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Characterization of *Drosophila melanogaster* Manf – an evolutionarily conserved neurotrophic factor

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ACADEMIC DISSERTATION

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Abstract

Among neurotrophic factors MANF/CDNF family is unique as their protein sequences are evolutionarily conserved between multicellular organisms. Still, little is known about their mechanism of action and interacting molecules. At the time of initiation of this study there were no known neurotrophic factors in invertebrates. According to the protein sequence homology there was an uncharacterized homologue to the novel neurotrophic factor MANF in *Drosophila melanogaster*. We found that *Drosophila* Manf (*DmManf*) is an essential gene in a fruit fly development. *DmManf* represents a true orthologue to mammalian MANF as its mutant lethality is rescued by human MANF. We have generated *DmManf* deletion mutant surviving to second instar larval stage with maternal contribution. When the maternal contribution of *DmManf* is abolished, the mutants die at the end of embryogenesis before hatching. In *DmManf* mutant the dopaminergic neurites degenerate and the dopamine level is extremely low. Ultrastructural analysis reveals nonapoptotic cell death in the embryonic ventral nerve cord and neuropile decomposition together with cell body glia activation taking place. In secretory cells like gastric caeca or fat body the visible loss of rough endoplasmic reticulum and drastic accumulation of vesicles, some filled with cellular debris, occur. According to microarray expression analysis data, expression of genes involved in vesicular transport and metabolism were altered in *DmManf* mutants. The expression of several genes implicated in pathology of Parkinson's disease (PD) was also altered. The degeneration of dopaminergic neurons is the hallmark for PD and this thesis work makes an effort to enlighten the mechanisms how the neurotrophic factor MANF protects these degenerating neurons.

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List of Original Publications

This thesis is based on the unpublished data and following original publications referred to in the text by their Roman numerals. The articles are reproduced with kind permission from the copyright owners.

I Mari Palgi, Riitta Lindström, Johan Peränen, T. Petteri Piepponen, Mart Saarma, and Tapio I. Heino (2009). Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. Proceedings of the National Academy of Sciences of the United States of America 106:2429-2434.

II Mari Palgi, Dario Greco, Riitta Lindström, Petri Auvinen, and Tapio I. Heino (2012). Gene expression analysis of *Drosophila Manf* mutants reveals perturbations in membrane traffic and major metabolic changes (2012). BMC Genomics, 2012, 13:134, in press.

Abbreviations

6-OHDA	6-hydroxy-dopamine
Abl	Abelson kinase
AD	Alzheimer's disease
AEL	after egg laying
ALS	amyotrophic lateral sclerosis
ARP	arginine rich protein
ARMET	arginine-rich, mutated in early stage tumors
ARMETL1	arginine-rich, mutated in early stage tumors-like 1
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X
bp	base pair
BiP/GRP78	Binding immunoglobulin protein/ Glucose Regulated Protein 78
Bcl-2	B cell lymphoma-2
BDNF	brain derived neurotrophic factor
BH_4	tetrahydrobiopterin cofactor
BSC	Bloomington Stock Centre
bZIP	basic leucine zipper
cAMP	3'-5'-cyclic adenosine monophosphate
Catsup	Catecholamines up
Cbl	Casitas B-lineage Lymphoma, E3 ubiquitin ligase
CDNF	cerebellar dopamine neurotrophic factor
CHK-1	checkpoint kinase 1
CNS	central nervous system
crc	cryptocephal
DA	dopamine
Dab	disabled
DAT	dopamine transporter
DEBCL	death executioner Bcl-2 homologue
Ddc	dopamine decarboxylase
dia	diaphanous, a formin
DJ-1	PARK7; peptidase C56 family
dlg	disc large
DNA	deoxyribonucleic acid
DNT-1, 2	*Drosophila* neurotrophin 1, 2
DopEcR	Dopamine/Ecdysteroid receptor
DSHB	Developmental Studies Hybridoma Bank
EDEM	ER degradation enhancing α-mannosidase-like protein
EGF	epidermal growth factor
eIF-2α	elongation initiation factor subunit 2 alpha
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated protein degradation

ERK	extracellular-signal-regulated kinase
Ero-1	ER oxidoreductin-1
ERSE	ER stress-response element
ERSE-II	ER stress-response element II
fas II	fasciclin II
FGF	fibroblast growth factor
GDNF	glial derived neurotrophic factor
GEF64C	guanidine nucleotide exchange factor
Gfr	glial cell derived neurotrophic factor receptor
GO	gene ontology
grh	grainyhead
GSH	reduced glutathione
GSSG	oxidized glutathione
Hac1	yeast bZIP protein binding to the CRE motif
HD	Huntington's disease
Hsp83	Heat shock protein 83; human HSP90 homologue
Htra2	mitochondrial HtrA serine peptidase 2, also known as OMI and PARK13
IGF	insulin growth factor
InR	Insulin receptor
IRE1	inositol-requiring protein-1
KDEL	Lys-Asp-Glu-Leu, ER retention signal
KEGG	Kyoto Enzyclopedia of Genes and Genomes
Klp68D	kinesin-like protein at 68D
klu	klumpfuss
kkv	krotzkopf verkehrt, chitin synthase
knk	knickkopf
Kr	Krüppel
Ku70	Lupus Ku autoantigen protein p70 homologue
Ku80	Lupus Ku autoantigen protein p80 homologue
L-DOPA	L-dihydroxyphenolalanine
lola	longitudinals lacking
MANF	mesencephalic astrocytes-derived neurotrophic factor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGF	nerve growth factor
Nrk	Neurospecific receptor kinase
NTF	neurotrophic factor
NT-3, 4	neurotrophin 3, 4
odd	oddskipped
PD	Parkinson's disease
PDI	protein disulfide isomerases
PERK	pancreatic ER KR-like kinase/ eIF-2α kinase
ple	pale; tyrosine hydroxylase
PNS	peripheral nervous system
pros	prospero
pst	pastrel

Pu	Punch
pum	pumilio
$p75^{NTR}$	low affinity neurotrophin receptor
Rb	retinoblastoma protein
repo	reversed polarity
Ret	rearranged during transfection; proto-oncogene tyrosine-protein kinase receptor
rl	rolled, ERK kinase
RNA	ribonucleic acid
ROP	Ras opposite
ROR	receptor tyrosine kinase-like orphan receptor
ROS	reactive oxygen species
RSEL	amino-acid sequence Arg-Ser-Glu-Leu
RTDL	Arg-Thr-Asp-Leu; putative ER retention signal
RTK	receptor tyrosine kinase
SAP	scaffold attachment factor A and B, Acinus and PIASmotif
SAPLIP	saposin like protein
Saposins	Sphingolipid Activator PrO[S]teINs
SIL1	nucleotide exchange factor
siRNA	silencing RNA
SNAP29	Synaptosomal-associated protein 29
SOD	superoxide dismutase
sol	small optic lobes
spz	spätzle
Stx17, 18	Syntaxin 17, 18
Syx1A, 6	Syntaxin 1A, 6
TGF β	transforming growth factor beeta
TH	tyrosine hydroxylase
TNFR	tumor necrosis factor receptor
Trk	tropomyosin receptor kinase; high affinity neurotrophin receptor
Trkl	tropomyosin receptor kinase-like
UPR	unfolded protein response
UPRE	unfolded protein response element
VMAT	vesicular monoamine transporter
VNC	ventral nerve cord
Xbp1	X box binding protein 1

1 Review of the literature

1.1 The concept of neurotrophic factors and their evolution

1.1.1 The family of neurotrophic factors

Neurotrophic factors (NTFs) are a subset of growth factors acting on neuronal tissue. They are endogenous soluble proteins regulating survival, growth, morphological plasticity, or synthesis of proteins for differential functions of neurons (Hefti et al., 1993). The basis for 'neurotrophic concept' was made by the early descriptive work of Santiago Ramon y Cajal (Ramon y Cajal, 1909/1995) and advanced at molecular level by Rita Levi-Montalcini (Levi-Montalcini, 1982). According to the classical neurotrophic theory during development the neurons are made in excess and the neuronal numbers are controlled by the limited amount of target derived neurotrophic factors. Only the neurons that reach their targets and make the appropriate contacts obtain NTF and survive. Since then NTFs have been found to have multiple other roles than survival. Several well known growth factors can behave also as NTFs, for example fibroblast growth factors (FGFs), epidermal growth factors (EGFs), insulin-like growth factor (IGF), transforming growth factor β (TGF β) family members, and cytokines (Loughlin and Fallon, 1993). The well-established mammalian family of NTFs is the family of neurotrophins (NGF, BDNF, NT3, and NT4). Neurotrophins act via Trk receptors, belonging to the receptor tyrosine kinase (RTK) family and $p75^{NTR}$, the member of tumour necrosis factor receptor (TNFR) superfamily. Second large family of NTFs is the glial cell line-derived neurotrophic factor (GDNF) and its homologues (artemin, neurturin, and persephin). These NTFs bind to specific receptors Ret and Gfrs, reviewed by Airaksinen and Saarma (, 2002). All the data of above mentioned NTFs, their receptors, and signalling have been gained from studies of vertebrates.

1.1.2 The conservation of neurotrophic factors in evolution

Despite recent rapid development in whole genome sequencing surprisingly few homologues to neurotrophic signalling counterparts have been found in primitive vertebrates and invertebrates. In addition, the homologues show rather low homology to NGF and GDNF family members. Nevertheless, increasing amount of sequencing data from different genomes has revealed that at sequence level neurotrophins and their receptor homologues exist in primitive deuterosomes, like sea urchin, acorn worm, and sea squirts (Bothwell, 2006). In protostomes neurotrophin receptor homologues also exist such as LTrk in molluscs (Benito-Gutierrez et al., 2006). In the snail *Lymnaea* Trk and sea snail *Aplysia* Trk-like (Trkl) are so far the most similar proteins to Trk receptors but the exact phylogenetic relationship of mollusc Trks to each other and to vertebrate Trks is unknown. Recently, in the crustacean *Daphnia pulex* genome, a neurotrophin, $p75^{NTR}$ and Trk orthologue together with Trkl, Ror, and Nrk were described (Wilson, 2009). By taking advantage of cysteine knot motif of neurotrophins, the search by cystein knot homology through the whole *Drosophila* genome result in NT1 and 2 belonging to the spätzle (spz) family (Zhu et al., 2008). The genome searches of *Daphnia pulex* and *Capitella, Helobdella* and *Lottia* genomes reveal spätzle family and other neurotrophin signalling components in these protostomes as well (Wilson, 2009).

There is a possibility that neurotrophin, Trk, and $p75^{NTR}$ existed at the protostome/deuterostome split. In protostomes a "neurotrophin superfamily" includes spz family and neurotrophins as two paralogous families. Trks and Trkl proteins form closely related paralogous families within the protostomian RTKs, and Trkl are absent in deuterostomes. The recent finding of $p75^{NTR}$ in several protostomes suggests that death domain tumour necrosis factor receptor (TNFR) superfamily proteins appeared early in evolution. However, there are little functional data about these recently identified invertebrate neurotrophic systems. Definitely more studies are needed to clarify are those *in silico* found homologues to mammalian neurotrophic signalling components conserved in functional level as well.

1.2 Neurotrophic factors in *Drosophila*

1.2.1 Special features of the embryonic neuronal development of *Drosophila*

During almost half a century after the establishment a neurotrophic concept no NTFs were found in protostomes, including nematodes and arthropods. The absence of NTFs in invertebrates has been explained by fundamental differences in the development and maintenance of vertebrate and invertebrate nervous systems. In *Drosophila*, the neurogenesis is very fast and commences during eight hours. The future fate and identity of the neuroblast is determined already before the delamination by spatial gene expression (Bossing et al., 1996). *In vitro* analysis of neuroprogenitors in culture compared to dye injected neuroprogenitors *in vivo* allowed determining the developmental capacities of individual neural precursors of defined spatial and temporal origin (Luer and Technau, 2009). The neuroepithelial cells when isolated at the pre-mitotic and pre-delamination stage express characteristics of the progenitor type appropriate to their site of origin in the embryo. There are slight differences between mesectodermal midline progenitors and lateral neuroepithelial future neuroblasts, the last exhibit a higher degree of autonomy in generation of their lineages (Luer and Technau, 2009). In *Drosophila* as in vertebrates neurons and glia are made in excess. Approximately 30% of neurons generated during embryonic neurogenesis are further eliminated by programmed cell death (Rogulja-Ortmann et al., 2007). In *Df(3R)H99* embryos where proapoptotic genes *reaper*, *hid* and *grim* are deleted (Zhou et al., 1997), supernumerary neurons are produced (Rogulja-Ortmann et al., 2007). In *Drosophila* embryo, the neuronal proliferation, survival and programmed cell death are under control of segment specific Hox genes (Prokop et al., 1998; Bello et al., 2003; Miguel-Aliaga and Thor, 2004). Furthermore, there are several transcription factors acting together in a manner of a combinatorial code to define the fate of different neuronal precursors (Miguel-Aliaga and Thor, 2009). However, the exact targets of these transcription factors acting in combinations remain elusive.

