDNF in the rat peripheral nervous system.

3.3.1.1. MANF and CDNF administration to the central nervous system

Administration of proteins to the brain is challenging since in many cases they are unable to penetrate the blood brain barrier (BBB) and their tissue diffusion is limited. Thus, therapeutic proteins have to be delivered close to the target site. Although MANF and CDNF have shown therapeutic effects in PD models after single injections (Lindholm et al. 2007, Voutilainen et al. 2009, Airavaara et al. 2012, Voutilainen et al. 2017), in the clinical situation of chronic disease pathology, long-term administration may be necessary. Long-term infusion of MANF and CDNF has been studied in rats (Voutilainen et al. 2011), and CDNF in non-human primates (Garea-Rodríguez et al. 2016). Currently, CDNF has reached clinical studies in PD patients (ClinicalTrials.gov identifier: NCT03295786). In this study paradigm, intermittent CDNF protein injections are given to the patients’ brain via intrastriatal injection catheters.

In addition to protein infusion and gene therapy, long-term protein delivery can be implemented by cells that secrete the therapeutic protein to the target site (Emerich et al. 2014). Intrastriatally injected genetically engineered CDNF-expressing stem cells showed neuroprotective functions in the 6-OHDA rat model (Jiaming & Niu 2015, Mei & Niu 2015). However, as the fate of the injected engineered cells was not addressed, the contribution of CDNF to the reported outcome is unclear. In addition, the cells were transfected only transiently. Hence, the expression of CDNF would most probably be negligible one week after cell transplantation when the dopamine toxin was applied, as plasmids that are not integrated to the genome would not be passed to the daughter cells at division.

In encapsulated cell biodelivery, the therapeutic cells are not intended for grafting, instead, they are protected by a membrane in order to avoid contact with host cells. Encapsulated cell biodelivery has some positive features compared to the aforementioned protein delivery methods: compared to injection of free cells or viruses, encapsulated cell biodelivery is more
controllable, e.g., in terms of location and retrieval. Compared to protein administration, the cell encapsulation method can provide a constant supply of in-place synthesised and correctly folded protein. This is not always the case with recombinant proteins produced by bacteria (Gupta & Shukla 2017). Even proteins produced by mammalian cells can be aberrantly modified by separation and purification steps. The effects of striatal delivery of encapsulated cells secreting GDNF has been studied in the rat 6-OHDA model of PD (Shingo et al. 2002, Sajadi et al. 2006), but the method failed to produce a sufficient amount of GDNF in preclinical testing (The Michael J. Fox Foundation for Parkinson’s Research 2004). The delivery of encapsulated cells secreting NGF to the basal forebrain has been studied in Alzheimer’s disease patients (Eriksdotter-Jönhagen et al. 2012, Eyjolfsdottir et al. 2016). No adverse events were observed related to the device carrying encapsulated human retinal pigment epithelial cells (ARPE-19) when implanted to the brain of altogether 10 patients for a period of 6–12 months.

3.3.2. MANF in diabetes

In humans, T1D is most commonly caused by an autoimmune attack against pancreatic β-cells (Evans-Molina et al. 2013). The consequential insulitis, i.e., inflammation of the pancreatic islets of Langerhans, is accompanied by oxidative stress, mitochondrial dysfunction, and chronic ER stress in the β-cells. During disease development, β-cells die through apoptosis leading to insulin deficiency. Currently, there is no disease-modifying treatment for T1D, and the patients are destined to use exogenous insulin daily throughout their lives (Aghazadeh & Nostro 2017).

One approach in the search for a cure to T1D is the reduction of β-cell ER stress (Cnop et al. 2017), making MANF an interesting therapeutic candidate. As described earlier, the deletion of MANF in mice resulted in the loss of β-cells, probably due to chronic activation of the UPR (Lindahl et al. 2014). Similarly, knock down of MANF in a human β-cell line (EndoC-βH1) caused increased ER-stress triggered by cytokines (Hakonen et al. 2018). In non-obese diabetic (NOD) mice, a defect in MANF induction in response to hen egg lysozyme expression–induced ER stress in β-cells was linked with increased diabetes susceptibility (Dooley et al. 2016), further strengthening the idea of MANF’s important role in the resilience of β-cells. Indeed, pancreatic overexpression of MANF in mice by gene therapy was cytoprotective against a β-cell death–inducing toxin, streptozotocin, as indicated by a reduced number of TUNEL-positive β-cells compared to controls (Lindahl et al. 2014).

In addition to being important for the survival of β-cells, MANF seems to have mitogenic effects on these cells. Extracellularly administered MANF (100 ng/ml = 5.5 nM) promoted β-cell proliferation in mouse pancreatic islets in vitro (Lindahl et al. 2014). Encouragingly, recombinant MANF (5.5 nM) increased β-cell proliferation in human islets as well (Hakonen et al. 2018). However, the effect was only seen when the islets were co-incubated with an
inhibitor for transforming growth factor-β, whose action has been linked with repressed β-cell proliferation (Dhawan et al. 2016). The mitogenic properties were evident also in vivo in adult mice, where AAV6-MANF-treated animals showed enhanced proliferation of β-cells after streptozotocin, as studied by Ki67-staining (Lindahl et al. 2014), suggesting that it might have a regenerative effect in diabetes.

As the autoimmunity against β-cells persists in human subjects with T1D, possible regenerative therapy should be applied together with immune suppression (Aghazadeh & Nostro 2017). Interestingly, MANF might be effective towards the immune response as well. Extracellular human MANF (5.5 nM) showed partial protection of cultured mouse islets and EndoC-βH1 cells against cytokine-induced apoptosis (Cunha et al. 2017, Hakonen et al. 2018). The protective effect was associated with repression of NF-κB signalling as shown by reduced nuclear translocation and phosphorylation of the p65 subunit of NF-κB in EndoC-βH1 cells (Hakonen et al. 2018). The addition of MANF also reduced GRP78 mRNA levels, implying attenuated ER-stress, although reduction in other UPR-markers was not observed.

Similarly to T1D, type 2 diabetes (T2D) is a disorder where blood glucose levels rise, although the aetiologies of the two diseases differ. T2D is not an autoimmune disease, instead, it is a metabolic disease caused by reduced effects of insulin in the peripheral target tissues and dysfunction of β-cells exhausted by the increased insulin demand (Cnop et al. 2012). Since ER stress and inflammation are linked to the development of insulin resistance and to the β-cell failure and death in T2D, MANF could be associated with T2D as well. Interestingly, a human subject with a homozygous mutation in MANF was found suffering from T2D, suggesting a role for MANF in human diabetes (Yavarna et al. 2015). However, whether the mutation affected MANF protein concentration or biological activity was not addressed. Differently from what could have been expected from the mice with overexpression or deletion of MANF, the subject was reported to be suffering from obesity, which is a well-known risk factor for T2D development (Cnop et al. 2012). In fact, the subject was listed with multiple disabilities, some of which are interlinked, making it difficult to judge the role of mutated MANF in one clinical feature.

3.3.3. MANF in ischemia

Insufficiency in tissue blood supply, ischemia, is usually due to an occlusion in an artery by for example blood clot or atherosclerotic plaque. When occurring in the brain or heart, it is life-threatening. The lack of oxygen and nutrients, provided by constant blood flow, causes injury to the tissue as the cells are unable to conduct their normal functions (McMichael & Moore 2004). First-line treatment aims in restoring blood flow to the ischemic tissue. The reperfusion of ischemic tissue, however, causes additional damage to the tissue by oxidative stress and inflammatory response (McMichael & Moore 2004). Novel therapies are currently being investigated for their potential to
protect and recover from the ischemic damage (George & Steinberg 2015, Spath et al. 2016).

Pretreatment with human MANF administered either as extracellular protein (Airavaara et al. 2009) or by gene therapy (Airavaara et al. 2010) reduced infarct area in the brain and promoted neurological and locomotoric recovery after transient middle cerebral artery occlusion in rats. MANF has also been reported to have similar activities when given two to three hours after ischemia (Yang et al. 2014b, Wang et al. 2016). As well, intravenously administered recombinant mouse MANF reduced the infarct size in a mouse experimental model of cardiac ischemia (Glembotski et al. 2012).

MANF seems to protect the injured cells from ischemia-induced apoptosis, as shown by reduced TUNEL-positive cell number compared to controls (Airavaara et al. 2009, Yang et al. 2014b), but its mechanism of action is unknown. MANF could exert its cytoprotective actions by alleviating chronic ER stress induced by ischemia (Yang et al. 2014b). MANF might also act against inflammation, as shown in vitro in primary astrocytes under oxygen and glucose deprivation (Zhao et al. 2013). MANF has been shown to induce neural progenitor cell migration to the infarct zone in rat cortex (Tseng et al. 2018), indicative of a regenerative potential. However, differently from rodents, the capacity of endogenous neural progenitor cells to repair injured brain tissue in humans is uncertain (Barkho & Zhao 2011).

3.3.4. MANF and CDNF in inflammation
In addition to the inhibitory effect of MANF and CDNF on pro-inflammatory cytokine expression and secretion (Cheng et al. 2013a, Zhao et al. 2013, Nadella et al. 2014, Zhao et al. 2014, Chen et al. 2015, Zhu et al. 2016), MANF might be involved in immune modulation. Injection of recombinant human MANF to the vitreous of mice was linked with a phenotype switch of macrophages from proinflammatory to anti-inflammatory in damaged retina (Neves et al. 2016). Further, MANF treatment promoted integration of transplanted photoreceptors to the damaged retina by inducing a supportive anti-inflammatory microenvironment. Thus, MANF might have potential in tissue transplantation and regenerative medicine based on cell replacement. In addition, the possible involvement in the regulation of inflammation has implications on the pathology of multiple diseases, including T1D and other autoimmune diseases, ischemia, and neurodegenerative diseases.
4. MANF and CDNF as potential biomarkers

Biological markers, i.e. biomarkers, are defined as objectively measurable characteristics that can be used as indicators of normal biological process, pathological process, or therapy response (Biomarkers Definitions Working Group 2001). Thus, biomarkers can be used in the prediction and diagnosis of diseases, and in the monitoring of disease progression or treatment effects. In disease prediction the aim is to characterise people at risk of developing a disease, which can possibly be prevented by early intervention (Mayeux 2004). In the case of diagnostics, a biomarker can be valuable in terms of improving the probability of correct diagnosis, in subclassification of diseases, or in the choice of optimal therapy. Monitoring changes in the biomarker can be used in the analysis of risks or benefits of applied treatments. In drug development, biomarkers are used as surrogate endpoints, reflecting the disease response to investigational therapy (Biomarkers Definitions Working Group 2001).

Biomarker discovery can be approached in two ways (Chahine et al. 2014). It can be obtained by unbiased discovery–based findings from screenings of multiple variables in a specimen by the means of proteomics, metabolomics, or gene expression profiling. Candidate variables are then selected based on their difference in relation to the control population. The other way is based on the knowledge about possible biomarker functions in a certain health or disease state, whereafter studies are conducted in order to find out whether the target can serve as a biomarker.

As described earlier, the expression of MANF increases in ER stress (see chapter 2.2. Relation to cellular ER stress). Since ER stress has been linked to the pathology of various disorders, including neurodegenerative diseases and diabetes (Oakes & Papa 2015), MANF is an interesting biomarker candidate for several diseases. More importantly, MANF has been shown to be secreted in relation to cellular stress in vivo, which is a prerequisite for biomarker detection in body fluids such as blood, urine, or cerebrospinal fluid (CSF). For example, MANF was observed in the urine of mice in relation to tubular or podocyte ER stress (Kim et al. 2016). In addition, MANF staining was found in the extracellular matrix of cartilage tissue immunohistochemical sections from mice expressing ER-retained mutated matrilin-3 but not from wild type mice (Hartley et al. 2013), implying increased MANF secretion in relation to ER stress in a model of skeletal disease.

There is not only the possibility that the expression levels or secretion of MANF or CDNF are altered in a certain physiological state, but also that their putative binding partners change, which adds another layer of variability in protein biomarker diagnosis. For example, in the diagnosis of prostate cancer, the fraction of prostate-specific antigen complexed with $\alpha_1$-antichymotrypsin, rather than its circulating levels overall, is suggested to be more informative of the pathology (Stenman 2010). That being said, no truly extracellular binding partners have been identified for MANF or CDNF so far, besides sulfatides for MANF (Mirzaian et al. 2015, Bai et al. 2018).
4.1. Possible applications

4.1.1. Biomarker for neurodegenerative diseases

The diagnosis of many sporadically occurring neurodegenerative diseases is largely based on standardised clinical criteria and histological findings, which in many cases can be achieved only post mortem for verification (Beach 2017). At the time of overt clinical symptoms, the disease has often already progressed significantly. For example, it is estimated that at the time of the onset of the cardinal motor symptoms in PD, such as bradykinesia, rigidity and tremor, approximately 30% of midbrain dopaminergic neurons and 50-60% of their striatal axons are lost (Cheng et al. 2010). However, early interventions would be more beneficial when aiming at slowing, halting, or even reversing the disease pathology. This holds true especially for the neuroprotective and neurorestorative treatments, which are currently under intense investigation. Biomarkers carry a potential to indicate neurodegenerative pathology at an early, subclinical stage where only low number of neurons are lost.

As both MANF and CDNF have shown neurotrophic properties in animal models of neurodegeneration, the role of their endogenous levels in disease pathology is intriguing. However, the expression of neither MANF or CDNF has been studied systematically in neurodegenerative animal models or in patients with neurodegenerative diseases. Nadella et al. (2014) reported a 2-fold increase in the endogenous rat Cdnf mRNA in the striatum and substantia nigra 30 days after 6-OHDA injection, along with no change in protein level. Decreased expression of endogenous MANF was associated with the death of cerebellar Purkinje cells in a neurodegenerative disease model of spinocerebellar ataxia (Yang et al. 2014a). The decrease in MANF was linked with lower activity of the transcription factor XBP1 in this transgenic mouse model expressing mutant TATA box–binding protein (TBP). The accumulation of mutant TBP was found especially in aged mice in relation to decreased chaperone expression. Thus, the result implies that the expression of MANF might decrease in old age, playing a role in neuronal death. This makes MANF a possible target for early detection of neurodegenerative diseases.

The endogenous levels of MANF and/or CDNF in neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis are interesting both in pathogenetic and therapeutic aspects. However, as brain biopsies are not a feasible choice for biomarker detection in living subjects, it remains to be investigated whether the possible intracellular changes in MANF and/or CDNF levels are reflected in the extracellular space, and could be investigated from CSF, that is in proximity to the brain tissue.

4.1.2. Biomarker for diabetes

Reduced expression of transcription factor GLIS3 was associated with lower MANF levels in the pancreatic islets of NOD mice, a model of T1D (Dooley et al. 2016). The positive relationship between islet GLIS3 and MANF levels was
also found in healthy humans (Dooley et al. 2016). As GLIS3 has been identified as a susceptibility gene for both T1D (Barrett et al. 2009) and T2D (Dupuis et al. 2010), it raises a question whether reduced MANF expression in β-cells is associated with the susceptibility of these diseases in humans. However, a preliminary investigation including 14 islets from T2D patients failed to reproduce a relationship between MANF and GLIS3 (Dooley et al. 2016).

On the other hand, endogenous MANF was found upregulated in the β-cells of Akita mice carrying a mutation in the insulin gene resulting in misfolding and aggregation of the nascent proinsulin in the ER (Mizobuchi et al. 2007). In addition, a robust increase in MANF expression and secretion was observed in EndoC-βH1 cells in response to a cytokine cocktail composed of IL-1β, IFN-γ, IL-17, and TNF-α (Hakonen et al. 2018). These data suggest that β-cell MANF levels could increase under stress situations. However, it is currently unsure whether the local changes in MANF levels can be monitored conveniently from, for example, blood circulation.

Interestingly, increased circulating MANF levels were found in T2D patients and in prediabetic subjects with impaired glucose tolerance compared to non-diabetic controls (Wu et al. 2017). In prediabetic subjects, serum MANF levels correlated positively with biological variables reflecting insulin resistance, suggesting that increased circulating MANF levels could indicate impaired insulin signalling at the early stages of disease development.

4.1.3. **Biomarker for stroke and heart attack**

As ischemia activates ER stress, it is plausible that MANF levels increase at the site of ischemic injury. Indeed, induction in Manf mRNA and MANF protein expressions has been shown in mice and rats after experimental cerebral (Apostolou et al. 2008, Lindholm et al. 2008, Yu et al. 2010, Shen et al. 2012) and myocardial ischemia (Tadimalla et al. 2008).

Virally transduced MANF showed an altered staining pattern in the rat brain six hours after transient middle cerebral artery exclusion (Airavaara et al. 2010). Instead of the perinuclear staining in the control hemisphere taken as ER localization, MANF was found in a punctate pattern that was not colocalising with neuronal (NeuN) or astrocytic (GFAP) markers, suggesting possible release of overexpressed MANF in response to ischemia. Induction in MANF expression in brain has been reported to take place already four hours after the occlusion and even before the injury could be detected (Yu et al. 2010), making it a potential stroke biomarker candidate. As ischemia has been reported to disrupt the BBB (Jiang et al. 2017), brain-derived MANF could possibly be monitored in the blood.

Increased MANF expression has been found in the hearts of mice after experimental myocardial ischemia (Tadimalla et al. 2008). Indication of increased MANF release from cardiac myocytes was observed when the cells were subjected to conditions simulating ischemia/reperfusion *in vitro* (Glembotski et al. 2012). Thus, circulating MANF could have potential for the
diagnosis of myocardial infarction, although additional benefit over currently used troponin is only speculative (Park et al. 2017). However, in contrast to troponin, which simply gets released from necrotic cardiac myocytes after infarction damage, mouse MANF levels were found increased for several days in the surviving cells adjacent to the ischemic zone (Tadimalla et al. 2008). Thus, the analysis of circulating MANF could have potential in the monitoring of tissue restoration after heart attack, taken that its cardiac levels and secretion attenuates with time.

4.1.4. Biomarker for inflammatory diseases
Accumulating evidence indicates that MANF expression is induced in non-immune (Hakonen et al. 2018) and immune cells in response to inflammation. Manf mRNA levels were upregulated in fruit fly hemocytes, macrophage-like cells, and mouse microglia in an inflammatory model of light-induced retinal damage (Neves et al. 2016). In humans, increased MANF transcript levels were measured in the circulating white blood cells of autoimmune patients with rheumatoid arthritis (Chen et al. 2015) or systemic lupus erythematosus (Wang et al. 2014b, Chen et al. 2015). The difference to controls was statistically significant, albeit the observed major overlap in measured levels between patients and controls may reduce its diagnostic potential. However, the majority of the patients were on anti-inflammatory medication, which might affect MANF levels. Were they even higher in patients without the medication, MANF might have potential in autoimmune disease diagnostics.

4.1.5. Biomarker for kidney disease
MANF was observed in mouse urine after acute kidney injury caused by tubular ischemia/reperfusion or intraperitoneal administration of tunicamycin and in transgenic mice expressing mutated protein in kidney podocytes leading to misfolding and ER stress (Kim et al. 2016). MANF was present in urine even before histological changes were evident and in cases where albuminuria was not observed, implying that the excretion of MANF to urine is an early and specific response to ER stress-related kidney injury. Future studies will reveal whether MANF can be used in the prediction, diagnosis, or monitoring of ER stress-related kidney diseases in humans.

4.1.6. Biomarker for retinal damage
MANF is present in the vitreous of human eye, as measured by ELISA (Gao et al. 2017). Higher average vitreous MANF levels were found in patients with proliferative diabetic retinopathy or retinal detachment, than those with macular holes, whereby MANF was suggested to serve as a possible biomarker for retinal disorders (Gao et al. 2017). However, as the concentration of MANF in the vitreous of a healthy eye is currently unknown, the conclusion of changed MANF levels in retinal disorders is uncertain.
4.2. Methods for analysing protein biomarkers

Most protein biomarkers in clinical use are measured from the blood (Geyer et al. 2017). Other sample sources include CSF, urine, and tissue biopsies (Mayeux 2004). Some biomarkers are analysed by in vivo imaging with the aid of a probe that selectively targets the biomarker in order to visualise it (Saji 2017). All in all, biomarkers need to be analysed from complex biological matrices in a specific, sensitive, and reproducible manner. Currently used clinical protein diagnostic tests include chemical assays measuring protein function, most commonly enzymatic activity, and immunoassays measuring protein concentration, such as ELISA (Geyer et al. 2017). Mass spectrometry (MS) holds the possibility for screening dozens of biomarkers at once and is thought to be emerging in clinical protein diagnostics. In this paragraph, I will describe only MS and immunoassay techniques as neither MANF nor CDNF has been demonstrated to possess enzymatic activity which could be utilized in its analysis. Furthermore, as this work describes the construction of ELISAs for MANF and CDNF, the greatest emphasis is laid on this particular method.

4.2.1. Mass spectrometry

In MS, ionised molecules are identified by their mass-to-charge ratio. In theory, all proteins and their post-translational modifications can be identified by MS-based proteomics, which makes it an efficient tool for unbiased biomarker discovery (Geyer et al. 2017). However, MS in not inherently quantitative, due to issues in, for example, the ionisation process and detection. Hence, most biomarkers discovered by MS have traditionally been measured by immunoassays (Rifai et al. 2006). Further evolved MS methodologies, such as “selected reaction monitoring” with targeted selection of analytes by mass filters and addition of internal standards, enables reproducible, selective, and sensitive protein quantitation (Vidova & Spacil 2017). Thus, MS itself can be employed in biomarker diagnostics. MS, with the possibility of screening multiple analytes simultaneously, might give more specific readout of a person’s health state compared to the analysis of single biomarkers (Geyer et al. 2017). On the other hand, large data sets place challenges on data processing and analysis.

In complex biological samples, such as blood, high abundance proteins can mask the signal of low abundance analytes (Geyer et al. 2017). Therefore, samples need to be processed by, for example, fractionation or depletion of the non-interesting proteins before analysis on MS. This raises concerns whenever the analyte-of-interest can be bound to the depleted proteins: the analyte may be lost completely or if retained partially, cannot be used for quantitative analysis. In addition, pre-treatments increase variability, cost, and complexity of the analysis. Currently, no MS-based blood protein analysis is in routine clinical laboratory use, although it is used in the analysis of small molecules, such as drugs and metabolites (Geyer et al. 2017). However, diagnostic protein assays based on MS are expected as the methods develop further.
4.2.2. Immunoassays

4.2.2.1. History of immunoassays

Immunoassays comprise a variety of biochemical tests that utilize the high specificity and stability of the antigen-antibody reaction (Cox et al. 2014). They can be used in the detection of either antigens or antibodies. In common to all of these tests is that one of the reactants is attached to a solid surface, such as microtiter plates or magnetic beads, allowing for easy washing and separation of the desired analyte in a sample. A label, that is coupled to one of the reacting components, enables the detection of the analyte. Quantification of the analyte in study samples is based on the label readout of simultaneously analysed samples containing a known analyte amount.

The first immunoassay was described in 1960 by scientists Solomon Berson and Rosalyn Yalow for the measurement of insulin in human plasma (Yalow & Berson 1960). The assay was based on the competition of plasma insulin over radiolabeled purified insulin for the attachment to an insulin antibody. After incubation, the ratio of bound to free radioactive insulin in the sample was measured, giving an indirect estimate of the amount of insulin in a plasma sample when compared to standards with radiolabeled insulin added with a known amount of unlabeled insulin. The method gave a Nobel price to Yalow in 1977 (Berson became deceased in 1972), denoting its important output in science.

Due to the impracticalities related to radioactive labels, such as issues with stability and safety, the immunoassay method was developed further. The cardinal change to enzyme-based detection was first described by Eva Engvall and Peter Perlmann in 1971, who named the method as enzyme-linked immunosorbent assay (ELISA). Validity of the method was demonstrated by quantification of rabbit serum immunoglobulin (Ig)G using the alkaline phosphatase label (Engvall & Perlmann 1971). In the same year, an independent report from Dutch scientists described the quantification of human chorionic gonadotropin (hCG) hormone in urine using the horseradish peroxidase (HRP) label (van Weemen & Schuurs 1971).

The most sensitive and specific immunoassay design is the so-called sandwich ELISA, which is based on the detection of an antigen by two antibodies simultaneously (Cox et al. 2014). This design was first described by Ishikawa and Kato in 1978 (Ishikawa & Kato 1978). The sandwich assay is built on a capture antibody, attached on a solid surface, and a labelled detection antibody, between which the analyte is “sandwiched” (Fig. 1 A). The detection antibody can also be unlabelled. In this case a secondary antibody with a label and specific binding of the detection antibody is required (Fig. 1 B). The increased assay specificity comes from use of two antibody preparations that recognise different epitopes on the same antigen. Increased sensitivity is achieved by the capture antibody, which enriches the analyte from a sample prior to detection with the second antibody. Sensitivity levels of low pg/ml...
concentrations are consistently achievable with sandwich ELISA format using conventional enzymatic labels such as HRP (Rifai et al. 2006). Sensitivity can often be increased by fluorescent or chemiluminescent labels. With further modifications to the detection method, even fg/ml-ranges of sensitivity can be reached, as in the case of single-molecule ELISA, where isolated analyte-antibody complexes are detected individually, allowing signal readout from single labelled immunocomplexes (Rissin et al. 2010).

![Diagram of ELISA](Image)

**Figure 1**  A schematic representation of a sandwich ELISA based on A) direct measurement of the labelled detection antibody, or B) indirect measurement of the bound detection antibody by a labelled species-specific secondary antibody with no reactivity to the capture antibody. Variable region is the antigen-binding region of an IgG molecule. Constant region is identical in all antibodies of the same class within a species, i.e., IgA, IgD, IgE, IgG, or IgM.

### 4.2.2.2. Usage of immunoassays

Since the invention of the ELISA technique in the 70’s, it has become a widespread tool used in both academia and clinical diagnostics (Twyman 2005). The advantages of ELISA include high sample throughput, sensitivity, and specificity, which allows analysis to be performed on complex protein matrices, such as serum, without pre-treatments. Furthermore, the ELISAs used in research can usually be transferred into rapid and automated clinical laboratory tests for efficient and robust analysis of large sample amounts. On the downside, ELISAs are rather costly and laborious to develop and the development is limited by the availability of high-quality antibodies (Geyer et al. 2017). In addition, due to the inherent nature of antibodies, the assays are prone to cross-reactions with other proteins, to background from matrix components, and to a non-linear analyte response known as the hook effect. Although there is the possibility for multiple simultaneous analyte detection by multiplex immunoassays (Tighe et al. 2015), ELISA does not suit well for large proteomics studies.

Examples of ELISA-based clinical laboratory tests include assays for infectious diseases (such as salmonella typhi IgG, human immunodeficiency virus antigen/antibody), hormones (such as C-peptide, insulin), cancer mark-
ers (such as prostate specific antigen), and cardiac markers (such as troponin) (U.S. Food and Drug Administration 2018). In addition, the immunoassay format is used in simple home tests, such as pregnancy tests, for the measurement of hCG in urine (Bioplus 2017). The test is built on a capture antibody bound on the test strip and a freely moving detection antibody labelled with a dye. When the strip is merged into the sample matrix, it works as a chromatography paper to carry solutes through the strip. If the sample contains hCG, it is bound to the detection antibody and the complex is run through the test strip until it reaches the stationary capture antibody. When a sufficient amount of detection antibody is retained on the same place, the dye particles form a colour band visible to the eye. Although the intensity of the formed colour is relative to the bound hCG, the test is primarily qualitative rather than quantitative which, however, is usually sufficient for its intended use.