1.2.2 Trophic interactions during *Drosophila* neural development

Decade ago emerging evidence appeared about trophic and reciprocal interactions between fruit fly glia and neurons (Booth et al., 2000; Sepp and Auld, 2003). It became evident that if glial cells are depleted by expressing in specific glial cells proapoptotic

proteins or toxins, neurons die and *vice versa*. Furthermore, EGF receptor signalling in response to secreted ligands Vein and Spitz from neurons has been shown crucial for glial survival in the embryonic ventral nerve cord (Hidalgo et al., 2001; Bergmann et al., 2002). The embryonic midline glia expresses platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) receptor homologue PVR. Appropriate ligands PVF1, 2 and 3 are expressed by midline neurons interacting with midline glia. When *PVR* is mutated, the midline glial number, migration and axonal enwrapment are affected (Learte et al., 2008). Midline glia also express fibroblast growth factor receptor (FGFR) breathless controlling glial migration (Klämbt et al., 1992). In summary, signalling via different TRK receptors, EGFR, FGFR, and PVR regulate the survival, differentiation, and migration of the midline glia (Klämbt et al., 1992; Bergmann et al., 2002).

Searches by structural cysteine knot homology to vertebrate neurotrophins through *Drosophila* genome allowed to identify *Drosophila* neurotrophins 1 and 2 (DNT1, DNT2) (Zhu et al., 2008). DNT1 and DNT2 belong to spz family. This family has been known in *Drosophila* to act via Toll and Toll-like receptors mediating the immune response. Already in 1998 *Drosophila* spätzle was shown to share homology with cysteine knot of neurotrophins, but recent detailed analysis and proofs of the functional neurotrophin homology was finally provided (Mizuguchi et al., 1998; Hoffmann et al., 2008; Zhu et al., 2008). DNT1 and DNT2 together with spätzle are expressed in midline glia and lateral muscles of *Drosophila* and are responsible for the survival of peripheral neurons (Zhu et al., 2008). Do the spätzle and DNTs mediate the neurotrophin properties via Toll-like receptors still remain to be proven. Flies lack Trk receptor homologues mammalian neurotrophins bind to with high affinity, but have orphan receptors closely related to Trk - Ror (Wilson et al., 1993) and Nrk (Oishi et al., 1997). Mammalian closest homologues to ROR and NRK bind to Wingless related ligands (Oishi et al., 2003). The ligands to *Drosophila* Ror and Nrk remain to be found. Besides Trk receptors, mammalian neurotrophins bind with low affinity to $p75^{NTR}$ receptor, belonging to TNFR family (Bothwell, 2006). In protostomes the representative found is *Drosophila Wengen*. Wengen and its known ligand cytokine Eiger when overexpressed are known to signal death via JNK pathway inducing small eye phenotype (Igaki et al., 2002; Kanda et al., 2002). The other function for *Drosophila* Eiger is combating extracellular pathogens (Babcock et al., 2009). Thus, similarly to vertebrates, invertebrate TNF family ligands have both immunological and neuromodulatory functions (Linker et al., 2009). Yet there is no evidence that Wengen is involved in neurotrophin signalling in *Drosophila* similarly to mammalian $p75^{NTR}$. It is worth of studying because in developing visual system of *Drosophila* glia provide still unknown cues to prevent neuronal apoptosis (Dearborn and Kunes, 2004).

Intriguingly, *Drosophila* has an orphan receptor homologous to the mammalian neurotrophic factor GDNF receptor Ret (Sugaya et al., 1994) and Gfr-like receptor called munin, but lack direct homologues to GDNF family ligands (Airaksinen et al., 2006).

1.3 MANF/CDNF family of novel proteins

Mesencephalic astrocyte-derived neurotrophic factor (MANF), previously called ARMET (Arginine Rich, Mutated in Early Stage Tumours), was first identified as a gene mutated in renal cell carcinomas (ATG_{50} to AGG, SNP rs13091931) and because of the arginine rich amino terminal region named arginine rich protein (ARP) (Shridhar et al., 1996b). The same mutation, deletion of codon 50 in *ARP* gene, was also reported in lung, breast, prostate, pancreatic cancers and in squamous cell carcinoma (Shridhar et al., 1996a; Shridhar et al., 1997). However, these polymorphisms (PMIDs 9230196 and 10767373) were later shown to exist in normal tissues as well and are thus unrelated to tumours (Evron et al., 1997; Tanaka et al., 2000; Piepoli et al., 2006). Furthermore, transcriptional data indicate a shorter open reading frame with no arginine tract. The previously implied start codon occurred in a trinucleotide repeat region and was polymorphic, being ATG in some individuals versus AGG in the reference genome allele. This allele of ATG is restricted to higher primates with a better conserved start codon occurring three triplets downstream. According to Gene Bank there is no experimental evidence indicating which start codon is used *in vivo*.

Independently of these studies, MANF was found from a screen in a search for novel factors supporting dopaminergic neurons (Petrova et al., 2003). Another close paralog to *MANF* was found from vertebrate genomes called *CDNF* (Cerebral Dopamine Neurotrophic Factor; also known as ARMET-like1, ARMETL1) (Lindholm et al., 2007).

1.3.1 The structure of MANF and CDNF

MANF/CDNF proteins have a secretion signal but no pro-sequences to be cleaved for protein maturation (Petrova et al., 2003). Neither their protein sequence nor structure resemble of any known growth factors. Characteristic feature for MANF and CDNF proteins is eight cysteines with highly conserved spacing among multicellular organisms (Shridhar et al., 1996b; Petrova et al., 2003; Lindholm et al., 2007).

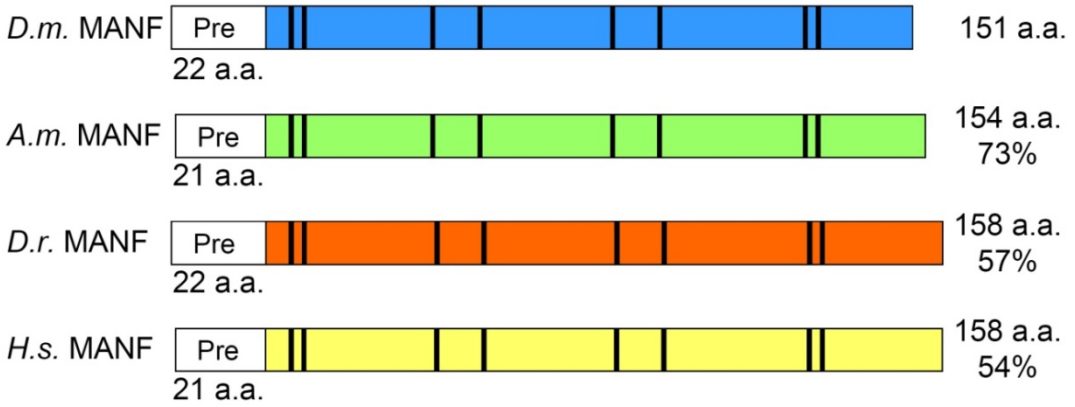

Figure 1. MANF protein family is evolutionarily conserved. Schematic organisation of MANF proteins of different species - *D.m.*,*Drosophila melanogaster*, fruit fly; *A.m.*, *Apis mellifera*, honeybee; *D.r.*, *Danio rerio*, zebrafish; *H.s.*, *Homo sapiens*, human. Black bars indicate conserved cysteines. Numbers ofamino acids are shown. Pre indicates the ER target signal sequence that is cleaved off before secretion.

The protein structure consists of seven α-helixes and four disulfide bridges. There are two patches of conserved lysines and arginines. For human MANF and CDNF both the crystal structure and solution structure has been solved (Parkash et al., 2009; Hoseki et al., 2010; Hellman et al., 2011). Commonly the structure of MANF and CDNF proteins contain two domains with positively charged surfaces and each domain fluctuates independently (Hoseki et al., 2010). The N-terminal domain resembles saposin-like proteins (SAPLIPs). The C-terminal domain with an intradomain disulphide bridge in a CXXC motif resembles the motif found in thiol/disulfide oxioreductases while its 3D structure resembles SAP (SAF-A/B, Acinus and PIAS) domain of saposins (Parkash et al., 2009; Hoseki et al., 2010; Hellman et al., 2011). Saposins (Sphingolipid Activator PrO[S]teINs) are small lysosomal proteins that serve as activators of various lysosomal lipid-degrading enzymes (Munford et al., 1995).They are thought to act by isolating the lipid substrate from the membrane surroundings, thus making it more accessible to the soluble degradative enzymes. CDNF and MANF are secreted highly soluble monomeric proteins at neutral pH 7 (Lindholm et al., 2007; Mizobuchi et al., 2007). In acidic pH they may oligomerize similarly to SAPLIPs (Ahn et al., 2006), at least CDNF at pH 4.6 crystallized as a dimer (Parkash et al., 2009). Differently from acidic saposins, MANF and CDNF are slightly basic proteins with pI values 8.5 and 7.7 respectively (Lindholm and Saarma, 2010). By definition saposins are small SAPLIPs known to interact with lipids and membranes (Bruhn, 2005) suggesting that the N-terminal domain of MANF/CDNF proteins may do it as well.

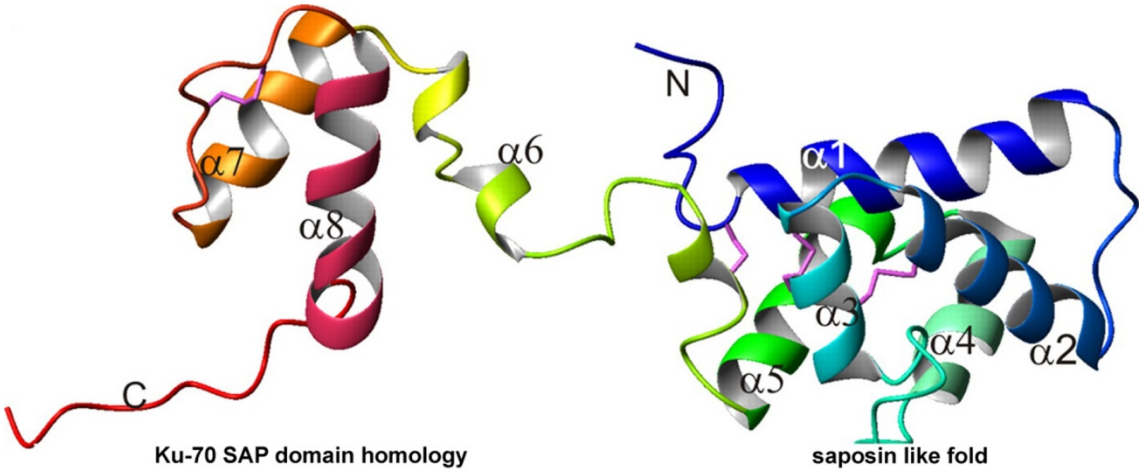

Figure 2. Schematic ribbon presentation of overall fold of MANF, colour-coded from N to C terminus starting from blue via green to red. N- and C-terminal domains are connected with flexible linker region. Disulphide bridges are shown in magenta. Modified from Hellman et al. (, 2011).

The backbone structure of MANF C-terminus is more flexible than that of N-terminus (Hoseki et al., 2010; Hellman et al., 2011). The C-terminal domain of MANF (C-MANF) is structurally similar to the SAP domain of Ku70 although their amino acid sequences show no clear homology. Ku70 in complex with Ku80 forms heterodimeric Ku-protein essential for non-homologous DNA double strand break repair (Walker et al., 2001). SAP domain is a short basic-helix-loop-helix like putative DNA/RNA binding

domain found in diverse nuclear and cytoplasmic proteins. SAP domain is thought to be involved in chromosome organisation but the exact role of their helical bundle is still a question mark (Aravind and Koonin, 2000). Importantly, the Ku70 SAP domain is known to bind in the cytoplasm to proapoptotic Bax (Bcl-2-associated X) keeping Bax in inactive form (Sawada et al., 2003). In apoptotic cells Ku70 dissociates from Bax which allows Bax activation and triggers mitochondrial cell death pathway (Sawada et al., 2003; Amsel et al., 2008; Gama et al., 2009). Recent study using cultured mouse dorsal root ganglia neurons in NGF deprivation induced cell death model confirm that the C-terminal part of MANF protected neurons as efficiently as Ku70 from death (Hellman et al., 2011).