4.2.2.3. **ELISA development and validation**

Antibody selection is an essential part of the ELISA development, as the binding affinity of the antibody to the antigen in big part determines the sensitivity and dynamic range of the assay (Cox et al. 2014). The antibody pair used in a sandwich ELISA has to recognise two non-overlapping epitopes in the antigen. In addition, binding of the antigen to the capture antibody should not cause steric hindrance for the detection antibody or alter the epitope recognised by it. Both polyclonal and monoclonal antibodies can be used as the capture and detection antibody in sandwich ELISA. Often the best result is achieved with the detection of multiple epitopes by a polyclonal antibody, used for more robust pull down of the analyte, while the detection of a single epitope by a monoclonal antibody adds specificity to the assay (Crowther 2001). Nevertheless, monoclonal antibodies are favoured because they can be produced theoretically infinitely with no changes in the recognised epitope.

The main objective of validation is to demonstrate the fitness of the developed assay to its intended use (Cox et al. 2014). Results obtained from a validated assay can be considered meaningful, reliable, and reproducible. In addition, validation may reveal limitations for the assay usage. Validation comprises several tests for the evaluation of critical assay performance parameters. Relevant parameters for a quantitative assay validation are: the calibration curve, accuracy, precision, sensitivity, specificity, and stability of the analyte in samples (DeSilva et al. 2003). Validation of the calibration curve includes determination of lower and upper quantification limits, defining the assay dynamic range where the analyte can be quantitated reliably. Accuracy describes the closeness of the results to the true values, while precision refers to reproducibility of the results. Specificity of ELISA is defined as the ability to measure the analyte without interference from other components in a sample (Smolec et al. 2005). Even though the guidelines often define thresholds for the validation parameters, it is more important to justify that the validation results are appropriate for the intended use of the assay (Smolec et al. 2005).
4.2.2.4. Matrix interference on ELISA

Biological samples contain factors that may interfere with immunoassays by, for example, binding to the assay components, or masking the analyte from the assay antibodies, thus leading to wrong results (Sturgeon & Viljoen 2011). Matrix effect can be evaluated by studying the linearity of diluted samples. The idea is that the interfere, caused by factors having possibly different affinity to the assay than the antigen, or exhibiting multivalency, will be revealed by serial sample dilutions while keeping the assay reagent amounts constant (Ismail 2007). The results may, however, be misleading as some interfering factors give linear responses upon dilution. On the contrary, some analytes do not show linearity upon dilution. Calculating the recovery of added analyte in the sample is another way to study matrix interference (Sturgeon & Viljoen 2011).

In particular, blood samples cause matrix effects on ELISA. Antibodies that bind animal immunoglobulins are present in human blood circulation, and therefore can cause interference in assays built on animal Ig-preparations (Bolstad et al. 2013). Interfering antibodies can be divided into two classes based to their specificity and origin: 1) heterophilic antibodies (HA) and 2) human anti-animal immunoglobulin antibodies (HAIA) (Kaplan & Levinson 1999). HAs react with antibodies of various species with low avidity (Levinson & Miller 2002). They are considered a natural step in the maturation of antibodies against specific epitopes, and thus, could be found in everyone. HA titers are especially high in certain disease states. For example, high HA titers are found in patients with mononucleosis, a feature that has been employed in the diagnosis of this disease. Also, rheumatoid factor can be counted as a HA. Even though the IgM-type antibodies are primarily against human IgG, they also recognise animal IgG (Kaplan & Levinson 1999).

In spite of their high prevalence, most HAs will not create problems in immunoassays due to their rather low avidity. Instead HAIA, which are developed as a result of immunisation, bind only one antigen with high avidity and can be present in the blood circulation at high titers (Kricka 1999). Consequently, they can interfere in the immunoassay with high intensity. The prevalence of human anti-mouse antibodies is probably highest of all HAIA. One reason for this may be the immunisation against mouse monoclonal antibodies used in therapeutics and diagnostic imaging.

On a sandwich ELISA, the effect of interfering antibodies may cause either falsely high or low results depending on the nature of the interference (Sturgeon & Viljoen 2011). Falsely low results may occur upon interfering antibody attachment to the capture antibody causing steric hindrance for the binding of the analyte or detection antibody (Fig. 2 A). Falsely high results may originate from the bridging the two assay antibodies together by interfering antibody without the presence of the actual antigen (Fig. 2 B). When the capture and detection antibodies originate from the same species, most often mouse mAb, they are likely to share common epitopes, thus, the chance for antibody interference increases (Bolstad et al. 2013). However, due to the nature of HAs, interference is also possible on assays built on antibodies...
originating from two species. As well, despite the preceding blocking by an unspecified protein, interfering antibodies may attach to the assay solid surface unspecifically and capture the sequential detection antibody. In a research context, the interference produces confusing results and false conclusions due to the stochastic occurrence of the interfering antibodies. Instead, in clinical laboratories false assay results can have detrimental effects on individuals when leading to incorrect diagnoses and treatments (Cole et al. 1999).

Neutralising the assay-interfering antibodies can be achieved by the addition of unspecific “blocker” immunoglobulins (Bolstad et al. 2013). The idea is that the blocker antibodies, preferably originating from the same species as the assay antibodies, adsorb the reactive antibodies prior to adding the sample on a capture antibody coated plate. This can be achieved either by the addition of non-immune animal serum or purified animal antibodies. Commercial blockers are mostly based on reactive antibodies. For example, commercially available immunoglobulin inhibiting reagent (IIR) is a mixture of antibodies produced against human heterophilic and anti-mouse antibodies in mice (Reinsberg 1998). Another possibility is to deplete the interfering antibodies from the sample by e.g., affinity extraction by protein A/G, antibody precipitation by propylene glycol, or size exclusion when the analyte’s molecular weight is considerably different from the Igs (Bolstad et al. 2013). However, the antibody depletion methods all involve extensive sample processing, which can affect the analyte concentration, or its availability for the assay antibodies. Since most interfering antibodies bind to the tail region (Fc) of the antibody (Bolstad et al. 2011), the interference can be prevented by enzymatic digestion of the assay antibodies by papain or pepsin and using only the antigen-binding fragments (Fab’ or F(ab’)_2) (Vaidya et al. 1992). Alternatively, antibody fragments can be produced by recombinant protein technology (Ma & O’Kennedy 2017).

![Figure 2](image-url) Interfering antibodies, attaching to the assay IgGs can lead into either A) false negative results, when by binding to the capture antibody, cause steric hindrance for binding of the analyte or detection antibody, or B) false positive results by cross-binding the two assay antibodies even if the analyte molecule is not present. Fab’ = fragment antigen-binding, Fc = fragment crystallisable.
AIMS OF THE STUDY

The aim of this study was to develop tools for the specific and sensitive quantification of MANF and CDNF proteins. The developed immunoassays were used to measure MANF and CDNF protein levels in biological samples related to type 1 diabetes in humans and gene therapy in animal models of Parkinson’s and Alzheimer’s disease. In addition, this study concentrated on establishing CDNF secreting cell clones. Efficient secretion of the therapeutic protein is essential when considering possible cell-based therapies for CDNF delivery into the central nervous system. The developed ELISAs were used to quantify the amount of secreted CDNF from the clones.

The aims of the study can be divided into three sections:

1) To develop, optimise, and validate sandwich ELISAs for sensitive and specific quantification of human and mouse MANF and CDNF (I-IV)

2) To use the developed ELISAs for the analysis of endogenous MANF concentration in human serum in relation to T1D (I) and CDNF transgene expression levels after viral gene delivery to rodent brain modelling Parkinson’s (II) and Alzheimer’s disease (III), as well as for the quantification of secreted CDNF from cell clones grown on culture plates or in polymeric microcapsules (IV)

3) To optimise CDNF coding sequence for improved secretion from cell clones, aiming at efficient delivery of CDNF by encapsulated cell therapy (IV)
MATERIALS AND METHODS

The main methods listed in Table 3 and explained in this chapter are the ones that I have personally used for this thesis. Detailed descriptions of all the used methods are presented in the original publications (I-III) and in the manuscript (IV). Unpublished methods are presented more closely in this chapter.

<table>
<thead>
<tr>
<th>Method</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunological methods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandwich ELISA</td>
<td>Design, optimisation, validation, sample analysis</td>
<td>I-IV, u</td>
</tr>
<tr>
<td>Multiplex immunoassay</td>
<td>Magnetic-bead based multiplex assay</td>
<td>I</td>
</tr>
<tr>
<td>Western blot</td>
<td>Tissues, <em>in vitro</em> cell samples</td>
<td>IV, u</td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>Followed by epifluorescence or confocal microscopy</td>
<td>IV</td>
</tr>
<tr>
<td>Cell culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammalian cell lines</td>
<td>ARPE-19, HeLa, EA.hy926, SH-SY5Y, U-78 MG</td>
<td>IV</td>
</tr>
<tr>
<td>Transfection</td>
<td>Transient and stable cell clone production</td>
<td>IV</td>
</tr>
<tr>
<td>Biomaterials</td>
<td>Cell encapsulation in cross-linked alginate</td>
<td>IV</td>
</tr>
<tr>
<td>Viability assays</td>
<td>Calcein AM/ethidium homodimer-1 staining and resazurin test</td>
<td>IV</td>
</tr>
<tr>
<td>DNA techniques</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer design, PCR</td>
<td>Transfection vector construction</td>
<td>IV</td>
</tr>
<tr>
<td>DNA sequencing analysis</td>
<td>Verification of transfection vector sequence</td>
<td>IV</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td>Fractionation of human serum</td>
<td>u</td>
</tr>
</tbody>
</table>
5. Development, optimisation, and validation of MANF and CDNF ELISAs

During the ELISA development process, several antibodies were tested in pairs for their ability to detect recombinant human (h) and mouse (m) MANF or CDNF protein. A summary of the antibody pair testing for pAb hCDNF ELISA is presented in Table 4. The antibody pairs that showed a good concentration-response curve for the analyte in the initial screenings were further optimised for improved detection. Optimised features included antibody dilutions, incubation temperature and time, blocking and sample buffers. The optimal conditions were determined empirically. Validations of the ELISAs were done as described (I). Validation included the determination of assay dynamic range, sensitivity, specificity, and intra- and interassay reproducibility. Matrix effect was analysed by quantitating the analyte recovery after sample dilution and the recovery of added recombinant protein. The antibodies and concentrations used in the final ELISA kits are listed below.

1) hMANF: Goat anti-hMANF pAb, 1 μg/ml, R&D Systems (AF3748), & Mouse anti-hMANF-HRP mAb, 1 μg/ml, Icosagen (908-100).

2) m/hMANF: Goat anti-hMANF pAb, 2 μg/ml, R&D Systems (AF3748), & Rabbit anti-hMANF pAb, 0.5 μg/ml, LSBio (LS_B2688), & Anti-rabbit-HRP pAb, 1:2 000, GE Healthcare (NA9340V).

3) hCDNF, mAb: Mouse anti-hCDNF mAb, 1 μg/ml, Icosagen (301-100), & Mouse anti-hCDNF-HRP mAb, 1 μg/ml, Icosagen (302-100).

4) hCDNF, pAb: Goat anti-hCDNF pAb, 1 μg/ml, R&D Systems (AF5097), & Rabbit anti-hCDNF pAb, 0.1 μg/ml, (Lindholm et al. 2007), & Anti-rabbit-HRP pAb, 1:2 000, GE Healthcare (NA9340V).

5) mCDNF: Goat anti-mCDNF pAb, 1 μg/ml, R&D Systems (AF5187), & Rabbit anti-hCDNF pAb, 0.2 μg/ml, (Lindholm et al. 2007), & Anti-rabbit-HRP pAb, 1:2 000, GE Healthcare (NA9340V).

5.1. Calibration of standards

The following recombinant proteins were used in the standard curves of the developed ELISAs: human MANF (P-101-100, Icosagen) in hMANF and m/hMANF ELISAs, human CDNF (P-100-100, Icosagen) in mAb and pAb hCDNF ELISAs and mouse CDNF (5187-CD, R&D Systems) in mCDNF ELISA. Human MANF and CDNF proteins were produced in the Chinese hamster ovary (CHO) cell line. Mouse CDNF protein was produced in a mouse myeloma cell line.

Concentrations of recombinant proteins were determined by modified Lowry method (DC Protein Assay, Bio-Rad) and BCA protein assay kit (Thermo Fisher) using bovine IgG as standard. Additionally, NanoDrop™ spectrophotometer was used in the determination of protein concentration by absorbance at 280 nm taking into account the calculated molar extinction coefficients and molecular weights of the purified proteins (Table 5).
Table 4.  Antibody-pair testing for hCDNF ELISA. Altogether four anti-hCDNF antibodies were tested in different combinations. Combination of goat (R&D Systems) and rabbit anti-hCDNF (Lindholm et al. 2007) antibodies showed a good concentration-response curve and sensitivity in the first screening.