The C-terminal motif of MANF and CDNF resembles classical endoplasmic reticulum (ER) retention signal KDEL – in human proteins RTDL and KTEL respectively, and RSEL in *Drosophila* Manf. It may function as an ER retention signal (Raykhel et al., 2007; Apostolou et al., 2008). There is evidence that in rat cardiomyocytes MANF is inefficiently retained in the ER and secreted during ER stress (Tadimalla et al., 2008) suggesting the weaker retention by RTDL signal for MANF than for KDEL-containing proteins.

1.3.2 MANF and CDNF protect dopaminergic neurons

MANF was identified from cultured rat ventral mesencephalic astrocyte type-1 cell line medium as promoting specifically the survival of dopaminergic neuronal cultures (Petrova et al., 2003). Importantly, both MANF and CDNF were shown to protect dopaminergic neurons in rat 6-OHDA (6-hydroxy-dopamine) PD model *in vivo* (Lindholm et al., 2007; Lindholm et al., 2008; Voutilainen et al., 2009). The 6-OHDA model is commonly used for preclinical studies to test novel therapeutic agents against PD (Ungerstedt and Arbuthnott, 1970). In this model 6-OHDA is injected unilaterally into striatum leading to retrograde degeneration of nigrostriatal pathway consisting of dopaminergic neurons (Sauer and Ortel, 1994). CDNF and MANF, injected prior the 6-OHDA injection into striatum were able to significantly reduce amphetamine-induced rotational behaviour in rats and protected TH-positive neurites in striatum and as well as TH-positive neuronal bodies in substantia nigra (Lindholm et al., 2007; Lindholm et al., 2008; Voutilainen et al., 2009). Furthermore, MANF and CDNF showed neurorestorative effect when injected 4-weeks after striatal 6-OHDA lesion. Importantly the MANF and CDNF treated animals show behavioural recovery of motor function after 6-OHDA lesion (Lindholm et al., 2007; Voutilainen et al., 2009). Recently CDNF has proven to protect and restore DA neurons also in MPTP treated mouse model of PD (Airavaara et al., 2011). Thus, MANF and CDNF efficiently keep the remaining DA neurons alive and restore their neurite network. In experimental stroke model the administration of recombinant MANF protein into the cerebral cortex before the lesion significantly reduced the infarction volume and apoptotic cell death in ischemic cortex (Airavaara et al., 2009). *Manf* has been shown to become transiently upregulated in adult rat brain after status epilepticus and global forebrain ischemia *in vivo* (Lindholm et al., 2008), suggesting the protective role of MANF for cortical and hippocampal neurons as well. The comparison of all three NTFs – MANF, CDNF and GDNF after the chronic infusion was performed (Voutilainen et al., 2011). After chronic infusion for three days

diffusion of MANF was better and it spread to significantly broader area around the infusion spot than GDNF. Both CDNF and GDNF were retrogradely transported from the striatum to the substantia nigra (Voutilainen et al., 2011). After 6-OHDA lesion chronically infused CDNF but neither GDNF nor MANF has a neurorestorative effect (Voutilainen et al., 2011). During the chronic infusion no behavioural signs of toxicity were observed with MANF, CDNF, or GDNF. These results imply that out of these three NTFs compared, CDNF's potential as a neuroprotective or even neurorestorative agent for the therapy of PD is most promising.

1.3.3 MANF in unfolded protein response (UPR)

1.3.3.1 Overview of the ER stress and UPR

ER is the cellular organelle responsible for synthesis, folding and modification of proteins destined to the secretory pathway and endosomal compartments. The data obtained from advanced microscopy 3D technique has revealed that ER is a dynamic and highly variable huge membrane network starting from nuclear envelope through interorganelle contacts to the peripheral ER (Friedman and Voeltz, 2011). ER is also an important site of Ca^{2+} storage and synthesis of sterols and lipids (Ron and Walter, 2007). Correct folding and modification of proteins in ER is susceptible for perturbation by various insults like oxidative stress, hypoxia, viral infection, and aging (Marciniak and Ron, 2006; Malhotra and Kaufman, 2007). Misfolded proteins in ER launch the ER-associated degradation pathway (ERAD) to translocate incorrectly folded proteins to the cytosol for proteasomal degradation. If the accumulation of misfolded proteins in the ER exceeds the capacity of ERAD, ER stress is induced to initiate UPR and restore ER homeostasis by activating the transcription of chaperones (McCracken and Brodsky, 2003). There are several excellent reviews written about ER stress and UPR (Mori, 2009; Hotamisligil, 2010; Walter and Ron, 2011).

In mammals there are three known ER stress sensors, the single-pass transmembrane proteins in ER membrane: PKR-like kinase (PERK), inositol-requiring protein-1 (IRE1) and activating transcription factor-6 (ATF6) (Malhotra and Kaufman, 2007). Invertebrates, exemplified through *Caenorhabtitis elegans* and *Drosophila*, have both IRE1 and PERK pathways participating in UPR. Even though ATF6 transcription factor is evolutionarily well conserved, its impact on UPR in *C. elegans* is minor (Mori, 2009) and in *Drosophila* unexplored. In normal stress-free ER the intraluminal domains of these ER stress sensors are in complex with several chaperone binding proteins (Bip/Gpr78). The release of the complex occur when misfolded proteins accumulate and compete for binding with the chaperone proteins (Bertolotti et al., 2000; Shen et al., 2002; Malhotra and Kaufman, 2007). The cytosolic domains of the sensors transduce the stress signal to elicit responses.

All these three branches of UPR function in parallel and have a unique signal transduction mechanism. The UPR branches are variable represented in different cell types (Walter and Ron, 2011). The IRE1 branch is the most ancient and the only branch in yeasts (Mori, 2009). IRE1 has a dual function as a kinase and ribonuclease. In response to misfolded protein accumulation in the ER, IRE1 oligomerises and this

induces the activation of its ribonuclease domain causing the cleavage of an intron out of mRNA of *Xbp1*, the bZIP transcription factor homologous to yeast Hac1 (Yoshida et al., 2001). Followed by ligation the Xbp1s (spliced) become activated to induce transcription of target genes. IRE1 undergoes autophosphorylation in response to misfolded proteins to form a dimer and further via transphosphorylation assembles oligomers (Korennykh et al., 2009). Activated IRE1 is able to destroy bulk of mRNAs bound to the ER membrane to avoid the mRNAs being translated in order to reduce the influx of proteins into ER (Hollien and Weissman, 2006).

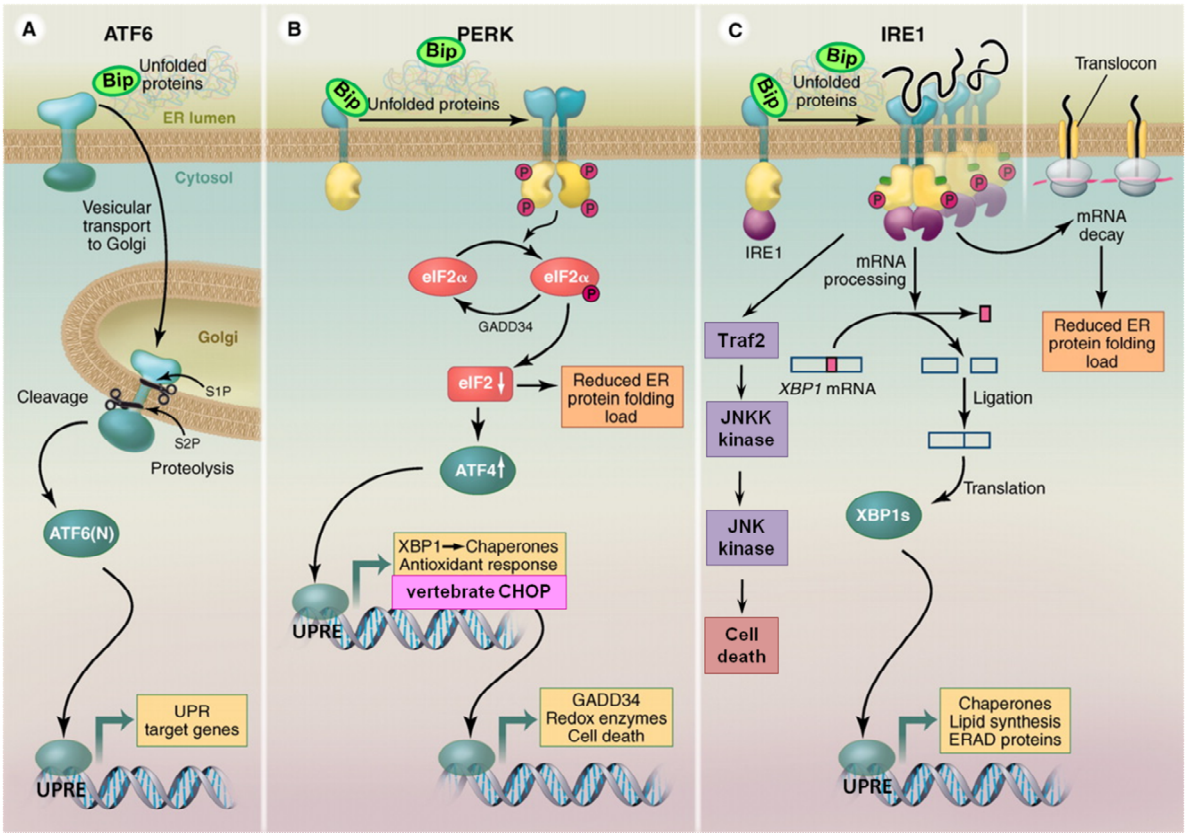

Figure 3. The three branches of the UPR (A to C). Under normal conditions metazoan three ER membrane bound signal tranducers (ATF6, PERK, and IRE1) are bound to Bip in the ER lumen. When the unfolded protein load exceeds, Bip is preferentially binding unfolded proteins and the sensors are released to activate bZIP transcription regulators. Activated ATF6(N), ATF4 and XBP1s move to nucleus to drive the transcription of UPR target genes. The target genes are encoding several chaperones increasing the protein folding capacity of the ER. Each sensor has a different strategy to proceed: ATF6 by regulated proteolysis, PERK by translational control and IRE1 by nonconventional mRNA splicing. In addition, PERK and IRE1 reduce the ER protein folding load by downregulating translation and degrading ER-bound mRNAs, respectively. In mammals, both PERK via CHOP and IRE1 via Traf2/JNK kinase can induce cell death. Modified from the review by Walter and Ron (, 2011).

ATF6 is synthesised initially as an ER transmembrane protein with large ER-lumenal domain (Figure 3.). Accumulated misfolded proteins induce the transport of ATF6 in vesicles from the ER to the Golgi apparatus where it is proteolytically

cleaved (Schindler and Schekman, 2009). The released amino terminal fragment ATF6(N) then moves to nucleus to induce transcription of UPR target genes. The third PERK branch after ER stress is commenced by PERK oligomerisation and autophosphorylation resulting in phosphorylation of translation initiation factor eIF2α (Figure 3.). The phosphorylation inactivates eIF2 and inhibits mRNA translation to reduce the novel protein load to ER. But the mRNAs with short open reading frames like *ATF4* become preferentially translated at low levels of eIF2 (Figure 3.) ATF4 in turn activates the transcription of CHOP (transcription factor C/EBP homologous protein) and several chaperone genes to release UPR. CHOP is inducing the proapoptotic genes thus leading to cell death.

There are three known cis-acting elements capable of binding to XBP1, ATF6 or both: unfolded protein response element (UPRE), ER stress-response element (ERSE) and ERSE-II respectively (Yamamoto et al., 2004). When activated these response elements activate the expression of chaperone genes to enhance the protein folding capacity and release the load of unfolded proteins.

1.3.3.2 MANF expression is induced by UPR

Several microarray studies have identified MANF as an unfolded protein response (UPR) upregulated gene (Holtz and O'Malley, 2003; Lee et al., 2003; Girardot et al., 2004). *In vitro* MANF is upregulated in several mammalian cell lines after induced UPR (Lee et al., 2003; Mizobuchi et al., 2007; Apostolou et al., 2008; Tadimalla et al., 2008).

MANF is induced mostly by ERSE-II promoter element (Mizobuchi et al., 2007) and 10 fold less efficiently by ERSE element (Tadimalla et al., 2008). Protein folding in the ER is an oxygen-dependent process and in hypoxia the protein folding becomes disturbed (Tu and Weissman, 2002). Therefore it is expectable that ischemia both in the brain and in the heart induces UPR leading to the accelerated transcription of *MANF* (Apostolou et al., 2008; Lindholm et al., 2008; Tadimalla et al., 2008). In cultured rat cardiomyocytes *MANF* is induced in response to ER stress by both ATF6 and Xbp1. When ERSE-II element in *MANF* promoter region was mutated the induction of MANF was 10 fold decreased suggesting the importance of ERSE-II element over ERSE in *MANF* induction (Tadimalla et al., 2008). In the same cell type endogenous MANF showed retention in the ER in stress free conditions but became robustly secreted once ER stress was induced (Tadimalla et al., 2008). The secretion of MANF protected the cultured cardiomyocytes effectively from apoptosis in stimulated ischemia conditions (Tadimalla et al., 2008). In several mammalian cell lines when ER stress was induced by various agents *MANF* but not *CDNF* was induced and upregulated in time-dependent manner (Apostolou et al., 2008). Silencing *MANF* by siRNA oligonucleotides in HeLa cells made the cells more susceptible to ER stress–induced death and surprisingly increased cellular proliferation. At the same time MANF overexpression inhibited proliferation and improved cellular viability under glucose-free conditions and tunicamycin treatment (Apostolou et al., 2008).

Interestingly, in the growth plate dysplasia model of matrilin-3 mutant (V194D) mice, when the chondrocytes develop ER stress, *MANF* is upregulated together with

well characterised ER stress chaperone genes *i.e. BiP* (Nundlall et al., 2010). Simultaneously to the mutant matrilin-3 retaining to ER causing ER stress, these mutant mice show decreased proliferation of chondrocytes and dysregulation of apoptosis leading to short-limb dwarfism (Nundlall et al., 2010).

Upregulation of *DmManf* has been shown after feeding tunicamycin to adult fruit flies indicating the involvement of DmManf in chemically induced UPR in *Drosophila* as well (Girardot et al., 2004).

1.3.3.3 ER- stress induced cell death

When the ER stress becomes excessive and prolonged overcoming the cellular capacity to neutralize it by ERAD and/or by UPR, the cell death pathways are activated. The exact molecular mechanisms regulating ER stress induced cell death are poorly studied. All three known UPR branches are bifunctional and can lead to both activation of protective or apoptotic signalling pathways. There are several general reviews dealing with the issue (Malhotra and Kaufman, 2007; Schröder, 2008; Hotamisligil, 2010; Walter and Ron, 2011). In mammals, during ER stress there is a delicate balance between proapoptotic (BH3 only, Bax) and anti-apoptotic proteins (Bcl-2 family) regulated by CHOP and JNK kinase pathway (Schröder, 2008). Once the executive caspases are activated the cell death commences fast, even in ten minutes. The preceding decisive step of regulation by the balance between pro- and antiapoptotic proteins is relatively slow and can take days. In addition, oxidative stress can contribute to ER stress induced apoptosis via IRE1/Traf2/JNK pathway (Malhotra and Kaufman, 2007).

1.3.3.4 Cellular redox homeostasis and protein disulfide bridge formation

In result of both oxidative and ER stress the reactive oxygen species (ROS) are generated, which increase the release of Ca^{2+} from ER to the cytoplasm. Ca^{2+} leak stimulates in turn the mitochondrial ROS generation and release of Cytochrome C from mitochondria pushing the cell towards apoptosis (Malhotra and Kaufman, 2007). Approximately 25% of the cellular ROS generation may be a consequence of disulphide bond formation in the ER called oxidative protein folding. Cellular redox homeostasis is maintained by interaction between reduced glutathione (GSH) and protein thiols with ROS. The cellular redox state is characterised by the ratio GSH to oxidised glutathione (GSSG); being in cytoplasm over 1:50 but in the ER lumen 1:1 to 3:1 (Hwang et al., 1992). The highly oxidative environment in the ER promotes the disulfide bond formation. In eukaryotes the disulfide bond formation is catalysed by ER oxidoreductases like thioredoxin-like protein disulfide isomerase (PDI) (Freedman, 1989). The active site of PDI contains cysteine residues CXXC resembling the motif found in MANF proteins (Parkash et al., 2009). In cysteine bond formation assisted by chaperones, the cysteines within CXXC of PDI accept two electrons from the substrate of polypeptide chain and become reduced. The reduced form of PDI is in turn oxidized by ER oxioreductin 1 (Ero1). The flavin-dependent reaction catalysed by Ero1 passes electrons directly to the final electron acceptor molecular oxygen with high potential to generate ROS (Malhotra and Kaufman, 2007). ROS may also be generated when glutathione reduces unstable and improper disulfide bonds. This would lead to glutathione depletion and return of thiols able to be reoxidised again by PDI/Ero1. In

consequence, an ineffective cycle of disulfide bond breakage and formation is generated, with each cycle ROS would be generated and GSH consumed. Does the PDI-like CXXC motif of MANF have any role in these processes remain to be proven.

1.4 UPR in *Drosophila*

Up to date most of the research on UPR has been carried on in yeast model and by using mammalian cell lines. Only recently the studies exploiting *Drosophila* have emerged reviewed by Ryoo and Steller (, 2007) and Rasheva and Domingos (, 2009).

The homologues to the main components of the UPR pathway are found in *Drosophila* genome (Table 1.). Pancreatic eIF-2α kinase showing 32% homology to human PERK (Sood et al., 2000), phosphorylates eIF2α *in vitro* and mediates attenuation of translation in yeast supporting its potential involvement in the UPR of *Drosophila* (Pomar et al., 2003). The overexpression of *Drosophila* PERK during eye development resulted in severely reduced and defective eye (Malzer et al., 2010). The screen for modifiers of PERK induced eye phenotype revealed *grapes*, the *Drosophila* orthologue of checkpoint kinase 1 (CHK-1) responsible for G2 cell cycle delay (Malzer et al., 2010). The knockdown of *grapes* rescued eye phenotype induced by PERK overexpression despite ongoing PERK activation suggesting CHK-1 as a link between ER stress and cell cycle arrest. In mammals, after PERK activation the synthesis of *ATF4* mRNA is induced (Harding et al., 2000). *Drosophila* has a homologue to *ATF4, cryptocephal* (*crc*), but lacks its downstream target CHOP involved in triggering UPR-induced apoptosis in mammals (Zinszner et al., 1998). Crc has been shown to be involved in ecdysis and molting behavior of the fruit fly (Hewes et al., 2000) but its involvement in UPR in *Drosophila* has yet to be shown.

There are homologues in *Drosophila* to mammalian chaperones BiP/GRP78, calnexin and calreticulin (Table 1.). The later form another class of chaperones specifically promoting the folding of glycosylated proteins (Ellgaard and Helenius, 2003). When the glycoproteins after several rounds of binding to calreticulin and calnexin still fail to fold correctly, they are recognized by EDEM (ER degradation enhancing α-mannosidase-like protein) interacting with translocation channel in ERAD protein degradation pathway (Hosokawa et al., 2001; Molinari et al., 2003). There are two EDEM homologues in *Drosophila*, but no data about their involvement in ERAD. Together with chaperones several other enzymes are involved in protein folding processes. Both in mammals and *Drosophila* there are several cis–trans peptidyl–prolylisomerases, which catalyze the isomerisation of peptidyl–propyl bonds (Price et al., 1991). *Drosophila* homologue of Ero1, Ero1L, when mutated leads to the accumulation of misfolded Notch in the ER and activates UPR (Tien et al., 2008).

There is little known about ER stress induced cell death in *Drosophila*. The mammalian transcription factor CHOP activating apoptosis downstream PERK signalling has no homologues in *Drosophila*.

Table 1. *Drosophila* homologues to known human proteins involved in UPR according to Flybase (http://www.flybase.org)

Human homologue	Fruit fly	Flybase Gene ID	Description
IRE-1 α/β	IRE-1	*CG4583*	ER membrane bound endoribonuclease
Xbp-1	Xbp-1	*CG9415*	bZIPtranscription factor
PERK	PEK (Sood et al., 2000)	*CG2087*	ER membrane bound protein kinase
ATF6 α/β	ATF6	*CG3136*	ER membrane bound transcription factor
ATF4	cryptocephal	*CG8669*	bZIP transcription factor
GADD34	Gadd34	*CG3825*	Protein phosphatase 1 binding
S1P, S2P	S2P	*CG8988*	proteases cleaving ATF6
CHOP	-	-	CCAAT/enhancer binding protein, bZIP transcription factor
BiP/GRP78	Hsc70-3	*CG4147*	Glucose RegulatedProtein 78
Calnexin	CG9906 (Christodoulou et al., 1997)	*CG9906*	chaperone promoting the folding of glycosylated proteins
Calnexin	Calnexin 99A (Rosenbaum et al., 2006)	*CG11958*	chaperone promoting the folding of glycosylated proteins
Calreticulin	Calreticulin (Bourbon et al., 2002)	*CG9429*	chaperone promoting the folding of glycosylated proteins
EDEM	EDEM1 EDEM2	*CG3810* *CG5682*	ER degradation enhancingα-mannosidase-like protein
96 genes		27 genes	cis–trans peptidyl–prolylisomerases catalyze the isomerisation of peptidyl–propyl bonds
Ero-1	Ero1L	*CG1333*	ER oxidoreductin-1 , thiol oxidase, catalyze disulfide bondformation
PDI	Pdi	*CG6988*	catalyze disulfide bondformation
Bcl-2	DEBCL Buffy	*CG33134* *CG8238*	roapoptotic Bcl-2 family members
Traf2 (Traf 1-6)	Traf-like (Traf3) Traf4 (Traf1) Traf6 (Traf2)	*CG4394* *CG3048* *CG10961*	TNF receptor associated factor

The two *Drosophila* Bcl-2 family members, DEBCL and Buffy, are nonessential during developmental cell death but mediate irradiation induced apoptosis (Sevrioukov et al., 2007; Galindo et al., 2009). In *Drosophila* these proteins localise to distinct intracellular

surfaces - DEBCL to mitochondria and BUFFY to ER (Doumanis et al., 2007). Their role in ER stress induced apoptosis is still unknown.

In both human and *Drosophila* the apoptosis in photoreceptors as a consequence of permanent ER stress is caused by certain mutations in *rhodopsin* gene (Humphries et al., 1992; Colley et al., 1995; Davidson and Steller, 1998). The accumulation of mutated misfolded Rhodopsin-1 activates Ire1/Xbp1 branch of UPR (Ryoo et al., 2006). Retinal degeneration commences even further when Xbp1 levels are simultaneously reduced showing that Ire1 via Xbp1 pathway protects photoreceptors from degeneration (Ryoo et al., 2006). Accumulation of misfolded Rhodopsin-1 activates the JNK pathway and caspase-dependent apoptosis (Davidson and Steller, 1998; Galy et al., 2005). Mammalian IRE1 binds to Traf2 and induces cell death via Jun kinase pathway (Urano et al., 2000). There are six Traf factors in mammals and three in *Drosophila*. The signalling pathways downstream *Drosophila* Traf1 and Traf2 are leading to endogenous JNK activation and NF-κB mediated immune response respectively (Cha et al., 2003). The impact of *Drosophila* Trafs in UPR induced apoptosis is yet unknown. Instead, independently of the three UPR sensing pathways the inactivation of *cdk5* and *mekk1* genes resulted in delay of the age related retinal degeneration and prevented JNK phosphorylation (Kang et al., 2012). The exact upstream signals for CDK5 remain to be identified, but Ca^{2+} signalling and ROS have been previously implicated.

The third branch of Ire1 promotes the degradation of ER bound mRNAs to diminish the protein synthesis during ER stress (Hollien and Weissman, 2006). Altogether, all three known branches downstream of Ire1 are present in *Drosophila*.

In conclusion, the genes coding for proteins involved in UPR are well conserved in evolution between mammals and *Drosophila* with the exception of transcription factor CHOP regulating apoptosis. Still, for many conserved components of UPR in *Drosophila* the functional data is missing. Both Ire1 and PERK pathways are well established in *Drosophila* UPR as the impact of ATF6 remains elusive.