<table>
<thead>
<tr>
<th>Capture ab</th>
<th>Detection ab</th>
<th>Secondary ab</th>
<th>Standard curve</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit pAb, in-lab</td>
<td>mouse mAb, Enzo Life Sci.</td>
<td>HRP-linked anti-mouse</td>
<td>No slope</td>
<td>Low background</td>
</tr>
<tr>
<td>rabbit pAb, Storkbio</td>
<td>mouse mAb, Enzo Life Sci.</td>
<td>HRP-linked anti-mouse</td>
<td>No slope</td>
<td>Low background</td>
</tr>
<tr>
<td>mouse mAb, Enzo Life Sci.</td>
<td>rabbit pAb, in-lab</td>
<td>HRP-linked anti-rabbit</td>
<td>No slope</td>
<td>Low background</td>
</tr>
<tr>
<td>mouse mAb, Enzo Life Sci.</td>
<td>rabbit pAb, Storkbio</td>
<td>HRP-linked anti-rabbit</td>
<td>No slope</td>
<td>Low background</td>
</tr>
<tr>
<td>mouse mAb, Enzo Life Sci.</td>
<td>goat pAb, R&amp;D Systems</td>
<td>HRP-linked anti-goat</td>
<td>No slope</td>
<td>High background (Abs&gt;1)</td>
</tr>
<tr>
<td>rabbit pAb, Storkbio</td>
<td>goat pAb, R&amp;D Systems</td>
<td>HRP-linked anti-goat</td>
<td>No slope</td>
<td>Low background</td>
</tr>
<tr>
<td>rabbit pAb, in-lab</td>
<td>goat pAb, R&amp;D Systems</td>
<td>HRP-linked anti-goat</td>
<td>Slight slope</td>
<td>High background (Abs&gt;0.9)</td>
</tr>
<tr>
<td>goat pAb, R&amp;D Systems</td>
<td>mouse mAb, Enzo Life Sci.</td>
<td>HRP-linked anti-mouse</td>
<td>Linear slope,</td>
<td>Abs. 0.082 to 2.239 for CDNF conc. of 0 to 5 000 pg/ml</td>
</tr>
<tr>
<td>goat pAb, R&amp;D Systems</td>
<td>rabbit pAb, Storkbio</td>
<td>HRP-linked anti-rabbit</td>
<td>Linear slope,</td>
<td>Abs. 0.191 to 2.032 for CDNF conc. of 0 to 5 000 pg/ml</td>
</tr>
<tr>
<td>goat pAb, R&amp;D Systems</td>
<td>rabbit pAb, in-lab</td>
<td>HRP-linked anti-rabbit</td>
<td>Linear slope,</td>
<td>Abs. 0.294 to 2.679 for CDNF conc. of 0 to 5 000 pg/ml/ml</td>
</tr>
</tbody>
</table>

Table 5.  Extinction coefficients (εM) and molecular weights used for the determination of recombinant protein concentrations by NanoDrop™. Nanodrop™ applies the Beer-Lambert equation of Absorbance = εM (l/mol*cm) * path length (cm) * protein concentration (mol/l) for the calculation of protein concentration (mol/l) based on absorbance and molar absorbity and gives the result as weight per volume (g/l) according to the molecular weight (g/mol) of the protein. The εM was calculated as: N_{tryptophanes} * 5500 + N_{tyrosines} * 1490 + N_{disulphide bonds} * 125 (Pace et al. 1995). The correctly folded proteins contain four intra-molecular disulphide bonds (Parkash et al. 2009). Recombinant mouse CDNF consists of amino acids Leu27 – Leu187 and a C-terminal 10-His tag, as described by the manufacturer.

<table>
<thead>
<tr>
<th></th>
<th>N_{tryptophanes}</th>
<th>N_{tyrosines}</th>
<th>N_{disulphide bonds}</th>
<th>εM (l/mol*cm)</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MANF (Icosagen)</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>16 430</td>
<td>18.17</td>
</tr>
<tr>
<td>Human CDNF (Icosagen)</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>14 940</td>
<td>18.32</td>
</tr>
<tr>
<td>Mouse CDNF (R&amp;D Systems)</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>21 930</td>
<td>19.73</td>
</tr>
</tbody>
</table>
6. Biological samples

6.1. Study samples
The constructed ELISAs were used for the analysis of MANF and CDNF levels in various biological samples (Table 6). hMANF ELISA was used for the quantification of MANF in human serum samples from T1D patients (I). hMANF ELISA was also used in the analysis of endogenous MANF concentration in the cell lysates and conditioned media of several cell lines of human origin (IV). pAb hCDNF ELISA was used in the quantification of hCDNF levels in rodent brain after viral gene delivery in Parkinson’s disease (II) and Alzheimer’s disease (III) model settings. pAb and mAb hCDNF ELISAs were used in the quantification of overexpressed hCDNF in the ARPE-19 cell line after transient transfection and in the stable cell clones (IV) and for the quantification of endogenous CDNF in various cell lines of human origin (IV). Sample details are discussed in the original publications (I-III) and manuscript (IV). In addition, several mouse and human tissues were used in the analysis of endogenous MANF and CDNF levels using respective ELISAs. The same tissues were analysed on reducing western blots for comparison with ELISA.

All experimental procedures involving humans or animals adhered to the principles of the Declaration of Helsinki (I) or directive from European Communities Council (86/609/EEC) (II-III), respectively, and the study protocols were approved by the local Ethical Committees for Research (I-III).

6.2. Optimisation of tissue lysate preparations
Three different lysis buffers were tested for the most efficient extraction of endogenous CDNF in tissue samples as analysed by mCDNF ELISA. The compositions of the buffers are presented in Table 7. Recovery of recombinant mouse CDNF, added to the lysis buffer simultaneously with a tissue sample, was analysed for the investigation of the analyte stability and detectability in the chosen lysate buffer and lysis protocol.

Short-time acidification of tissue lysate has been reported to enhance the detection of some proteins, such as NGF (Zettler et al. 1996) and GDNF (Okragly & Haak-Frendscho 1997), possibly by dissociating from their binding partners, thus liberating the analyte for detection in ELISA. The effect of low pH on the detectable CDNF and MANF concentrations was evaluated by adding 1M HCl to an aliquot of sample in order to decrease the pH<2 for 30 minutes, followed by re-neutralisation with 1M NaOH. Parallel aliquots were supplemented with similar volumes of water to equalise sample volumes.
Table 6. Samples analysed by the constructed ELISAs and included to the thesis. Sample replicates are indicated as n. T1D = type 1 diabetes, 6-OHDA = 6-hydroxydopamine, PD = Parkinson’s disease, AAV = adeno-associated virus, APP = amyloid precursor protein, PS1 = presenilin-1, AD = Alzheimer’s disease, ctrl = control, u = unpublished.

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Analysed for</th>
<th>Sample details</th>
<th>n/ctrl n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1D serum</td>
<td>Endogenous MANF</td>
<td>Recent onset and long-term patients</td>
<td>206/225</td>
<td>I</td>
</tr>
<tr>
<td>Human tissues</td>
<td>Endogenous MANF &amp; CDNF</td>
<td>Testing / assay validation</td>
<td>2</td>
<td>u</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-OHDA model of PD</td>
<td>hCDNF transgene expression</td>
<td>Titer and time-dependent hCDNF expression in striatum (site of AAV-injection) and substantia nigra</td>
<td>28/18</td>
<td>II</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP/PS1 model of AD</td>
<td>hCDNF transgene expression</td>
<td>hCDNF expression in hippocampus (site of AAV-injection) and cortex</td>
<td>34/33</td>
<td>III</td>
</tr>
<tr>
<td>Mouse tissues</td>
<td>Endogenous MANF &amp; CDNF</td>
<td>Testing / assay validation</td>
<td>6</td>
<td>u</td>
</tr>
<tr>
<td>Cell culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell clones</td>
<td>Overexpressed CDNF</td>
<td>Cell lysate, medium</td>
<td>23</td>
<td>IV</td>
</tr>
<tr>
<td>Cell lines</td>
<td>Endogenous MANF &amp; CDNF</td>
<td>Cell lysate, medium</td>
<td>5</td>
<td>IV</td>
</tr>
</tbody>
</table>

Table 7. Composition of the tested lysis buffers. The tested lysis buffers included “High salt” and “High salt and high detergent” buffers optimised for NGF extraction (Zettler et al. 1996), and a lysis buffer described in Promega GDNF Emax® ImmunoAssay System manual (Technical bulletin no. 221). BSA = bovine serum albumin. Protease inhibitor cocktail from Roche (04693159001). BSA = bovine serum albumin, NaN₃ = sodium azide, EDTA = ethylenediaminetetraacetic acid, Na₃VO₄ = sodium orthovanadate.

<table>
<thead>
<tr>
<th>Name</th>
<th>Buffer/salt</th>
<th>Detergent</th>
<th>Stabilisation</th>
<th>Preservative</th>
<th>Chelator</th>
<th>Protease inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>High salt</td>
<td>100 mM Tris-HCl (pH 7.0) / 1 M NaCl</td>
<td>0.2% Triton-X</td>
<td>2% BSA</td>
<td>0.02% NaN₃</td>
<td>4 mM EDTA</td>
<td>Cocktail</td>
</tr>
<tr>
<td>High salt &amp; detergent</td>
<td>100 mM Tris-HCl (pH 7.0) / 1 M NaCl</td>
<td>2% Triton-X</td>
<td>2% BSA</td>
<td>0.02% NaN₃</td>
<td>4 mM EDTA</td>
<td>Cocktail</td>
</tr>
<tr>
<td>Promega</td>
<td>20 mM Tris-HCl (pH 8.0) / 0.137 M NaCl</td>
<td>1% Triton-X</td>
<td>10% glycerol</td>
<td>-</td>
<td>2.5 mM EDTA</td>
<td>Cocktail + 0.5 mM Na₃VO₄</td>
</tr>
</tbody>
</table>
6.3. **Comparison of ELISA with western blotting**

Human and mouse tissue lysates were analysed on western blot (WB) in order to compare the ELISA results with another method. The lysates (50 μg) were reduced (5% β-mercaptoethanol), boiled (95°C, 5 min), and separated on SDS-PAGE together with recombinant proteins. Proteins were blotted on nitrocellulose membranes and incubated with rabbit anti-CDNF (0.4 μg/ml, Lindholm et al. 2007) for staining of human and mouse CDNF, with goat anti-hMANF (0.5 μg/ml, R&D Systems) for human MANF, rabbit anti-MANF (1 μg/ml, Icosagen) for mouse MANF, and mouse anti-β-actin or mouse anti-α-tubulin (both from Sigma), for staining the reference proteins. Following incubation with an appropriate HRP-linked secondary antibody, bound antibodies were visualised by ECL Western Blotting Substrate kit (Pierce).

6.4. **Detection and identification of background interference in human sera**

When analysing human serum samples by hMANF ELISA, we occasionally observed extremely high absorbance results in some individuals (I, Fig. 1 B). For studying MANF in serum samples more closely, its elution profile was analysed in size exclusion chromatography in two serum pools from three individuals each. A total of 300 μl serum was supplemented with a protease inhibitor cocktail (Roche), filtered through 0.45 μm Whatman filter, loaded to HiLoad 16/60 Superdex 200 column (GE Healthcare) and eluted with 120 ml of elution buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0). Fractions of 2 ml were analysed for absorbance at 280 nm for the detection of IgG and albumin, and by hMANF ELISA for detection of immunoassay reactivity. The fractions showing a response peak in ELISA were pooled and concentrated 80-fold using Amicon Ultra centrifugal filters with a 10 kDa cut-off. The concentrated and pooled fractions were analysed on WB as described in chapter 6.3 (Comparison of ELISA with western blotting).

Fractions showing a response in hMANF ELISA overlapped with the elution peak of IgG. This raised our suspicion of an immunoglobulin interference in the immunoassay. The possible interference was studied by a control ELISA, as described (I). The control ELISA was run similarly to the hMANF ELISA with a sole change of the coating antibody into a non-MANF recognising antibody (goat anti-hCDNF instead of goat anti-hMANF, both from R&D Systems). The control ELISA was also used for the verification of interference elimination by a commercial blocking agent (IIR).
7. Generation of CDNDF secreting cell clones for encapsulated cell biodelivery

The third aim of the thesis was to generate CDNDF secreting ARPE-19 cell clones for encapsulated cell biodelivery purposes, and to test CDNDF secretion in 2D-cultures and after microencapsulation, as described (IV). The human-derived ARPE-19 cell line was chosen as the parental cell line because its use in clinical applications is approved by regulatory authorities (U.S. Food and Drug Administration, FDA, and European Medicines Agency, EMA).

Since low CDNDF secretion was observed from the produced clones expressing wild type CDNDF, transfection plasmids with modified CDNDF coding sequences were prepared (Table 8). CDNDF expression and secretion from the new constructs was analysed after transient transfection and from stable cell clones, as described (IV).

<table>
<thead>
<tr>
<th>Construct</th>
<th>pCI-neo</th>
<th>pCR3.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human wild type CDNDF</td>
<td>(Galli 2009)</td>
<td>(Lindholm et al. 2007)</td>
</tr>
<tr>
<td>KTEL-deleted human CDNDF</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Codon optimised human CDNDF</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Codon optimised + KTEL-deleted human CDNDF</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

8. Reliability and validity of ELISA results

8.1. ELISA validation (I-IV, unpublished)

The development of ELISAs originated from the need for a reliable method for CDNF and MANF quantification. No such methods were on the market at the time of starting this work. A commercial mouse MANF ELISA tested later (SEC300Mu, Wuhan USCN Business Co., lot L150401046) gave positive results for \( \text{Manf}^- \) tissue lysates, further emphasising the importance and value of stringent validation of the tools used in research. A summary of validation results of the developed ELISAs is given in Table 9.

8.1.1. Analysis of recombinant proteins used in the standard curves (unpublished)

Quantitation of an analyte in a sample by ELISA is based on a standard protein of known concentration. Differences in the folding and post-translational modifications between the analyte in a biological sample and the recombinant protein used in the standard curve, may result in differences in antibody detection and consequently false quantification results (Twyman 2005). Thus, the quality and proper determination of the standard curve protein concentration is the foundation of the whole assay (Porstmann & Kiessig 1992).

Correctly folded MANF and CDNF contain four intramolecular disulphide bonds (Parkash et al. 2009). In this study, all the proteins used in standard curves of the developed assays originated from mammalian cells, which are known to be able to form disulphide bonds efficiently. However, different mammalian cells may produce slightly different glycan structures. Human CDNF has two confirmed glycosylation sites (Apostolou et al. 2008, Sun et al. 2011). It has not been studied whether hamster cells, in which the used recombinant hCDNF was produced, are able to glycosylate human CDNF. On the other hand, it is unknown at what extent human CDNF is glycosylated in vivo, and thus, whether endogenous CDNF differ from the recombinant.

We did not rely on the given recombinant protein concentrations. Instead, we measured their concentration by different methods, which all gave consistent results, although with some variation from the concentration given by the manufacturers (Table 10). However, it should be noted that in all the methods used, protein quantification was based on the amount of tyrosine and tryptophane residues, containing aromatic structures, and to some extent on disulphide bonds. Thus, the methods are prone to give consistent results. Also, as the determination is mainly based on amino acids, possible fractionated or degraded products will also affect the protein concentration, although not necessarily detectable by the ELISA.
Table 9. Summary of the main validation results of the developed ELISAs. Within the assay dynamic range, the individual back-calculated accuracy values were within 20% relative error (RE = derived concentration / expected concentration x 100%) and precision within 15% coefficient of variation (CV = SD / mean x 100%). Precision data are averages of several independent values. The matrix effect was studied by determining the linearity of dilution and recovery of spiked analyte in all biological study matrices used. The results imply that the studied biological matrices do not interfere with the detection of MANF or CDNF by the assays and that they behave essentially similarly as the simple blocking buffers used for the dilution of standard curve samples. AAV = adeno-associated virus, n.d. = not determined. Superscripts indicate published (I-HII) and unpublished data (IV, u).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity (pg/ml)</th>
<th>Dynamic range (pg/ml)</th>
<th>Intra-assay precision (%)</th>
<th>Interassay precision (%)</th>
<th>Sample matrix</th>
<th>Linearity-of-dilution, average (range)</th>
<th>Spike-and-recovery, average (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMANF ELISA</td>
<td>45^{(i)}</td>
<td>62.5–2 000^{(ii)}</td>
<td>8.1^{(i)}</td>
<td>5.5^{(i)}</td>
<td>Human serum</td>
<td>107% (96-116), n=3^{(i)}</td>
<td>102% (94-110), n=6^{(i)}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human cell lines</td>
<td>106% (83-114), n=5^{(i)}</td>
<td>91% (90-93), n=3^{(i)}</td>
</tr>
<tr>
<td>m/hMANF ELISA</td>
<td>29^{(u)}</td>
<td>62.5–1 000^{(u)}</td>
<td>9.8^{(u)}</td>
<td>8.6^{(u)}</td>
<td>Mouse tissues</td>
<td>103% (92-114), n=3^{(u)}</td>
<td>94% (86-102), n=5^{(u)}</td>
</tr>
<tr>
<td>mAb hCDNF ELISA</td>
<td>31^{(iv)}</td>
<td>31.3–2 000^{(iv)}</td>
<td>7.1^{(iv)}</td>
<td>8.8^{(iv)}</td>
<td>Human cell lines</td>
<td>106% (81-119), n=3^{(iv)}</td>
<td>106% (98-116), n=3^{(iv)}</td>
</tr>
<tr>
<td>pAb hCDNF ELISA</td>
<td>10^{(iii)}</td>
<td>15.6–1 000^{(iii)}</td>
<td>8.2^{(iii)}</td>
<td>9.7^{(iii)}</td>
<td>AAV2-CDNF rat brain</td>
<td>104% (85-118), n=2^{(iii)}</td>
<td>93% (89-98), n=2^{(iii)}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AAV2-CDNF mouse brain</td>
<td>98% (83-111), n=3^{(iii)}</td>
<td>n.d.</td>
</tr>
<tr>
<td>mCDNF ELISA</td>
<td>6^{(iv)}</td>
<td>15.6–2 000^{(iv)}</td>
<td>10.3^{(iv)}</td>
<td>6.8^{(iv)}</td>
<td>Mouse tissues^{(iv)}</td>
<td>99% (83-114), n=5^{(iv)}</td>
<td>99% (85-114), n=4^{(iv)}</td>
</tr>
</tbody>
</table>

Table 10. Summary of recombinant human MANF and CDNF concentrations measured by different methods. The result is shown as mean±SD (ng/ml). For the preparation of reference standards, the protein concentration values analysed by NanoDrop™ were used. n.d. = not determined. Unpublished data.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Batch</th>
<th>Modified Lowry</th>
<th>BCA kit</th>
<th>NanoDrop™</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MANF (Icosagen)</td>
<td>16.11.10</td>
<td>4.06 ± 0.22 (n=4)</td>
<td>n.d.</td>
<td>3.93 ± 0.15 (n=3)</td>
<td>5.0</td>
</tr>
<tr>
<td>Human CDNF (Icosagen)</td>
<td>5.11.10</td>
<td>2.32 ± 0.13 (n=4)</td>
<td>2.41 ± 0.19 (n=5)</td>
<td>2.28 ± 0.06 (n=4)</td>
<td>4.0</td>
</tr>
<tr>
<td>Mouse CDNF (R&amp;D Systems)</td>
<td>RRU0110101</td>
<td>0.52 ± 0.06 (n=2)</td>
<td>n.d.</td>
<td>0.56 ± 0.01 (n=3)</td>
<td>0.50</td>
</tr>
</tbody>
</table>
8.1.2. **Specificity of the ELISAs (I-IV, unpublished)**

Specificity of the ELISAs was tested by purified recombinant proteins and biological samples (Table 11). For mouse MANF and CDNF ELISAs, samples from respective KO mice served as ideal controls for assay specificity. Neither serum nor several different tissue lysates from the KO mice gave signal on the mouse ELISAs, implying absolute specificity to the analyte. All the human biological samples were positive for MANF, hence, the background noise could not be analysed in a straightforward manner for hMANF ELISA. Therefore, we constructed a control ELISA to reveal unspecific background (I). Human tissue samples were negative in the control ELISA, but serum samples gave a signal. After addition of IIR, the signal was reduced in control ELISA, implying attenuation of unspecific background in hMANF ELISA also. Depletion of MANF from biological samples, by the means of e.g. affinity column, would be another alternative in the analysis of background signal in a biological sample matrix. However, as the ELISA is very sensitive, even minor remaining amounts of MANF could lead to wrong assumptions on the background.

The assays were highly selective to the homologs and species (Table 11). Despite the high amino acid sequence homology of human (sequence ID NP_006001.4) and mouse MANF (NP_083379.2, 99% mature sequence homology), mouse MANF could not be detected with hMANF ELISA, implying the mAb used in the hMANF ELISA detected a region significantly different between human and mouse MANF. In contrast, m/hMANF ELISA detected both human MANF and mouse MANF. hMANF ELISA did not detect human CDNF (NP_0.001025125.2, 63%) and mMANF ELISA did not detect mouse CDNF (NP_808315.1, 64%), or vice versa. pAb hCDNF ELISA, but not mAb hCDNF ELISA, gave a slight response to recombinant mouse CDNF (80%). mCDNF ELISA did not detect human CDNF but gave a response to rat tissue lysates (NP_001032632.1, 87%).

Due to the characteristic structure of MANF and CDNF, it is possible that the N- and C-terminal domains are separated by proteolytic cleavage of the short connecting sequence. Selective detection of either of the domains may turn out to be important in future studies imply their presence and independent actions in organisms. The hCDNF ELISA constructed on two mAbs detected only the full-length human CDNF and not the separate N- (Parkash et al. 2009) or C-terminal (produced in E.coli, 8.9.2009, M. Hellman) domains, indicating that one of the antibodies used detects the N-terminal and the other the C-terminal domain of CDNF. pAb hCDNF ELISA detected 6.1% of the C-terminal CDNF domain and 28.0% of the N-terminal domain from a 1 ng/ml concentration. Other purified C- and N-terminal domains, beside those of human CDNF, were not available for the comparative analysis. The epitope-reactivity of the antibodies used in the rest of the constructed ELISAs could be studied on peptide arrays, implicative of domain recognition.
Table 11.  Tested cross-reactivities of the assays to structural homologs. wt=wild type. Superscripts indicate published (HII) and unpublished data (IV, u).

<table>
<thead>
<tr>
<th>Homolog</th>
<th>Source</th>
<th>Human MANF</th>
<th>Mouse MANF</th>
<th>Human CDNF</th>
<th>Mouse CDNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Recombinant (Icosagen)</td>
<td>Mouse tissue lysates &amp; serum</td>
<td>Recombinant (Icosagen)</td>
<td>Recombinant (R&amp;D Systems) &amp; mouse tissue lysates &amp; serum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Sample</th>
<th>Acid treated (pg/ml)</th>
<th>Non-acid treated (pg/ml)</th>
<th>Acid treated vs. non-acid treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMANF</td>
<td>Human lung</td>
<td>351.4</td>
<td>341.8</td>
<td>103%</td>
</tr>
<tr>
<td>m/hMANF</td>
<td>Mouse pancreas</td>
<td>11 082.9</td>
<td>11 016.0</td>
<td>101%</td>
</tr>
<tr>
<td>mAb hCDNF</td>
<td>Human brain</td>
<td>51.4</td>
<td>49.0</td>
<td>105%</td>
</tr>
<tr>
<td>pAb hCDNF</td>
<td>Mouse brain</td>
<td>1 357.0</td>
<td>1 311.0</td>
<td>104%</td>
</tr>
<tr>
<td>mCDNF</td>
<td>Mouse brain</td>
<td>500.0</td>
<td>504.0</td>
<td>99%</td>
</tr>
</tbody>
</table>

Table 12.  pH treatment did not affect the detectable MANF or CDNF concentration in tissue lysates. The pH in the acid-treated samples was lowered below 2 for 30 minutes, after which the samples were re-neutralised. Water was added to the non-acid treated sample to equalise sample volume. Unpublished data.
8.1.3. Efficient analyte recovery from tissue homogenates (unpublished)

Three different lysis buffers were tested. Both high salt buffers showed precipitation of salt, and BSA used in these buffers interfered with the total protein analysis of the lysates. In addition, the “High salt and high detergent” buffer caused problems in sample processing due to excessive lathering. “High salt buffer” gave a background in KO mouse tissues. Instead, KO tissues lysed with “Promega” lysis buffer were negative in mCDNF ELISA, whereas, complementary wild type tissues were positive. Thus, the “Promega” lysis buffer was chosen for the following experiments. Recovery of spiked CDNF, applied at lysis, was 80% with the chosen buffer. pH treatment did not affect the detection of MANF or CDNF in the ELISAs (Table 12).

8.1.4. ELISA and western blot results are comparable (unpublished)

Both MANF and CDNF were detectable in various mouse and human tissue lysates as analysed by the ELISAs (data not shown). The presence of MANF and CDNF in the tissue lysates was confirmed by WB (Fig. 3 A & B). The quantitative ELISA results were in line with WB stainings, as compared to the recombinant protein stained on the same blot. Also, the differences in MANF and CDNF concentrations obtained by ELISA were evident on WB.

Unfortunately, the MANF concentration differences in blood samples could not be confirmed on WB despite multiple attempts. The two major blood protein fractions, albumin and IgG, caused background and loading problems on WB. Even with specific removal of both fractions by ProteoPrep® immuno-affinity devices (Sigma-Aldrich), the total protein concentration in samples remained too high to load sufficient sample volume for MANF detection. In addition, minor remains of IgG caused unspecific antibody binding on the membranes at 25 kDa (IgG light chain), which is close to the size of MANF.

![Figure 3](https://example.com/figure3.png)

**Figure 3** WB membranes with A) mouse and B) human tissues. The amount of MANF or CDNF in the loaded sample (ng / 50 μg total protein), based on ELISA results, is presented under each band. The band observed in the pancreas tissue lysate, when stained with anti-CDNF antibody, was unspecific as a similar band was found in a pancreas lysate of Cdnf<sup>−/−</sup> mouse (data not shown). For recombinant proteins M=MANF, C=CDNF (ng/lane). n.d.=not determined. Unpublished data.
8.2. Background in human serum samples

8.2.1. 150 kDa fractions give signal in hMANF ELISA (unpublished)

Human serum samples showed highly variable results in hMANF ELISA. To study more closely what the ELISA detected, molecules in human serum were separated by their size and the size fractions were analysed by the ELISA. The elution profile of the two major serum protein components, IgG (~150 kDa) and albumin (67 kDa), estimated by absorbance at 280 nm, were similar to those reported by the column manufacturer (GE Healthcare 2000) (Fig. 4 A), and were used as crude molecular size indicators in the experiment.

In both of the analysed serum mixes, hMANF ELISA gave positive signal at fractions 86 to 94 ml, eluting slightly before the theoretical peak of the 17 kDa myoglobin (Fig. 4 A). An additional peak overlapped the elution peak of IgG. Fractions around the peaks were pooled and analysed on WB. Both the staining with analyte specific antibody followed by HRP-linked secondary antibody (Fig. 4 B), or the staining with the secondary antibody alone (Fig. 4 C), showed band smears on WB at 50 to 150 kDa in pooled fractions containing IgG. The results indicated unspecific antibody detection leading to the suspicion that human serum IgG could give background in hMANF ELISA. An additional <20 kDa band was found in the 150 kDa protein fraction (Fig. 4 B), but whether MANF was present in this fraction was not studied further.

![Figure 4](image_url)

**Figure 4** Gel filtration of two human serum mixes. A) Fractions eluting at 86-94 ml gave signal in hMANF ELISA. Additional signal was found at fractions 60-68 ml overlapping IgG elution peak. *Elution of IgG (~60 to 69 ml), albumin (~72 to 78 ml), and myoglobin (~90 to 99 ml) reported by the column manufacturer. Pooled, concentrated and reduced fractions were analysed on WB and stained with B) goat anti-MANF followed by secondary antibody or C) the secondary anti-goat antibody only. Arrow indicates theoretical migration of MANF. Unpublished data.
Presence of MANF in the fractions eluting slightly before 17 kDa proteins was confirmed by WB (Fig. 4 B, arrow). The MANF antibody also recognised bands of roughly size 55, 90, and 125 kDa, although such high molecular weight proteins were unexpected in these fractions. It is unknown whether the higher molecular weight bands consisted of MANF, aggregated at the concentration step, or whether the bands occurred due to unspecific detection of another protein. A subtle 55 kDa band was also evident when staining with the secondary antibody alone, implicative of unspecific detection (Fig. 4 C).

Finding MANF in the approximately 20 kDa protein fractions imply that MANF eluted as an unbound monomer. However, the result should be verified on a higher resolution gel filtration, as it is possible that circulating MANF could be bound to a molecule of small weight, such as sulfatides (890 g/mol) (Bai et al. 2018), leading to minor size shift undetectable with the column used. Moreover, putative MANF complexes may be masked from the ELISA. To study possible complexation of MANF in human serum, the eluted fractions should be studied systematically on, for example, WB or MS.