1.5 Neurodegenerative diseases – an interplay between UPR and autophagy

The common hallmarks of the late phase of neurodegenerative diseases are the specific abnormal protein aggregates or inclusions in the brain tissue of patients, indicating defects in protein folding and proteasome degradation machinery. These diseases are PD (α-synuclein, inclusions called Lewy bodies), amyotrophic lateral sclerosis (ALS; mutated SOD aggregations), Alzheimer's disease (AD; neurofibrillary tangles of hyperphosphorylated Tau and β-amyloid aggregations), Huntington's disease (HD; huntingtin with over 35 polyglutamine repeats aggregates (Scheper et al., 2011), prion-related disorders, and many others (Matus et al., 2008; Matus et al., 2011). These abnormal protein aggregates in neurons cause neurological impairment and finally lead to neuronal loss. For AD it is established that UPR activation is an early phenomenon found in AD neurons caused by hyperphoshorylated tau (Scheper et al., 2011). In PD increasing evidence suggests chronic ER stress as a common feature associated with several PD-linked genes and sporadic PD models. UPR markers were reported in the post-mortem brain samples of PD patients (Hoozemans et al., 2007). Normally

aggregated misfolded proteins are sent for degradation to proteasome or destroyed in the cell by macroautophagy (hereafter referred as autophagy). For proteasome degradation the misfolded proteins need to be unfolded to fit the proteasome pore. So the only way to get rid of the aggregated and terminally misfolded proteins inside the cell is by autophagy. Autophagy occurs in all tissues at basal level (Ravikumar and Rubinsztein, 2004), but its activation preferentially occurs under starvation and when damaged organelles or terminally misfolded proteins are present (Levine and Klionsky, 2004; Martinez-Vicente and Cuervo, 2007). Autophagy is a catabolic process in which lysosomes degrade intracytoplasmic contents in double-membraned autophagosomes. Autophagosomes are formed by the elongation and fusion of phagophores derived from preautophagosomal structures originating from the plasma membrane and other membraneous structures like the endoplasmic reticulum and mitochondria. Autophagy has been found to be critical for the maintenance of neuronal homeostasis and has a role in elimination of misfolded, ubiquinated proteins. Mice defective for autophagy die soon after birth, and conditional neuronal knockouts of such genes cause neurodegeneration accompanied by formation of inclusions (Hara et al., 2006; Komatsu et al., 2006). Recently it has been shown that α-synuclein overexpression itself can inhibit autophagy in mutant mouse (Winslow et al., 2010). Autophagy is a key route for the degradation of intracytoplasmic aggregate-prone proteins (which is a feature of most neurodegenerative diseases) and is also a disposal route for dysfunctional organelles (for example mitochondria implicated in many diseases, including PD). One disease-associated autophagy substrate is mutant huntingtin. The proportions of cells with mutant huntingtin aggregates increase when autophagy is impaired (Ravikumar et al., 2002). Therefore the percentage of cells with mutant huntingtin aggregation can serve as a sensitive indicator of autophagic substrate clearance (Klionsky et al., 2008).

1.6 Dopamine in the fruit fly development

Despite the evolutionary distance between mammals and *Drosophila*, functional parallels exist between the fly and human dopaminergic systems – in both dopamine (DA) plays a role in modulating locomotor activity, response to stress, and response to drugs of abuse. The DA synthesis and signaling pathways are as well highly conserved between vertebrates and invertebrates. In a fruit fly, DA besides acting as a neurotransmitter, has multiple other roles and is essential for normal development (Neckameyer, 1996). *Drosophila*'s exoskeleton, the cuticle, is build up of layered glycosylated carbohydrate polymers cross linked to proteins via DA derived quinones (Wright, 1987). Mutations affecting the DA synthesis pathway in *Drosophila* (*pale, Ddc*) result in unpigmented cuticle and lethality during the late embryonic stages. Thus, DA and its precursor L-DOPA are important for cuticle sclerotisation and pigmentation (Wright, 1987; Neckameyer and White, 1993). There is subset of DA neurons present already at embryonic stages and later developing into adult network responsible for synaptic modulation, behavioural plasticity, memory retrieval, feeding and motor behaviour, and circadian rhythms (Neckameyer, 1996; Monastirioti, 1999; Hamasaka and Nässel, 2006). Depletion of DA delays the development of the fruit fly (Neckameyer, 1996).

1.6.1 The dopamine synthesis

DA is synthesized from tyrosine and the first rate limiting enzyme is tyrosine hydroxylase (TH), coded by *pale* (*ple*) in *Drosophila* (Neckameyer and White, 1993). TH converts L-tyrosine to L-dihydroxyphenolalanine (L-DOPA). L-DOPA is then turned into DA by L-aromatic amino acid decarboxylase (also dopamine decarboxylase; Ddc); at rates so rapid that normally the brain L-DOPA levels are negligible. Because endogenous levels of L-DOPA are normally low, the formation of DA can be enhanced dramatically by providing Ddc with the increasing amounts of L-DOPA. Normally the amount of tyrosine at the cell is relatively high and above the Km of TH, making the level and activity of TH crucial for the rate of DA synthesis. The activity of TH is regulated by binding to its cofactor tetrahydrobiopterin (BH_4) and by the end product DA together with other catecholamines competing for the binding site of the enzyme accomplishing end-product inhibition (Cooper et al., 2003). The available amount of BH_4 is also important, regulated by guanosine triphosphate (GTP) cyclohydrolase coded by *Punch* (*Pu*) locus in *Drosophila* (Mackay and O'Donnell, 1983). The regulation of the activity of TH to bind $BH_4$is thought to involve direct phosphorylation from low- to high affinity form of TH, depending on neuronal firing state (Haavik et al., 1990; Almas et al., 1992; Vie et al., 1999).

1.6.2 Dopamine as a neurotransmitter

Released from the nerve terminals DA binds to its G-protein coupled receptors. There are two types of dopamine receptors, D1 and D2 type with different outcomes – D2 type receptors inhibit adenylate cyclase as D1 type receptors stimulate adenylate cyclase. The DA receptors are further classified by their location as postsynaptic receptors (both D1 and D2 type) or presynaptic autoreceptors (D2 type). DA by acting via D1 type receptors stimulates adenylate cyclase via G protein and increases cAMP formation, which activates cAMP dependent protein kinase (protein kinase A, PKA).

In *Drosophila*, there are two D1 type receptors – DAMB1 and dDA1/DmDOP1 (Blenau and Baumann, 2001; Kim et al., 2003). In the fly CNS, DAMB displays highly restricted expression to the mushroom body involved in memory and learning (Han et al., 1996) while dDA1/DmDOP1 is expressed in larvae and adults both in mushroom body and in the ventral ganglion (Kim et al., 2003). The only D2-like receptor DD2R has multiple alternative splicing variants with predominant expression in the adult head (Hearn et al., 2002).

There is a third type of receptor for DA in arthropods and *Drosophila* responding to both DA and steroid hormone, Dopamine/Ecdysteroid receptor *CG18314* (DopEcR). Interestingly, DopEcR, another G-protein coupledreceptor, shows sequence homology with vertebrate β-adrenergic receptors and is activated by DA to increase cAMP levels and to activate the phosphoinositide 3-kinase pathway. Conversely, ecdysone and its active form ecdysone-20E show high affinity for DopEcR in binding studies and inhibit the effects of DA, coupling the receptor to a rapid activation of mitogen-activated protein kinase pathway (Babcock et al., 2009). DopEcR is expressed in adult eye and central nervous system. The temporal expression profile ranges from extremely low expression through of a peak of moderately high expression observed within 18-24 hour embryonic stages and during late pupal stages (http://www.flybase.org).

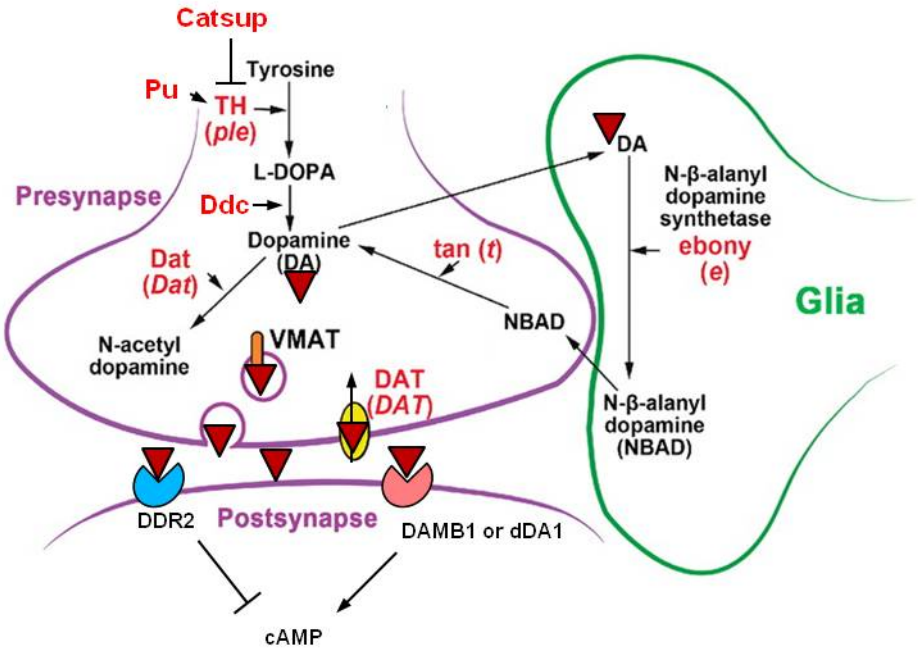

Figure 4. DA synthesis and metabolism in *Drosophila* synapse. DA synthesis takes place in presynaptic nerve terminal and depends on tyrosine taken in from hemolymph. Intracellular DA is packed via VMAT into vesicles released by synaptic activity. Released DA binds to different types of DA receptors of postsynaptic dendrites. The overspill of DA is taken in by surrounding glia and metabolised into less active metabolites then returned to the nerve terminal. Modified from Shao et al. (, 2011)

DopEcR may represent the *Drosophila* homologue of vertebrate γ-adrenergic receptors, which modulate various activities of the brain, blood vessels, and pancreas. Thus, in arthropods DopEcR couples the ecdysteroid molting regulation to the modulation of the nervous system and behaviour.

Released DA in nerve terminals must be up taken to terminate the action of neurotransmitter and restore cellular homeostasis. The uptake of DA is mediated by highly conserved in evolution DA transporter (DAT). DAT is a target of many drugs, for example cocaine binds directly to DAT and prevents DA uptake thus prolonging the influence of DA in the synaptic cleft. On the other hand, several neurotoxins like MPTP have a high affinity to DAT and are transported into neurons via DAT (Cooper et al., 2003). *Drosophila* DAT has also a high affinity towards cocaine and its preferred substrates are DA and tyramine whereas octopamine is transported less efficiently and serotonin not at all (Porzgen et al., 2001).

Intracellularily the level of free DA is kept low by another membrane transporter, vesicular monoamine transporter (VMAT), packing DA into intracellular vesicles. *Drosophila* has one gene coding for VMAT transporting monoamines (DA, serotonin, octopamine etc.) into synaptic vesicles (Greer et al., 2005). VMAT modulates the concentration of free DA in presynaptic nerve terminals and decreases the amount of free DA in the cytoplasm.

1.6.3 The localisation of DA synthesising cells in *Drosophila*

In order to study DA distribution in animal tissues, either TH or DAT has been broadly used. In *Drosophila*, the early embryos (0-8 hours after egg laying, AEL) lack TH expression and only at 12-16 hours AEL strong TH expression is detectable in tissues of ectodermal origin (foregut, hindgut, anal pads, part of proventriculus) and in certain midline neurons (8-12 hours AEL) (Neckameyer, 1996). Further studies have identified these neurons originating from unpaired mesectodermal midline precursor 3 (MP3) neurons (Wheeler et al., 2006) comparable to the grasshopper's and *Manduca sexta*'s DA synthesising H-cell (Goodman et al., 1981; Mesce et al., 2001). Recent studies mapping the developmental embryonic identity of the *Drosophila* H-cell showed that DA identity is under the control of transcription factors lethal of scute and islet activating the downstream genes as *DAT*, *ple*, *Ddc*, and *VMAT* (Stagg et al., 2011). In addition to the midline DA positive neurons, there are two dorso-lateral rows of DA positive neurons in each hemisegment in the ventral nerve cord, expressing TH from embryonic stage 16 onward. The presence of DA is detectable 18 hours AEL (Budnik and White, 1988). Majority of the DA neurons are interneurons found in bilateral clusters. In *Drosophila* larval CNS, the DA neurons have been characterised (Budnik et al., 1986; Budnik and White, 1988; Lundell and Hirsh, 1994) to consist of four clusters of 4-10 neurons in the brain lobes and three to five paired (lateral) or unpaired (medial) neurons per segment in the subesophagial, thoracic and abdominal ganglia (Friggi-Grelin et al., 2003). In 3rd instar larvae there are approximately 80 DA neurons detectable (Budnik et al., 1986). In adult fruit fly brain six to eight neuronal DA clusters have been identified projecting to specific regions of the mushroom body and to the central complex (Budnik and White, 1988; Nässel and Elekes, 1992; Hamasaka and Nässel, 2006). Recently, lineage tracing experiments revealed seven neuroblast lineages responsible for the generation of the larval central brain DA neurons during embryogenesis (Blanco et al., 2011).