### 8.2.2. Control ELISA reveals background in human sera (I, unpublished)

There were two possibilities for the observed reactivity of 150 kDa serum fractions in hMANF ELISA: either MANF was present in a complex of 150 kDa, or the fractions gave background on the immunoassay due to HA interference. For the evaluation of possible background, a control ELISA was constructed using goat anti-CDNF coating and mouse anti-MANF detection antibodies (I). By using a similar antibody pair (goat+mouse), the control ELISA bears equivalent possibility for the occurrence of HA interference as the original ELISA.

The 20 kDa fraction showed minimal absorbance in the control ELISA, indicative of specific detection of MANF by hMANF ELISA (Fig. 5). In contrast, the 150 kDa fraction gave signal in the control ELISA that was 64% of that observed in hMANF ELISA, suggesting considerable unspecific background. Purified human IgG (Sigma) gave similar absorbance readings in both hMANF and control ELISAs (data not shown), indicating that the background found in human serum could in fact represent non-specific antibody binding.

### 8.2.3. Efficient removal of the background caused by heterophilic antibodies (I, unpublished)

Out of 10 human sera, four gave an absorbance reading in control ELISA less than 15% of that observed in hMANF ELISA (range 0-13%), indicating low HA interference. The other six sera showed a background absorbance of 97±6% (mean±SD) from hMANF ELISA readings. Addition of a commercial HA blocker (IIR) efficiently reduced absorbance in the control ELISA, and less so in hMANF ELISA, implicative of efficient reduction of unspecific background allowing for the measurement of the actual MANF signal (Fig. 5). A concentration of 0.5 mg/ml IIR was always added when analysing human sera by hMANF ELISA. In a screen of 35 sera supplemented with IIR, a remaining
background absorbance of ≤11% (2.3±3.4%) was observed in 32 cases (I, Suppl. Table S5). This level of background can be taken as relatively small, as it falls within the normal variation of ELISA. In the remaining three samples, background level remained at 25-55%. It should be noted that all sera with a high hMANF ELISA reading (>16 ng/ml, n=21) were included to the screen, and thus verified that the signal originated from the presence of the actual analyte at high concentration rather than from unspecific background.

The control ELISA combining CDNF and MANF antibodies did not recognise either recombinant MANF or CDNF analysed separately, however, it does not rule out the possibility of detecting a theoretical MANF/CDNF heterodimer. Nevertheless, the occurrence of such is improbable, as the heterodimer would only be present in human blood circulation and not in tissues, which were negative in the control ELISA (Fig. 5). Also, the fact that a HA blocker reduced the signal in the control ELISA suggests recognition of interfering antibodies rather than a heterodimer.

![Figure 5](image_url)  
**Figure 5** Human sera gave high absorbance values in the control ELISA. Similarly, the 150 kDa serum fraction showed signal in the control assay, in contrast to the 20 kDa fraction which, however, was positive in hMANF ELISA. No background was observed in human tissue lysates. Immunoglobulin Inhibiting Reagent (IIR, Seralab) reduced the background efficiently, suggestive of background caused by heterophilic antibody interference. Figure modified from I.

8.3. Stability of MANF and CDNF in biological samples (I)

The samples were stored at -80°C and thawed before analysis. MANF showed good freeze-thaw stability in human serum (I, Fig. 1 C & D). After eight freeze-thaw cycles, 93.1±11.8% (n=8) from added 500 pg/ml concentration was detected. The stability of MANF and CDNF in tissue lysates was not tested, however, added analyte during processing of the lysate gave good recovery (refer to chapter 8.1.3. Efficient analyte recovery from tissue homogenates), implying that it was not degraded at lysis. Overall, both MANF and CDNF seem to be stable proteins, and excellent stability of recombinant MANF and CDNF has been demonstrated even at +37°C (Voutilainen et al. 2011).
9. **MANF and CDN in biological samples**

9.1. **MANF is present in human blood circulation**

The average concentration of serum MANF in a population of 14.6±12.0-year-olds (n = 113) was 7.0 ± 3.1 ng/ml (0.39±0.17 nM) (I). More than three times lower human serum MANF levels (2.1±1.4 ng/ml, n=71, age 60.5±8.6 years) were reported by a research group working in China (Wu et al. 2017). It is currently unknown, whether there was an actual difference in the MANF serum concentration between the populations of the two studies because of, for example, differences in age or ethnicity. Nevertheless, the difference might occur due to technical issues, such as differences in sampling or sample processing, in the fraction of total MANF detected by the ELISAs, differential detection of the endogenous MANF compared to the recombinant protein used in standard (produced in *E.coli* in the commercial ELISA, cat no. SEC300Hu/ RPC300Hu01), or in the actual concentration of the standard.

The control population in our study (I) was matched to the T1D population by age and gender. Based on the control population, serum MANF levels are similar between genders, and age does not affect its levels (I, 1 to 51 years). However, possible diseases, therapies, or fasting status, among others, were not addressed but could affect circulating MANF levels.

The origin(s) of circulating MANF is an open question. MANF is a ubiquitously expressed protein with high amounts found especially in professional secretory tissues (Lindholm et al. 2008). Although the highest MANF levels are found in pancreas, β-cells are unlikely a major source of serum MANF, as long-term T1D patients lacking these cells had normal serum MANF levels (I, Fig. 2 C). Circulating MANF could even originate from blood cells, such as leucocytes (Chen et al. 2015). Our preliminary studies indicate that MANF levels in serum and plasma are similar (unpublished data). Thus, it is improbable that serum MANF would derive to a great extent from activated platelets at blood coagulation in contrast to, for example, brain-derived neurotrophic factor, BDNF (Fujimura et al. 2002).

As well, the role of circulating MANF is currently unknown. MANF could have a functional role in serum, similar to serum albumin or lipoproteins. However, these proteins are usually much more abundant in the plasma (>1 μg/ml), and generally have molecular size of >45 kDa, which is the cut-off for kidney filtration. On the contrary, MANF serum concentration is higher than those for cytokines and other signalling molecules (<1 ng/ml) that act locally and get diluted in the blood. The concentration of circulating MANF falls into the range of so called tissue-leakage proteins (~100 pg/ml to 100 ng/ml), characterised as intracellular proteins which can be released to the blood circulation as a result of cell death or damage (Anderson & Anderson 2002). However, vast data indicate that MANF is a secreted protein. Hence, it could be secreted to the blood as an endocrine hormone to exert it actions in distant organs, although doubtful due to MANF's ubiquitous expression.
9.2. Increased serum MANF level in newly diagnosed T1D patients (I)

Because of the clear diabetic phenotype of Manf/- mice (Lindahl et al. 2014), we were interested to find out whether MANF is associated with the pathogenesis of T1D in humans. We aimed to address this by analysing MANF in the serum of T1D patients and their non-diabetic relatives. Serum samples were chosen because they are relatively easy to obtain compared to biopsies from pancreatic islets, the actual site of disease pathology. Differently from our hypothesis drawn from Manf/- mice, where the lack of MANF caused β-cell death, children with recent onset T1D had a 54% higher average serum MANF concentration compared to the age-matched controls (Fig. 6 A). In order to find out whether increased MANF is associated to inflammation, we analysed the levels of 10 cytokines by a multiplex immunoassay in the same serum samples. Samples with extremely high MANF levels (>95% cut-off limit determined from the control group) had generally higher cytokine levels compared to samples with lower MANF levels (I, Table 4). Differences in IL-10, IL-12, and IL-13 were statistically significant at the level of \( p<0.05 \), however, after Bonferroni correction for repeated tests, none of the comparisons reached statistical significance. Bonferroni correction is criticised for being extremely conservative in the control over type I error (false positives), thereby increasing the probability for type II error (false negatives) (Perneger 1998). However, the observed differences remained insignificant even with less stringent correction methods, namely Bonferroni-Holm or Hochberg (data not shown). Despite the fact that MANF expression and secretion has been shown to increase in a human pancreatic cell line in response to cytokines that are involved in the T1D pathology (Hakonen et al. 2018), it is unsure how well circulating cytokine levels reflect the local inflammation in pancreas (Svensson et al. 2012). After all, the systemic levels are diluted and can be influenced by other inflammatory diseases as well. Hence, the insignificant comparisons observed in sera do not necessarily exclude the possibility of increased circulating MANF levels occurring due to insulitis.

**Figure 6** Changes in the circulating MANF in T1D. A) High serum MANF concentrations were observed in children with recently diagnosed T1D. The grey line indicates a 95% cut-off limit for MANF based on the non-diabetic controls (13.5 ng/ml). B) Serum MANF correlated inversely with C-peptide, indicative of functional β-cell mass (\( r_s=-0.37, p=0.001, n=84 \)). Figures from I.
In the context of the therapeutic potential of MANF in T1D, a highly interesting question is whether the observed increase in MANF was related to β-cells. To address this, we measured the circulating C-peptide, a by-product of insulin production, for a crude estimate of the functional β-cell mass. MANF and C-peptide levels correlated inversely in the population of recent onset T1D patients (Fig. 6 B), suggesting that high serum MANF was related to worse β-cell function at the diagnosis. Although the correlation does not serve as direct evidence of the relationship, it is intriguing to hypothesize that MANF was released from the remaining ER stressed β-cells strained to produce high levels of insulin due to a low level of functional β-cell mass. Currently, the best attempt to monitor β-cell dysfunction and ER stress involves the analysis of the proinsulin/C-peptide ratio, which indicates the level of immature insulin molecule release from β-cells (Mirmira et al. 2016). Another alternative for investigating whether serum MANF levels relate to β-cell ER stress would be to study serum MANF levels from Akita mice, where pancreatic UPR and upregulation of MANF has been documented (Mizobuchi et al. 2007).

Differently from children below 10 years of age, the average serum MANF concentration in 10-17-year-old patients with recent onset T1D, or in in adults with longer-term T1D, did not differ from the controls (I, Fig. 2 B & C). Interestingly, young children are more likely to go through a “honeymoon” or remission phase of disease progression, characterised by a decreased need for exogenous insulin together with increased endogenous insulin secretion (Fonolleda et al. 2017). This is achieved by partial recovery of β-cell mass and improvement of peripheral insulin sensitivity, but the underlying mechanisms of the remission are unclear. Whether MANF has a role in the remission is an important question in relation to possible disease modifying therapy. Unfortunately, we did not have the data on insulin dosage, or C-peptide levels, at later time points, for the evaluation of the probability of the “honeymoon” phase in relation to measured MANF concentration serum at diagnosis.

The finding of MANF in human serum raises the possibility to use it as a biomarker. To find out the potential of MANF as a predictive biomarker for T1D, we analysed its serum levels in non-diabetic autoantibody positive first-order relatives (siblings or parents). First-order relatives positive to islet autoantibodies are under increased risk for developing T1D (Kulmala et al. 1998). Some of the non-diabetic autoantibody positive subjects show impaired β-cell function as a sign of preclinical state of T1D (Ferrannini et al. 2010). Serum MANF level in the control groups of autoantibody-negative and autoantibody-positive subjects were comparable to each other (I, Fig. 2 A - C), suggesting that circulating MANF levels do not change at the pre-clinical state of the disease, and that circulating MANF does not have diagnostic potential in T1D. It remains to be studied, whether after disease onset MANF could be used in the analysis of treatment response aiming at more individualised therapy. As chronic β-cell ER stress is acknowledged to be important in the pathology of both T1D and T2D (Cnop et al. 2017), the assessment of reduction in ER stress by possible novel therapies can turn out to be important.
In addition to MANF levels in T1D, we studied the possibility of antibodies raised against MANF in this autoimmune disease. Only one out of 217 tested samples gave positive results on a radiobinding assay using $^{35}$S-labelled recombinant hMANF. Although raising a possibility of MANF acting as an autoantigen, the result implies that MANF is not a major autoantibody target in T1D. The low incidence of MANF autoantibodies observed also decreases the probability that MANF levels in T1D patient sera could have been influenced by MANF-specific autoantibodies when measured by ELISA. Nevertheless, the method used to study MANF autoantibodies would not reveal antibodies raised against splice variants, products of translational infidelity, or putative changes in the post-translational modifications of MANF, which could all occur in stressed β-cells during disease development (Dunne et al. 2012).

9.3. Quantification of hCDNF levels after gene delivery in animal models of Parkinson’s and Alzheimer’s disease (II, III)

The exact dose of the therapeutic protein achieved by gene delivery cannot be easily assessed or controlled, although the level of protein expression can be altered by changing the virus titer. In addition, the stability of gene expression can be limited due gene silencing, as has been demonstrated, for example, for the cytomegalovirus (CMV) promoter in rodent CNS (Gray et al. 2011). Hence, the quantification of expressed therapeutic protein is important in the interpretation of its functional outcomes.

In this study, the time- and virus titer-dependent human CDNF protein expression was studied by ELISA after AAV2-hCDNF transduction in a rat PD model (II). CDNF transgene expression was also quantified in an Alzheimer’s disease mouse model (III). The species-selectivity of the immunoassay was utilized when analysing human CDNF levels in rodent brains. At the time the samples were analysed, pAb hCDNF ELISA was the only choice for human CDNF quantification. The more specific mAb hCDNF ELISA was constructed only thereafter. Although the used ELISA detects small amounts of mouse CDNF (Table 11), the control samples devoid of human CDNF expression did not give background with the used dilutions, implying specific quantification of the human CDNF transgene in the AAV-CDNF transduced samples. The ELISA analysis of tissue lysates does not differentiate whether the expressed protein is intracellular or extracellular, or which cell types are transduced — matters that can affect the therapeutic outcome in addition to the expression level. Instead, immunocytochemistry was used for the investigation of these aspects.
9.3.1. **hCDNF expression after AAV2-hCDNF transduction is titer- and time-dependent in the rat 6-OHDA model of PD (II)**

Previous studies had indicated neurorestorative and neuroprotective effects of intracranially injected CDNF protein in rodent models of PD (3.3.1. MANF and CDNF in neurorestoration). In this study, the neuroprotective effect of CDNF gene therapy was studied in the 6-OHDA-induced PD model in rats with viral vector transduction two weeks pre-lesioning. Rat striata, transduced with AAV2-hCDNF (4.0x10⁷, 2.0x10⁸, or 1.0x10⁹ virus genome (vg)/striatum), were positive on pAb hCDNF ELISA in contrast to control samples (intact, AAV2-GFP or vehicle treated), verifying successful gene transduction and CDNF expression. The analysed CDNF concentration in AAV2-hCDNF injected side increased along with the virus titer (Fig. 7 A). Compared to 4.0x10⁷ vg, a 5-fold higher virus titer (2.0x10⁸ vg) resulted in 8-fold higher hCDNF concentration (21±7 vs. 161±142 pg/mg total protein), and a 25-fold higher virus titer (1.0x10⁹ vg) resulted in 25-fold higher hCDNF concentration (529±169 pg/mg). The results suggest a rather linear titer-dependence of the transgene expression level four weeks post-injection.

![A](image1.png)
![B](image2.png)

**Figure 7** Titer- and time-dependency of hCDNF levels in rat striatum after gene therapy. A) hCDNF levels increased along with virus titer (n=4/group). B) hCDNF levels increased with time after the transduction (n=4/group). Error bars show the SD of the mean (column horizontal line). Modified figures from II.

Human CDNF was detected already one week after transduction, and its expression levels increased up to eight weeks (Fig. 7 B). At 12 weeks, the variation between animals became very high, implying variable stability of the expression with longer time. Some hCDNF was also observed in the substantia nigra of the AAV2-hCDNF injected side with the highest virus titer, but the levels were on average 7.5–fold lower compared to striatum (II, Fig. 2 D). Whether the CDNF in substantia nigra resulted from the transport of the virus (Paterna et al. 2004) or secreted CDNF protein (Mätlik et al. 2017), is unknown. In the striatum, CDNF expression was found along the injection tract. The staining seemed mainly intracellular and co-stained with NeuN-marker, indicating CDNF expression in neurons.

Rather modest behavioural and histological effects were obtained in our study compared to another study of AAV2-hCDNF striatal delivery in a rat PD
model, where viral transduction was performed six weeks after 6-OHDA lesioning (Ren et al. 2013). Ren and colleagues reported almost complete recovery in behavioural tests at four weeks post-transduction, unlike in our study even at 12 weeks (II, Fig. 4; 10 weeks post-lesioning). In addition, Ren et al. reported 2.5-times higher number of nigral TH-positive neurons 12-weeks post-lesioning, whereas our viral vector therapy resulted in only 1.7-fold increase 10-weeks post-lesioning, compared to respective controls (II, Fig. 5 C). In both studies, hCDNF expression was driven by the CMV promoter. The used 6-OHDA amounts were similar (20 vs. 18 μg divided into two injection sites), but the injection coordinates and the rat strains differed (Wistar vs. Sprague-Dawley). Unfortunately, CDNF protein concentration was not determined by Ren and colleagues. Even the titer was not reported unequivocally, making it difficult to compare the behavioural and histological results of the two studies in respect of the achieved CDNF concentration.

It is plausible that the CDNF concentration achieved by gene delivery did not reach to the level of its highest effect in our study. In a neurorestoration study in rats with CDNF protein infusion, an inverted U-shaped dose-response curve was observed, where 3 μg daily dose was found superior to that of 1.5 μg or 4.5 μg (Voutilainen et al. 2011). For example, with 3 μg/day the observed nigral TH-positive neuron number at 14 weeks after 6-OHDA was 2.7-times higher compared to controls, whereas no change was observed with 1.5 μg. In contrast, in our study a similar U-shaped response in the behavioural or histological outcome with different titers was not observed (II, e.g. Fig. 4, 5 D).

As the calculated half-life of CDNF in rat striatum is 5.5 hours (Mätlik et al. 2017), under chronic infusion of 1.5 μg/day (1 500 ng/24h = 62.5 ng/h) CDNF would reach to 344 ng/striatum at steady state ((62.5 ng/h*5.5 h+At-1) /2), where At-1 is the cumulative amount left from the previous half time). In contrast, in our study the maximum achieved striatal CDNF amount was 2.6 ng (17.3 ng/ml*0.15 ml, lysis volume of striata). The two studies differ in therapy length (2 weeks vs. 12 weeks) and in CDNF location (extracellularly administered vs. intracellular production). Although the studies are not completely comparable, it is plausible that the CDNF acquired by viral transduction is more efficient compared to recombinant protein, as better results were obtained with gene therapy resulting in less than 1% of the CDNF amount compared to protein infusion. Differences may arise from the quality of CDNF (folding, post-translational modifications) or mode of action (intracellular, activity-dependent).

The highest virus titer used in our study resulted in 776±346 pg/mg hCDNF concentration in the striatum eight weeks post-transduction. As compared to endogenous CDNF in mouse brain (761±141 pg/mg, n=4, unpublished data) or in human striatum (481±217 pg/mg, n=33, unpublished data), the amount was increased only 2-2.6-fold by gene therapy. This suggests that even small changes in CDNF concentration can result in a modest protective effect. However, additional positive effects of putatively induced endogenous Cdnf by gene therapy cannot be excluded (Nadella et al. 2014).
The positive neurorestorative and protective outcome of CDNF in rodent PD models raised our interest to test CDNF’s therapeutic effect in a mouse model of Alzheimer’s disease (AD). AD is a progressive disease where degeneration of neurons starts from the hippocampus and entorhinal cortex leading to gradual loss of memory and cognitive impairment. The pathogenesis involves formation of abnormal neuritic plaques and tangles consisting of amyloid beta and tau proteins (Scheltens et al. 2016). To study the effects of CDNF in AD, recombinant hCDNF protein or transgene in an AAV2 vector were injected bilaterally (10 μg or 1.4x10⁹ vg/side) to the hippocampi of 12–13-months-old wild type and APP/PS1 mice, which are characterised by the formation of amyloid plaques at around four months of age (Garcia-Alloza et al. 2006) and decline in spatial memory tests from 12 months (Minkeviciene et al. 2008).

Human CDNF concentration in the mouse brain was analysed by ELISA six weeks post-transduction. The ELISA results revealed high differences in CDNF transgene concentration (0.01-78 ng/mg) in the hippocampi of AAV2-hCDNF injected animals (Fig. 8 A). Due to the high variation in transduction efficiency, mice with hippocampal CDNF concentration lower than 1 ng/mg (on average 0.04±0.11 ng/mg, n=13) were excluded from the behavioural assay group analysis. However, all mice were included to the correlation analysis. Both aged APP/PS1 and wild type mice performed better in tests measuring long-term memory when treated with human CDNF compared to the controls (III, Fig. 2). The precise determination of CDNF protein expression after viral gene delivery led to the important observation that the measures of long-term memory, namely in the time spent in the platform zone (Fig. 8 B) and the number of platform crossings (III, Fig. 3 B) in the Morris swim task, correlated positively with the measured hippocampal human CDNF levels, strengthening the idea that CDNF could be involved in memory consolidation. In addition to gene therapy, single injection of recombinant human CDNF protein resulted in similar actions two weeks post-injection.

### Figure 8

**hCDNF expression in AD mouse model after gene therapy.** A) High variation of the transgene expression was observed in the transduction site (values in the left hippocampus). The groups were treated at different times and received different batches of the virus. Mean±SD are indicated by horizontal lines. B) hCDNF expression level correlated positively with the time spent in platform zone in Morris swim task (R=0.54, p=0.01). A) Modified from III, B) Figure from III.
The mechanism behind the observed effect of CDNF on improved long-term memory remained open, as no changes in hippocampal neurogenesis or dopaminergic neuron innervation were found. Also, no changes in the amyloid plaque load in APP/PS1 mice were observed with CDNF therapy. Long-term memory retention requires de novo protein synthesis (Jarome & Helmstetter 2014). Hippocampal ER stress and consequential reduction in protein synthesis is linked with impaired memory in AD (Duran-Aniotz et al. 2014). A potentially important effect of CDNF on the attenuation of ER stress and, therefore, more effective protein synthesis, was not studied. Indeed, incubation of recombinant CDNF in the culture medium of rat primary hippocampal neurons reduced the levels of UPR markers, including the translational attenuator phosphorylated eIF2α, induced by extracellular amyloid beta peptides (Zhou et al. 2016). Although reduced ER stress by CDNF therapy could improve the memory consolidation in APP/PS1 mice suffering from amyloid pathology, the observation of a similar effect in wild type mice raises doubts against the validity of this hypothesis.

9.3.3. Comparison of hCDNF concentrations after AAV2-hCDNF transduction in rodents (II, III)

The highest human CDNF concentrations found in the mouse hippocampi (III) were 100-fold higher than the average human CDNF measured in the rat striata (II) eight weeks post-injection (80 vs. 0.78 ng/mg), although the same viral vector was used with only 1.4-fold higher titer (1.4 vs. 1.0x10⁹ vg). The reason for such a difference in CDNF protein concentration is unknown. One possibility is that in the AD study the tissue piece analysed was more restricted in relation to transduced cells compared to that of the PD study. As the measured CDNF concentration was normalised to total protein, higher percentage of transduced cells in the sample would lead to a higher result. Supporting this, the immunocytochemical images revealed that CDNF protein was expressed evenly throughout the hippocampi (III, Fig. 1 A & B), whereas, in case of striatal transduction, CDNF expression was mainly restricted to the injection tract (II, Fig. 3 A). The differences could also be caused by differential detection of the transgene on ELISA depending on the brain area or species used. However, as the linearity of dilution was similar in both rat striatal and mouse hippocampal tissue lysates (Table 9), the validation results suggest no changes in the analyte detection between the two sample matrices, or due to different dilutions used in order to get the result on the dynamic range of the assay (up to 1:800 vs. 1:20).
9.4. Analysing CDNF secretion from engineered cell clones aimed for encapsulated cell biodelivery (IV)

Long-term administration of proteins to the CNS can be implemented by encapsulated cells secreting the therapeutic protein. Naturally, this method relies heavily on constant secretion of the protein. Despite the successful production of several ARPE-19 cell clones with high CDNF expression, its secretion was found to decrease markedly once the cells were confluent and stopped dividing on culture plates (Fig. 9). The intracellular levels of CDNF remained high in the non-dividing cells, implying no changes in the transgene expression. CDNF secretion decreased from the encapsulated cells as well, although metabolically stable (IV, Fig. 2 D & E). The observed halt in CDNF secretion differs from the reports of stable secretion of other proteins studied in the same cell line in relation to encapsulated cell therapy, such as ciliary neurotrophic factor (Tao et al. 2002) or NGF (Fjord-Larsen et al. 2010), suggesting that the mechanisms regulating CDNF secretion differ from these proteins.

![Graph](https://via.placeholder.com/150)

**Figure 9** Secretion of CDNF from stable cell clones. The secretion of wild type (wt)CDNF decreased substantially once the clones reached confluency on cell culture plates. In contrast, KTEL-deleted CDNF was stably secreted by non-dividing cells. *** p<0.001. Figure from IV.

9.4.1. Enhanced CDNF expression with codon optimisation (IV)

Although NGF was secreted stably from encapsulated ARPE-19 cells in vitro and in vivo, when implanted to the basal forebrain of pigs (Fjord-Larsen et al. 2010), enhanced expression was required for a clinical study in AD patients (Fjord-Larsen et al. 2012). This was achieved by transfecting cells with sleeping beauty transposase (Ivics et al. 1997), which integrates multiple copies of the transgene to the genome (Grabundzija et al. 2010). In addition, the insertions usually occur in loci that prevent silencing of the transgene. We used another method to enhance CDNF expression, namely codon optimisation (Quax et al. 2015). Most amino acids are encoded by multiple synonymous codons, but the level of complementary transfer (t)RNAs in cells differ. In codon optimisation, the codon triplets are changed to represent a more ubiquitous tRNA in the host while keeping the amino acid sequence
unchanged. Since tRNA levels differ from one species to another, codon optimisation is often used when expressing a protein of one species in a cell of another species (Gustafsson et al. 2004). However, it may also enhance the expression of allogenic genes, as all genes are not encoded by the most abundant tRNAs. The tRNA profile of a gene determines its translation rate, which contributes to the control of protein expression, but also influences protein folding (Pechmann & Frydman 2013).

In our case, where human CDNF was expressed in a human cell line, codon optimisation with 113 (20%) nucleotide changes in its coding sequence (from Icosagen) resulted in a 2-fold increase in the intracellular and a 3-fold increase in the secreted CDNF concentration after transient transfection (IV, Fig. 3 A). However, the secretion decreased in non-dividing cell clones expressing codon-optimised CDNF, similarly to the wild type CDNF clones (Fig. 9). In addition, the intracellular protein load was further increased with codon optimisation, which may lead to problems in the ER and protein folding. Hence, enhanced expression of CDNF achieved by codon optimisation did not solve the problem we encountered with the secretion of wild type CDNF.

9.4.2. Stable secretion of KTEL-deleted CDNF in confluent cultures (IV)

Intracellular CDNF localised heavily within the ER of the clones, as observed from confocal microscopy images of cells double labelled with antibodies against CDNF and ER-resident PDI (IV, 1 B). Thus, we wanted to test the effect of deleting the putative KDEL receptor binding site in the C-terminus of human CDNF (KTEL). The hypothesis was that by deleting the canonical KDEL sequence, CDNF secretion would increase as has been demonstrated for MANF (RTDL) (Glembotski et al. 2012, Oh-Hashi et al. 2012, Henderson et al. 2013, Mätlik et al. 2015) and mouse CDNF (QTEL) (Norisada et al. 2016). Accordingly, KTEL-deleted CDNF was secreted three times more efficiently compared to wild type CDNF under transient transfection (IV, Fig. 3 A). With the combination of codon optimisation and KTEL deletion, approximately 5-fold CDNF secretion was obtained compared to wild type CDNF (IV, Fig. 3 A). More remarkably, the secretion of KTEL-deleted CDNF did not decrease in confluent cultures (Fig. 9). The reason for this is puzzling but implies that KDEL receptors are involved in the substantial reduction of CDNF secretion in non-dividing cells. Are KDEL receptors more abundant in confluent cells? Or are the levels of other KDEL-receptor binding molecules lower in confluent cells? There are three different KDEL receptors with somewhat different affinities to the KTEL sequence found in human cells (Raykhel et al. 2007). Do the levels of these isoforms differ in dividing compared to confluent cells? The KTEL sequence has been shown to respond to ER-calcium depletion by increased secretion (Henderson et al. 2014). Is the calcium concentration different in dividing and confluent cell clones?