In adult females, TH mRNA expression is found in fly ovaries, in the nurse cell cytoplasm and in follicle cells through the first 12 stages of egg chamber development (Neckameyer, 1996). Later in mature eggs TH expression becomes abolished. When newly eclosed females are fed with DA inhibitors, the fertility and ovary development become altered (Neckameyer, 1996). Studies with TH inhibitors revealed that in *Drosophila* larvae the abolishment of DA resulted in behavioural akinesia and loss of exploratory behavior, which influences larval feeding and locomotion, and finally leads to the developmental delay and death of larvae (Neckameyer, 1996).

1.6.4 *Drosophila* mutants related to DA synthesis

The mutants of *ple*, *Ddc*, and *Pu* in *Drosophila* are all embryonic lethal and show loss of pigmentation in mouth parts (Mackay and O'Donnell, 1983; Wright, 1987; Neckameyer and White, 1993). In *Drosophila*, there is a negative regulator of TH activity, *Catecholamines up* (*Catsup*), named so because in its mutant the biogenic amines are upregulated (Stathakis et al., 1999). Catsup is a membrane protein with protein homology to human zinc transporters (http//:www.flybase.org). Biogenic amines including DA are also released into hemolymph acting as neurohormones far from the

site of release (Coleman and Neckameyer, 2004).In mammals the inactivation of biogenic amines is established by monoamine oxidase or catechol-O-methylase-transferase. In insects, these enzymes are in low abundance or absent and biogenic amines are inactivated mainly by N-acetylation or O-sulphation (Wright, 1987). In *Drosophila*, β-alanyl conjugation is mediated by ebony protein, the β-alanyl synthase (Borycz et al., 2002). In *ebony* mutants the body color of the flies is darkened, almost black. Interestingly, in adult fly CNS ebony is expressed by glia and is involved in circadian rhythm maintenance and regulation of DA metabolism (Shao et al., 2011). β-alanyl-dopamine is further degraded by β-alanylhydrolase (coded by *tan* gene in *Drosophila*) to β-alanine and dopamine. *Tan* mutants have also altered body color, brownish yellow. Thus in *Drosophila*, altered dopamine levels reflect in pigmentation – the larval mutants of lacking DA (*pale*, *Ddc*) have lighter coloured or colourless mouth parts. Furthermore, *Catsup* and *Pu* mutations cause melanotic salivary glands and cuticle defects (Stathakis et al., 1999). Cuticular proteins are crosslinked by DA derivates, synthesised by phenol oxidases (*e.g.* Yellow). Mutations of genes coding ABC transporters affect eye colour - *white*, and its binding partners *brown* and *scarlet* –result in reduced biogenic amine levels, including low levels of DA. Interestingly, in wild-type head homogenates most of the biogenic amines are found in the vesicle-rich fraction, whereas they are found in the supernatant fractions from *white*, *brown* and *scarlet* flies suggesting deficiency in transport of biogenic amines into vesicles (Shieh, 2011).

1.7 Development of the *Drosophila* cuticle

In arthropods, both the role of a skeleton and a protective skin is maintained by the exoskelton, the cuticle. Cuticle is secreted at the end of embryogenesis as an apical extracellular matrix by the epidermis and the epithelia of trachea, hind- and foregut (reviewed by B. Moussian, 2010). When the larvae grow, the cuticle is renewed by each molt - finally the adult cuticle is formed during metamorphosis. The insect cuticle has a horizontally layered structure made of polysaccharide chitin, proteins and lipids. The classical terminology divides the cuticle by biochemical and physiological properties into outer chitin-free layer epicuticle and the inner chitinous procuticle (Rewitz et al., 2006). By ultrastructure it is possible to divide epicuticle into two sublayers – inner and outer epicuticle or cuticulin; and the procuticle into endocuticle and exocuticle. According to the mode of establishment the cuticle can be divided into three layers: the envelope (outer epicuticle/cuticulin layer), the epicuticle (inner epicuticle) and the procuticle (Almas et al., 1992; Payre, 2004).

Drosophila embryonic epidermis is composed of a single layer of cells with apical part of well defined microvillae thought to enhance the secretory capacity of these cells. First, starting from the embryonic stage 16, the envelope is established on the surface of plasma membrane, above the tips of microvillae (Almas et al., 1992). Next, the epicuticle is assembled underneath of the envelope composed of proteins, lipids and quinones secreted to the valleys between microvillae. Towards the hatching the epicuticle is gradually thickening by constant secretion, synthesis, and traffic of cuticular components (Moussian, 2010). The mutants of genes involved in secretion – t-SNARE *Syntaxin-1A* or Sec23/24 homologues *ghost* and *haunted* – have disrupted epicuticle formation and in consequence thin cuticle with almost missing microvillae (Moussian et

al., 2007). These outer two non-chitinous layers are hydrophobic by nature and protect the animals against dehydration and swelling (Urano et al., 2000). In order to further improve the shielding power of the outer epicuticle, a wax and cement layer is added to the surface of cuticulin layer after hatching.

The thick procuticle provides the cuticle with strength and elasticity due to the helicoidal lamellar structure of chitin microfibrils. Chitin is a polysaccharide composed of polymerised N-acetylglucosamine (GlcNAc) residues. The central enzyme linking the GlcNAc residues together, chitin synthase, localises at the tips of microvillae and extrudes the synthesised chitin fibers across the plasma membrane (Merzendorfer, 2011). In *Drosophila*, the chitin synthase is encoded by *krotzkopf verkehrt* (*kkv*) (Moussian et al., 2006). Lack of chitin in the cuticle of *kkv* mutants causes specific embryonic bloated phenotype as a result of detachment of the cuticle from the epidermis and disintegration of the epicuticle (Moussian et al., 2006). So the formation of epicuticle is dependent of the correctly layered chitinous procuticle as the mutations altering chitin microfibrillae formation or organisation disrupt the uniformity of the

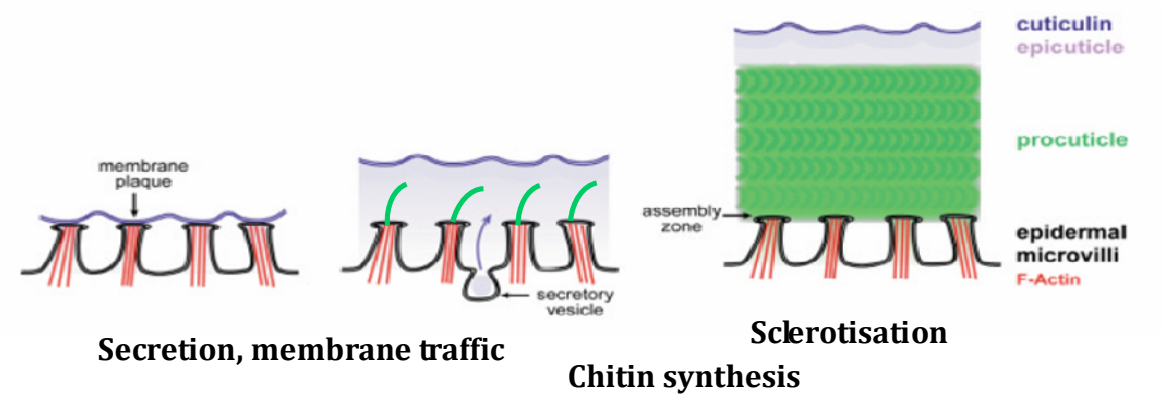

Figure 5. Scheme of arthropods cuticle developmentand formation of different layers.
The outer-most layer cuticulin (or envelope, in blue) is deposited first at the top of microvilli of the apical part of epidermis. Next the epicuticle (pale blue) consisting of proteins, lipids and quinones is formed by apical secretion of vesicles from the valley between the microvillae and deposited below the cuticulin. The most prominent layer, procuticle, is made of laminae of chitin microfibers (green). Adapted from Locke and Payre, (Locke, 2001; Payre, 2004).

epicuticle (Moussian et al., 2005; Moussian et al., 2006). Procuticular chitin microfibrillae are further stabilised by chitin binding proteins and quinone crosslinking. In *Drosophila*, important chitin-binding proteins are membrane bound knickkopf (knk) and retroactive (rtv). In both, *knk* and *rtv* mutants the chitin microfibrils are disorganised (Moussian et al., 2006). Interestingly, mutations in the ETS-type transcription factor *grainyhead* (*grh*) cause similar phenotypes as mutations in *knk* and *rtv* (Ostrowski et al., 2002) implying grh as one of the main regulators of cuticle development. Despite the fundamental differences between arthropods cuticle and mammalian skin, the role of grh in preserving epidermal integrity has been conserved between *Drosophila* and mice (Mace et al., 2005; Ting et al., 2005).

The strength of the cuticle is further improved by sclerotisation carried on by quinones crosslinking the proteins. Quinones are derived from cytoplasmic DA

precursors N-acetyldopamine (NADA; synthesising enzyme DA N-acetyltransferase) and N-β-alanyldopamine (NBAD; synthesising enzyme NBAD synthase encoded by *ebony*). NADA and NBAD together with other DA derivates are released to the developing cuticle by a still unknown mechanism (Moussian, 2010). Phenol oxidases residing in the cuticle use these DA derivates as substrates to catalyse the quinones for sclerotisation and pigmentation of the cuticle.

In order to grow the arthropods must shed the old cuticle and generate a new larger one. This molting process is regulated by the steroid hormone ecdysone synthesised and secreted by the ring gland. Ecdysone is synthesised from cholesterol, and there is a group of mutants involving mutations in components of ecdysone synthesis pathway called the Halloween group because of their ghost like phenotype with undifferentiated cuticle. Most of these genes (*phantom*, *spook*, *spookiest*, *disembodied*, *shadow*, *shade* and *ghost*) belong to the Cytochrome P450 family and catalyse different steps in the pathway from cholesterol to ecdysone synthesis (Rewitz et al., 2006).

Altogether, the correct construction and stabilisation of the procuticle in combination with the epicuticle formation is a highly complicated task for an epidermal cell. No wonder that several mutations disrupting the establishment of the cuticle are embryonic lethal.

2 Aims of the study

Neurotrophic factors have been characterised in vertebrates to control the neuronal numbers during development and to support the neuronal survival and modulate activity. In *Drosophila* and other invertebrates the clear homologues to mammalian neurotrophic factors have been missing. MANF/CDNF family of novel mammalian neurotrophic factors have been described as the first family of neurotrophic factors highly conserved in evolution. The aim of this work was to characterize the *Drosophila* homologue of MANF/CDNF family, to investigate its role in fruit fly development and to answer the question is DmManf functioning as an invertebrate neurotrophic factor.

The specific aims were:

- To characterise the expression of DmManf in fruit fly embryonic and larval development
- To generate the null mutants for *DmManf*
- To resolve the orthology between human and *Drosophila* Manf
- To study the functional homology between mammalian and fruit fly Manf in supporting the dopaminergic neurons
- To study and compare the alterations in gene expression in *DmManf* mutant and overexpression conditions

3 Material and methods

Methods applied in this work are described in detail in original publications as follows in Table 1.

Table 1. Methods used in this study.

Method	Applied in Publication		Unpublished
Nucleic acid methods	I	II	
PCR, cloning and DNA sequencing	I	II	
RNA isolation and reverse transcription	I	II	
Northern blotting	I		
in situ hybridisation	I		
Quantitative PCR		II	
aaRNA amplification and hybridisation		II	
Immunological methods	I	II	
Antibody production	I		
Immunohistochemistry	I	II	X
Western blotting	I	II	
Cell culture methods	I		
Transfection	I	II	
Generation of stable transfected line	I	II	
Imaging and image analysis	I	II	
Light microscopy	I		X
Laser confocal microscopy	I	II	X
Transmission electron microscopy	I	II	
Fixation for TEM of *Drosophila* embryos	I	II	
High pressure freezing of larvae		II	
***Drosophila* methods**	I	II	
Generation of transgenic flies	I		
P-element excision mutagenesis	I		
ovo^{D}female sterile technique	I	II	
FRT mitotic recombination	I	II	
GAL4/UAS ectopic expression system	I	II	X
Bioinformatical methods		II	
R platform, Bioconductor Limma Package		II	
DAVID annotation tools		II	
Dopamine measurements	I		

Filipin staining of embryos to visualise cholesterol was made by incubation of dechorionated by hand embryos in filipin (Sigma, F-9765) 0.05 mg/ml solution in PBS.