In conclusion, the wild type CDNF is not an optimal candidate for the delivery from encapsulated cells due to its poor secretion, at least from ARPE-
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19 cells. Instead, the KTEL-deleted CDNF showed prolonged secretion in the non-dividing cell clones. However, the biological activity of KTEL-deleted CDNF is currently unknown and needs to be tested before applications.

9.4.3. Is endogenous CDNF secreted? (IV)

Secretion of endogenous MANF has been reported in HeLa cell line and primary rat cardiac myocyte cultures (Apostolou et al. 2008, Glembotski et al. 2012). In contrast, only the secretion of overexpressed CDNF has been published (Lindholm et al. 2007, Apostolou et al. 2008, Sun et al. 2011, Norisada et al. 2016). To find out whether endogenous CDNF is secreted, the conditioned media from epithelial-like ARPE-19 and HeLa cells, endothelial-like EA.hy926 cells, neuronal-like SH-SY5Y cells, and glial-like U-78 MG cells were studied by ELISA. Although CDNF was expressed by all of the cell lines, no CDNF was found in the conditioned culture media incubated for 72 hours (IV, Table 1). Instead, MANF was present in all of the conditioned media with levels ranging from 0.41 to 4.20 ng/ml.

The absence of CDNF in the media could be a true observation. Alternatively, it could be explained by limited ELISA sensitivity. If both MANF and CDNF were secreted proportionately to their expression (the intracellular levels of MANF were 160-230 times higher than those of CDNF), the levels of CDNF in the media should have ranged from 2 to 24 pg/ml. These levels are below of mAb (31 pg/ml) hCDNF ELISA sensitivity, and therefore could have been left undetected. However, the same media were also negative when analysed by pAb hCDNF ELISA with sensitivity limit of 10 pg/ml. A cell line with higher CDNF expression could give insight to whether it can be secreted at all. Cells of the muscle or testis potentially have high CDNF expression, as the highest CDNF levels have been reported in these tissues in vivo (Lindholm et al. 2007).

CDNF may, however, be less readily secreted from cells than MANF. Insertion of the C-terminal sequence of human CDNF (ASA)KTEL to Gaussia luciferase led to less secretion of the reporter protein under chemically induced ER stress compared to a similar construct with human MANF C-terminal sequence (ASARTDL) (Henderson et al. 2014). This suggests that CDNF has a higher affinity for KDEL receptors compared to MANF and thus, could be secreted less efficiently. However, in the case of the full-length proteins, other factors beyond KDEL receptor binding are supposed to affect the outcome of secretion, such as binding to GRP78 (Oh-Hashi et al. 2012, Norisada et al. 2016). Norisada and colleagues (2016) reported equal secretion levels for flag-tagged mouse MANF and CDNF when overexpressed in HEK cells, but the secretion efficiencies might differ for endogenous proteins and expression levels. Further, the C-terminal QTEL sequence found in mouse CDNF might have different affinity to KDEL receptors than KTEL in human CDNF, although this has not been tested (Raykhel et al. 2007, Henderson et al. 2014).
10. Recommendations for future research

The cytoprotective and restorative effects of MANF and CDNF have been studied in animal models of various diseases, however, whether changes in their expression is related to disease pathologies, is largely unknown. The ELISAs developed in this work can be utilized in the quantification of changes in MANF and CDNF expression and secretion in various physiological and pathological stimuli in vitro and in vivo in experimental animals with the aim in increasing the knowledge of functions of these proteins in health and disease. In addition, quantification of MANF and CDNF in pathological human tissues can give insights to their associations with disease development and to their therapeutic potential. The constructed ELISAs could also be useful in the development and implementation of potential CDNF and/or MANF-based therapies, whether they be executed by the means of recombinant proteins, gene therapy, or encapsulated cells.

As a change in MANF expression and secretion has been linked to the pathology of diverse diseases, such as neurodegenerative diseases, ischemia, and diabetes, its levels should be studied in samples obtained from human subjects and animal disease models to find out whether it could be used as predictive, diagnostic or therapy response biomarker for the above-mentioned diseases. Since most biomarkers used in the clinics are analysed from the blood, the finding of circulating MANF further strengthens its potential in the biomarker field. Unfortunately, the expected absence of connection between MANF levels in the peripheral blood and brain lowers its biomarker potential in neurodegenerative diseases with CNS-restricted pathology. This holds true in case no active transport mechanism for MANF is proven, as large hydrophilic proteins are unable to diffuse efficiently through the BBB. However, MANF is also found in the CSF (unpublished data). As CSF is in close contact to the brain tissue, possible CNS-restricted changes in MANF expression could be determined from those samples. In addition to serum and CSF, MANF is present in human saliva (unpublished data), which could serve as a non-invasive sample source for extracellular MANF detection. The presence of MANF in human urine has not been studied, but it could possibly be exploited as another non-invasive source for MANF detection.

The rapid and robust secretion of MANF in response to ER stress could provide a tool for in vivo monitoring of changes in cellular stress in bodily fluids. We were interested in finding out whether circulating MANF levels reflected ER stress in T1D patients, but association could not be demonstrated with serum samples, as no well-established circulating marker for ER stress has been described so far. We attempted to analyse GRP78 by WB (Delpino & Castelli 2002), Fetuin-A levels by ELISA (Ou et al. 2012), and oxidative stress by nitrite detection using Griess reagent and ELISA quantification of HNE-protein adducts (Marrocco et al. 2017) (data not shown), but the outcome of these was either methodologically poor or unsatisfactory due to irreproducibility of results. Future studies will reveal whether the circulating levels of MANF can be utilized for reflecting ER stress in vivo. On the other
hand, if extracellular MANF is found to correlate with cellular ER stress in the human body, the lack of specificity for a certain pathological state may decrease its suitability as a disease biomarker. It could possibly qualify for monitoring of the progression or treatment responses for various diseases.

The biomarker potential of CDNF was not studied in this work. CDNF was detected in multiple mouse and human tissues but its levels were low/undetectable in human serum, CSF, and saliva as measured by hCDNF ELISAs (unpublished data). The undetectable basal levels could be an advantage in diagnostics whenever CDNF levels increase in these matrices in relation to a pathologic process. Whether the analysis of MANF or CDNF has value in the clinics is an open question. In many cases the determination of a single diagnostic marker is not reliable enough for accurate diagnostics (Twyman 2005). This is true especially when the marker is differentially expressed in multiple diseases. In addition, for a good marker the normal physiological variability needs to be rather narrow in order to distinguish changes reliably in populations (Cox et al. 2014). This holds not only for intersubject, but also intrasubject variability, especially if the marker is followed during disease progression, or in relation to therapy response. The stability of intrasubject MANF and CDNF levels in biological samples needs to be studied in repeated samples from the same subjects while keeping control on factors such as nutritional status, physical activity, circadian rhythm, and infections, as they can potentially affect MANF and CDNF levels.

In relation to possible CDNF therapy implemented by encapsulated cell biodelivery, an important question to address is how efficiently CDNF is secreted. This holds true to gene therapy as well when the transduced cells are not the ones affected by the disease pathology. The matters of secretion are also interesting in relation to CDNF’s potential as a fluid biomarker. Although the data presented in this thesis implies that CDNF is not readily secreted in a normal situation in vitro, it could be different in vivo especially in pathological situations, as what is known for MANF in relation to, for example, ER-stress and inflammation. However, despite the high structural similarity between MANF and CDNF, it should be kept in mind that they are individual molecules. Thus, they can — and in a biological sense they should — have diverse characteristics.
This thesis describes the development of ELISAs for sensitive quantification of MANF and CDNF proteins. The assays were validated for high performance when analysing biological samples including serum, tissue lysates, and in vitro cell samples. In this work, the assays were utilized in the analysis of MANF levels in human serum in relation to T1D (I), of human CDNF levels in rodent brains after gene therapy (II-III), and of CDNF secretion from cell clones in relation to possible delivery method of the protein in CNS-targeted therapy (IV). The main results achieved using the assays are the following:

I MANF was found in human serum. Extremely high concentrations were observed in a subpopulation of newly diagnosed type 1 diabetes patients. The circulating MANF levels were studied against functional β-cell mass and inflammation, but the exact mechanism and source of increased MANF remained open.

II Specific determination of human CDNF, transduced by AAV vector to the rat brain in a Parkinson’s disease model, showed titer- and time-dependent expression of CDNF in the transduction area, the striatum, and in a connected area, the substantia nigra. Expressed CDNF was mostly intracellular and the levels remained low, which could account for the rather modest histological and behavioural outcome observed.

III Specific determination of human CDNF, transduced by AAV vector to the mouse hippocampi in an Alzheimer’s disease model, revealed high variation in CDNF expression between experimental animals. The CDNF transgene expression level correlated positively with the outcome in behavioural tests measuring long-term memory. The results imply that CDNF could be involved in memory consolidation in the hippocampus.

IV Although robust secretion of overexpressed CDNF was observed in subconfluent cell cultures, the secretion decreased to negligible in non-dividing cells. KTEL deletion from the C-terminal domain of CDNF enhanced its secretion and resulted in stable CDNF secretion in non-dividing cell cultures, which is an important feature in encapsulated cell biodelivery.

CONCLUSIONS
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Bolstad, N., Warren, D.J. & Nustad, K. 2013, "Heterophilic antibody interference in
immunometric assays", Best Practice & Research Clinical Endocrinology & Metabolism, vol. 27, no. 5, pp. 647-661.


thrombospondin 1 and mesencephalic astrocyte-derived neutrotrophic factor (MANF)",
Delpino, A. & Castelli, M. 2002, "The 78 kDa glucose-regulated protein (GRP78/BIP) is
expressed on the cell membrane, is released into cell culture medium and is also present
& Celniker, A. 2003, "Recommendations for the bioanalytical method validation of ligand-
binding assays to support pharmacokinetic assessments of macromolecules"
Pharmaceutical research, vol. 20, no. 11, pp. 1885-1900.
Dhawan, S., Dirice, E., Kulkarni, R.N. & Bhushan, A. 2016, "Inhibition of TGF-beta Signaling
Promotes Human Pancreatic beta-Cell Replication", Diabetes, vol. 65, no. 5, pp. 1208-
1218.
Dooley, J., Tian, L., Schonefeldt, S., Delgingaro-Augusto, V., Garcia-Perez, J.E., Pasciuto, E.,
Di Marino, D., Carr, E.J., Oskolkov, N., Lyssenko, V., Franckaert, D., Lagou, V., Overbergh,
L., Vandenbussche, J., Allemeersch, J., Chabot-Roy, G., Dahlstrom, J.E., Laybutt, D.R.,
Petrovsky, N., Socha, L., Gevaert, K., Jetten, A.M., Lambrechts, D., Lintner, M.A.,
predisposition for beta cell fragility underlies type 1 and type 2 diabetes", Nature genetics,
vol. 48, no. 5, pp. 519-527.
Doroudgar, S., Thuerauf, D.J., Marcinko, M.C., Belmont, P.J. & Glenbotski, C.C. 2009,
"Ischemia activates the ATF6 branch of the endoplasmic reticulum stress response", The
Dunne, J.L., Overbergh, L., Purcell, A.W. & Mathieu, C. 2012, "Posttranslational modifications
of proteins in type 1 diabetes: the next step in finding the cure?", Diabetes, vol. 61, no. 8,
pp. 1907-1914.
Dupuis, J., Langenberg, C., Prokopenko, I., Saxena, R., Soranzo, N., Jackson, A.U., et al. 2010,
"New genetic loci implicated in fasting glucose homeostasis and their impact on type 2
Duran-Aniotz, C., Martínez, G. & Hetz, C. 2014, "Memory loss in Alzheimer's disease: are the
alterations in the UPR network involved in the cognitive impairment?", Frontiers in Aging
Neuroscience, vol. 6, no. 8.
therapy for neurodegenerative diseases: from promise to product", Advanced Drug
Engvall, E. & Perlmann, P. 1971, "Enzyme-linked immunosorbent assay (ELISA) quantitative
Eriksdotter-Jönhagen, M., Linderoth, B., Lind, G., Aladellie, L., Almkvist, O., Andreasen, N.,
Blennow, K., Bogdanovic, N., Jelic, V., Kadir, A., Nordberg, A., Sundström, E., Wahlund,
"Encapsulated cell biodelivery of nerve growth factor to the Basal forebrain in patients
18-28.
reticulum stress and the decline of beta-cell health in diabetes mellitus", Diabetes, obesity
& metabolism, vol. 15, suppl. 3, pp. 159-169.
Eyjolfsdottir, H., Eriksdotter, M., Linderoth, B., Lind, G., Juliusson, B., Kusk, P., Almkvist, O.,
Andreasen, N., Blennow, K., Ferreira, D., Westman, E., Nennesmo, I., Karami, A., Darreh-
growth factor to the cholinergic basal forebrain of Alzheimer's disease patients:
application of a second-generation encapsulated cell biodelivery device", Alzheimer's
Research & Therapy, vol. 8, pp. 30.


Kaplan, I.V. & Levinson, S.S. 1999, "When is a heterophile antibody not a heterophile antibody? When it is an antibody against a specific immunogen", *Clinical chemistry*, vol. 45, no. 5, pp. 616-618.


Lindholm, P., Voutilainen, M.H., Lauren, J., Peränen, J., Leppänen, V.M., Andressoo, J.O.,...


Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. 1995, "How to measure and predict the molar absorption coefficient of a protein", *Protein science*, vol. 4, no. 11, pp. 2411-2423.


References


Van Anken, E. & Braakman, I. 2005, "Versatility of the endoplasmic reticulum protein folding
References

factory”, Critical reviews in biochemistry and molecular biology, vol. 40, no. 4, pp. 191-228.