Table 2. Antibodies used in this study

Antigen/ code	Host	Source	Comments
BP102	mouse	DSHB	embryonic CNS
β-Galactosidase	rabbit	Cappel	for LacZ constructs
eIF2α Ser51-phospho	rabbit	Cell Signalling	
human MANF	rabbit	Mart Saarma's group	
human CDNF	rabbit	Mart Saarma's group	
cleaved Caspase 3	rabbit	Cell Signalling	batches 13, 17
cut	mouse	DSHB	PNS, Malpighian tubules
crumbs	mouse	DSHB	epithelial cells
Ddc	rat	Jay Hirsch	DA, 5-HT neurons, epidermis
dlg	mouse	DSHB	postsynaptic
DmManf	rabbit	Johan Peränen	
elav	mouse	DSHB	pan-neuronal
engrailed/invected	mouse	DSHB	specific neuronal
fasciclin II 1D4	mouse	DSHB	NCAM homologue in fly; neuronal fascicles
futsch 22C10	mouse	DSHB	Mab2 homologue; sensory neurons mostly
peroxidasin	mouse	John H. Fesler	hemocyte marker
phospho-Histone 3	rabbit	Uptake	Mitotic chromosomes
repo	mouse	DSHB	pan-glial marker except midline glia
islet	mouse	DSHB	motoneurons
TH	mouse	DiaSorin	DA neurons and epidermis
TH	mouse	PelFreez	DA neurons and epidermis
TH	rabbit	Wendy Neckameyer	DA neurons and epidermis
wrapper	mouse	DSHB	embryonic midline glia
tubulin	mouse	Sigma	ubiquitous loading control for Western blotting
twinfilin	rabbit	Gudrun Wahlström	ubiquitous loading control for Western blotting

Table 3. Fly strains used in this study

Genetic element	Source and/or reference
w^-; P{SUPor-P}KG0325	Bloomington Stock Centre (BSC), (Bellen et al., 2004)
w^-; ; *DmManf*$^{\Delta 96}$/TM6TbSb	I
C135-GAL4	Lori Hrdlicka,(Hrdlicka et al., 2002)
***da*^{32}-GAL4**	BSC, (Wodarz et al., 1995)
***Ddc*$^{HL8-3D}$-GAL4**	Jay Hirsh,(Li et al., 2000)
***elav*-GAL4**	Alicia Hidalgo
***gcm*-GAL4**	Angela Giangrande(Paladi and Tepass, 2004)
69B-GAL4	Jaakko Mattila, (Brand and Perrimon, 1993)
***repo*-GAL4**	Haig Keshishian, (Sepp and Auld, 1999)
TH-GAL4	Serge Birman, (Friggi-Grelin et al., 2003)
***tub*-GAL4**	BSC, (O'Donnell et al., 1994)
Dr^{Mio}/TM3, P{w^{+mC}=GAL4 twi.G}2.3, P{UAS-2xEGFP}AH2.3Sb^1 Ser^1	BSC(Halfon et al., 2002)
CQ2>T-LacZ	BSC (Fujioka et al., 2003)
w^-; ;UAS-*DmManf*^{133}	I
w^-;UAS-*DmManf*^{135}; UAS-*DmManf*^{133}	II
w-; UAS-*HsMANF*	I
w-, UAS-*HsCDNF*	I
UAS-mCD8-GFP	BSC
P{ry$^{+t7.2}$=neoFRT}82B P{w^{+mC}=ovoD1-18}3R/st^1 betaTub85D^D ss^1 e^s/TM3, Sb^1	BL2491, BSC(Chou and Perrimon, 1996)
y^1;P{ry$^{+t7.2}$=70FLP}3F/Dp(1;Y)y$^+$; TM2/TM6C, Sb^1	BL6941, BSC
***eagle*^{289}-LacZ**	Joachim Urban
Mz360 *eagle*-GAL4	Joachim Urban, (Ito et al., 1995)
UAS-*reaper*, *hid*, *grim*	Michael O´Connor, (Zhou et al., 1997)
***sqh*-EYFP-ER**	BSC
***sqh*-EYFP-mito**	BSC
***sqh*-EYFP-Golgi**	BSC

4 Results and discussion

4.1 Mapping *Drosophila Manf* expression

4.1.1 *Drosophila Manf* mRNA is expressed throughout the development and is maternally contributed

DmManf mRNA is present throughout *Drosophila* development and abundant already in very early embryos indicating high maternal contribution (I). Similar results were obtained by modENCODE Temporal Expression Profile microarray analysis showing that *DmManf* temporal expression profile ranges during *Drosophila* lifecycle from very high to moderately high expression with a peak within 6-18 hours AEL at embryonic stages and during early larval stages. (http://www.flybase.org/). *DmManf* is most prominently expressed in garland cells - the nephrocyte like cells with high endo- and exocytotic activity surrounding the proventriculus (I). Also salivary glands, the outer wall of the proventriculus, mesoderm, and CNS are *DmManf* positive (I).

4.1.2 In the embryonic nervous system DmManf is expressed in glia

We studied further the localisation of DmManf in the embryonic nervous system. DmManf antibody (I) recognised the same tissues as in situ RNA probe of *DmManf* and in addition the expression of DmManf was detectable at embryonic stage 15 onward in epidermis (I). In embryonic VNC, DmManf expression was highest in eagle-positive medial-most cell body glia surrounding TH-positive midline neurons (Urban and Technau, 1997) (I). Weaker expression of DmMANF was detected in longitudinal glia positive for transcription factor prospero and in engrailed-positive channel glia (I). We were unable to detect DmMANF expression in the midline glia positive for wrapper (I). DmManf protein was also present in hemocytes that express peroxidasin (Fig.6). During embryogenesis DmManf showed no co-localisation with any general or specific neuronal markers (elav, TH, engrailed) (I). In *Drosophila* embryonic CNS DmManf is expressed in cell body glial cells comparable to mammalian astrocytes (Ito et al., 1995). DmManf is a secreted protein. In mosaic *DmManf* null clones of ovarian follicle cells DmManf is detectable when secreted from neighbouring twin spot clone cells (I). The location of DmManf is in accordance to the cell nonautonomous neurotrophic factor theory, NTFs are secreted from neighbouring cells (glia or target cells *e.g.* muscles) to support neurons.

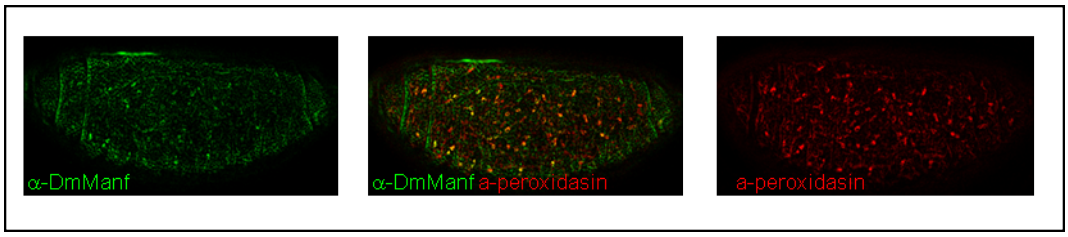

Figure 6. DmManf is expressed in embryonic hemocytes. DmManf expression (green; left image) at stage 16 embryos colocalises with hemocyte marker peroxidasin (red; right image), colocalisation of red and green channels shown in the middle image by yellow.

4.1.3 During larval stages DmManf is expressed in several tissues with secretory function

In line with MANF broader function in ER and UPR, DmManf is expressed in many tissues with secretory function – in salivary glands, garland cells, in glia, in some neurosecretory neurons, in the ring gland, gastric caeca, and Malphigian tubules (Figure 7 and unpublished data). In the ring gland, corpus cardiaca showed highest expression of DmManf (unpublished data). Furthermore, ubiquitous detectable amounts of DmManf are present in the fat body, imaginal discs, and muscles (Figure 7). According to FlyAtlas Anatomical Expression Data collected from microarray analyses *DmManf* mRNA expression is observed at high levels in the following larval organs or tissues: CNS, midgut, hindgut, Malpighian tubules, fat body, salivary gland, trachea, and carcass (http://flyatlas.org/atlas.cgi?name=CG7013-RA).

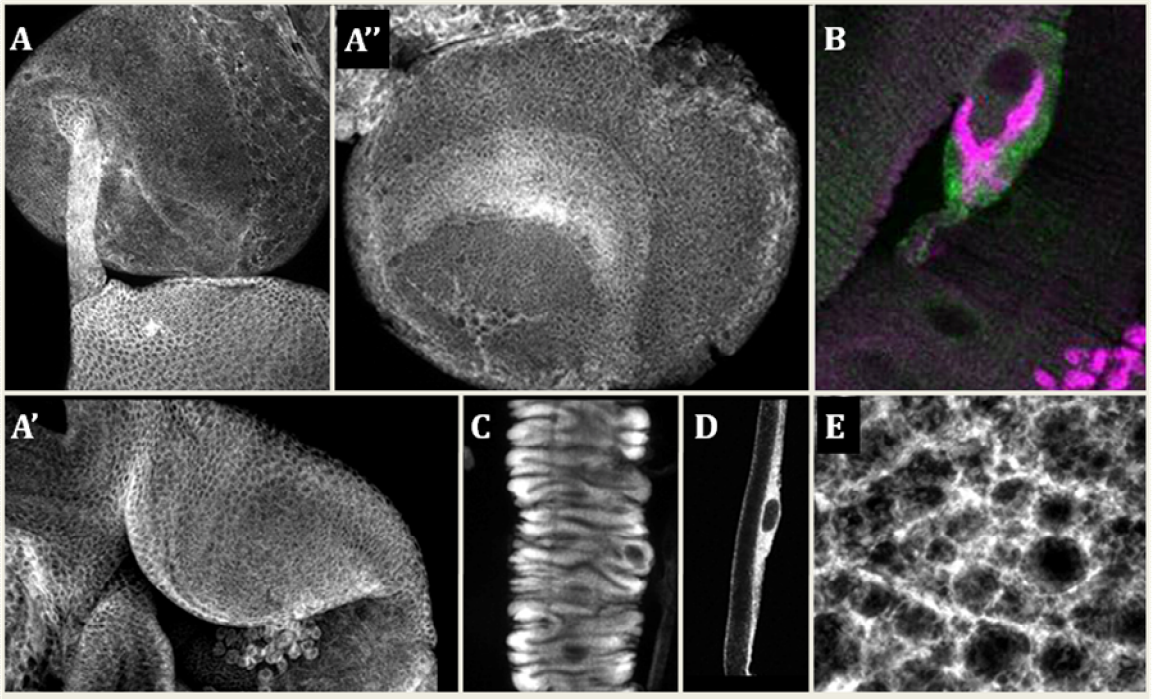

Figure 7. DmManf is expressed in several 3rd instar larval tissues. Anti-DmManf staining shown in white A,C-E and in green B. A-A'' larval brain lobes and eye disc A'. B – muscle with neuromuscular junction visualised by postsynaptic marker disc large (purple). Gastric caeca (C), trachea (D), and fat body.

4.1.4 Subcellular location of DmManf

To find out the subcellular localisation of DmManf in *Drosophila,* we used larval 2nd instar garland cells. Garland cells are nephrocytes with high rate of endocytosis and express several neuronal and exocytosis markers *e.g. pros*, mammalian *Prox-1* homologue, SNARE binding protein *Ras opposite* (*Rop*) facilitating neurotransmitter secretion, and *Syntaxin 1A* (*Syx1A*, a t-SNARE) (Wigglesworth, 1972; Forjanic et al., 1997). In the binuclear garland cells, DmManf is localised around the two nuclei, partially overlapping with ER-targeted marker (II).

Further to define the localisation of DmManf within different endosomal compartments we used 3rd instar larval salivary gland cells that are the largest cells found in *Drosophila* larvae. In the basal part, there was partial colocalisation of DmManf expression with recycling endosomal marker GFP-Rab11 as well as with early endosomal marker GFP-Rab5 (II). In the larval fat body cells with high secretory capacity GFP-clathrin light chain (Clc), a marker for clathrin coated vesicles, colocalised with DmManf in some structures (II). DmManf localised close to GFP-Rab7, an important player in trafficking between the early and late endosomes and lysosomes (II). Thus DmManf localises to the endosomal structures with markers Clc, Rab5, Rab7, and Rab11, but probably does not share the same protein complexes with them (II). When evaluating the colocalisation of DmManf with lysosomal compartment in DmManf transfected Schneider-2 cells no overlap was detected (II). For the Golgi complex and mitochondria, both used mito-EYFP and Golgi-EYFP markers showed partial overlap with DmManf (Figure 8 and unpublished data). Commonly there was no detectable expression of DmManf in the nuclei stained with DAPI.

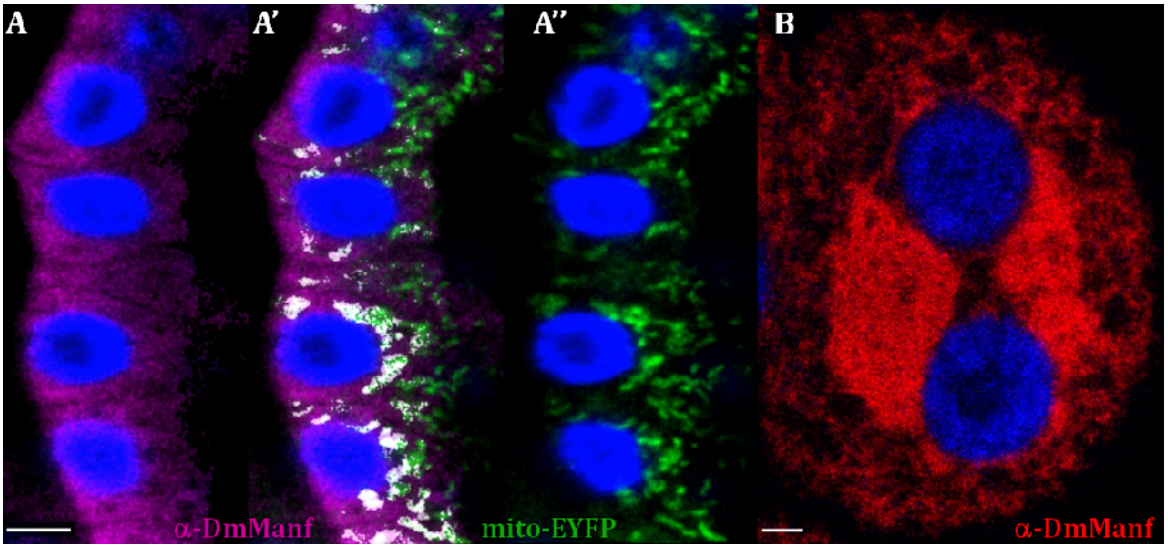

Figure 8. DmManf localises subcellularily around the nuclei and shows partial overlap with mitochondrial marker. A-A'''; a laser confocal section of larval salivary gland, nuclei are stained with DAPI (blue), DmManf (magenta) and mito-EYFP (green), overlap between the two channels is presented as white in A'. B – DmManf is localised massively around the nuclei in binuclear garland cell, nuclei are stained with DAPI (blue). Scale bars 5µm in A-A''' and 2µm in B.

4.2 Characterisation of *Drosophila DmManf* mutants

4.2.1 *DmManf* zygotic mutant is larval lethal

In order to generate DmManf mutants we used P-element excision method for the P-element insertion P{SUPor-P}KG03250 (Bellen et al., 2004) found 157 bp upstream from *DmManf* 5'UTR (I). In P{SUPor-P}KG03250 homozygous viable adult flies DmManf expression is unaltered, but the expression of the upstream neighbouring gene CG14879 was partially abolished (I). We made about 200 crosses of P{SUPor-

P}KG03250 females to Δ2–3 transposase line males resulting in five non-complementing homozygous lethal lines. Two of these lines were verified by sequencing — Δ96 had a deletion of 278 bp of the *DmManf* ORF deleting first two exons and part of the third exon (I). In Δ112, fifteen bp from 5'UTR of *DmManf* were missing (I). All five mutant lines share similar phenotype and are larval lethal. Initially, *DmManf* mutant larvae hatch and feed normally. Then they start to wander out of food as their heterozygote siblings continue to feed. Their overall development and growth is delayed in comparison to heterozygotes. Afterwards the movement of *DmManf* mutant homozygotes slow down and finally these larvae freeze immobilized but still respond to touch. There are differences between the individuals in the time scale of death but there are no survivors after the first molt. Still, during the embryonic neural development we were unable to find any abnormalities of $DmManf^{\Delta 96}$ mutants by several neuronal markers as fasciclin II (fas II), futsch, and BP102 (I).The early first instar $DmManf^{\Delta 96}$ mutant larvae still have traces of maternal DmManf protein left but before the death occurs as late first instars no *DmManf* mRNA or protein are visible (I, and immunostainings with anti-DmManf antibody). These results indicate that $DmManf^{\Delta 96}$ mutant larvae die gradually as soon as the maternally contributed *DmManf* gene products end up. At 25°C during 75 hours AEL all $DmManf^{\Delta 96}$ larvae die with attached half-shed old cuticle.

For the microarray expression analyses we collected larvae 29-50 hours AEL. When the expression profile of $DmManf^{\Delta 96}$ mutants was compared to the wild type larvae of exactly the same age, we found 690 genes significantly downregulated and 682 genes upregulated (II). Among downregulated genes the most enriched gene ontology (GO) terms fall into clusters related to (1) intracellular organelle lumen and nucleic acid metabolic processes, (2) cellular activities such as DNA replication, RNA processing and splicing. The 5th highly enriched cluster consisted of GO terms such as ER related genes (24 genes), proline and arginine metabolism (9 genes), and oxioreductases (9 genes) (II). Mitotic cell cycle, chromosomal segregation, and mitotic spindle organization were also clustered as significantly enriched.

The downregulation of genes involved in metabolic processes and cellular activities may be associated to ER stress, as one of the outcomes of UPR is general and unspecific downregulation of novel protein synthesis release protein load in ER. Moreover, according to Hotamisligil the ER is a central apparatus in metabolism coordination (Hotamisligil, 2010). In contrary to results of mammalian MANF knockdown in HeLa cells leading to increased cell proliferation (Apostolou et al., 2008), in *Drosophila* $DmManf^{\Delta 96}$ mutants the genes coding for replication, chromosome condensation, and mitotic spindle organisation are downregulated. Among the upregulated genes in $DmManf^{\Delta 96}$ larval mutants the most enriched functional clusters included GO terms like sugar metabolism and glucosidases, glycosyl hydrolases (18 genes), and hydrolases and carboxylesterases (23 genes), followed by cluster of monooxygenases, Cytochrome P450, iron, vesicular fraction, oxidation reduction and endoplasmic reticulum (49 genes) (II). Chitin and polysaccharide metabolism was among the highly enriched GO terms (40 genes). The 5th ranked cluster of GO terms was immune and defense response (19 genes) (II). The upregulation of genes involved in oxidation reduction, monooxygenases, cytochromes, and ER may indicate problems with oxidative processes in ER, *e.g.* in

protein folding and disulfide bond formation. The larvae were collected fairly before the first molt when failed would cause respiratory problems. Upregulation of genes implied in immune and defense response is notable and precedes the lethality observed more than twenty hours later. Upregulation of genes involved in sugar and polysaccharide (included chitin) metabolism needs further studies to enlighten the issue. Interestingly, there is evidence that sugar metabolism is transcriptionally regulated by UPR (Hotamisligil, 2010).

4.2.2 *DmManf* null mutant is rescued by ectopic overexpression of both *Drosophila* and human MANF

We confirmed that *DmManf*$^{\Delta 96}$ mutant lethality was solely due to the *DmManf* deletion since ubiquitous expression of DmManf in the mutant*DmManf*$^{\Delta 96}$ background resulted in complete rescue of larval lethality (I). With 69B-GAL4 driving the expression strongest in epidermis and CNS (Brand and Perrimon, 1993; and our observations) we were able to maintain the *DmManf*$^{\Delta 96}$ mutant flies as a living stock by DmManf ectopic expression only (I). When DmManf was expressed in dopamine producing cells in CNS and epidermis by TH-GAL4 line (Friggi-Grelin et al., 2003) to rescue the *DmManf*$^{\Delta 96}$ lethality, only partial rescue was observed (unpublished results). Several GAL4 lines with restricted expression patterns were used to drive DmManf expression – proventricular specific C135, pan-neuronal elav, and glial repo failed to rescue the lethality of *DmManf*$^{\Delta 96}$ mutants (I, and unpublished results). In conclusion, the sufficiently high expression of DmManf both in epidermis and CNS is essential for fruit fly viability.

In order to replace DmManf with human MANF or CDNF we carried out rescue experiments with UAS-*HsMANF* and UAS-*HsCDNF* transgenic flies. Both generated constructs contained additional stretch of sequence at the 3' end coding a tag of nine amino acids (I). Ubiquitous HsMANF expression was able to significantly rescue larval lethality of *DmManf*$^{\Delta 96}$ mutants (I) whereas HsCDNF construct gave no rescue despite the verified HsCDNF protein production in transgenic flies (I). Unfortunately our UAS-*HsCDNF* construct contained an alternative signal sequence cleaving site resulting in additional six amino acids in recombinant protein amino terminus. Recently Riitta Lindström has made novel transgenic flies with no extra tags for both human MANF and CDNF. These tag-free constructs of CDNF and MANF are both capable of rescuing fly *DmManf*$^{\Delta 96}$ mutant lethality (R. Lindström, unpublished results). These results demonstrate that both human MANF and CDNF are the fly functional orthologues and that fly and human MANF and CDNF may share the yet unknown cognate receptor. CDNF by sequence homology (47%, I) is evolutionarily slightly more distant than MANF from the *Drosophila* orthologue. CDNF by structure is apparently more sensitive for tagging in C' and/or N' terminus thus the tags disrupt its ability to rescue fruit fly *DmManf* mutant lethality.

4.2.3 Maternal and zygotic *DmManf* mutant is late embryonic lethal with severe phenotype

We abolished the maternal contribution by FLP/FRT recombination combined to the dominant female sterile technique (Chou and Perrimon, 1996). The method allows

obtaining heterozygous females whose germline is homozygous to the mutation. When these females are crossed to the heterozygous males, the maternal and zygotic null embryos are produced. Small population (less than 5%) of *DmManf*$^{\Delta 96}$ maternal and zygotic null (*DmManf*$^{mz\Delta 96}$) embryos are already severely malformed from early stages of embryonic development (unpublished observations), but most are undistinguishable from their heterozygote siblings until late embryogenic stage of 16 middle (I). Starting from stage of 16 to the end of embryogenesis *DmManf*$^{mz\Delta 96}$ embryos develop severe disorders and never hatch. The particular two features that tracheas show no air filling and the established cuticle enables immunostainings indicate severe cuticular defects. Indeed, ultrastructural analysis reveal that in *DmManf*$^{mz\Delta 96}$ mutants the all the cuticular layers are disorganised and disrupted (I). When these late stage 17 mutant embryos are devitellinised by hand, the normally well-defined anterior-posterior cuticular structures are malformed (Figure 9A). The yolk become adsorbed in wild type embryos, but persists in the gut of *DmManf*$^{mz\Delta 96}$. Starting from late stage 16 the ventral cord overcondenses (I, Figure 9B); several abdominal segments become broader and the peripheral nerves become misrouted and displaced from the abdominal anterior part of the embryo. The *DmManf*$^{mz\Delta 96}$ embryos obtain a phenotype with overshortened VNC protruding out of the defective cuticle (I). The phenotype of overshortened VNC is unusual since several other mutants *e.g. repo*, have uncondensed prolonged VNC (Halter et al., 1995; Olofsson and Page, 2005). Nevertheless, the glial cell number and positioning relative to the nerves are unaffected (I). To determine the impact of apoptosis to this phenotype we quantified cells positive for cleaved Caspase-3 in *DmManf*$^{mz\Delta 96}$ VNC at late stage 16 prior to the appearance of abnormalities. In comparison to the wild type there was no significant increase in the number of apoptotic cells (I).

The ultrastructure of *DmManf*$^{mz\Delta 96}$ at early stage 16 the neuropile still resembles that of the wild type (I). However, by late stage 17 we detected degradation of axonal membranes starting at the border of glia and neuropile (I). In addition, the cells defined by their location as cell body glia, look highly electron dense and contained remnants of cell debris (I). In mutant neuropile we detected also dying cells with poor and bleached cytoplasm, swollen nuclei and with dilated ER and other organelles atypical for the conventional apoptosis (I). Thus, the absence of DmManf caused non-apoptotic cell death of *Drosophila* neurons (I).

For microarray expression analysis embryos were picked during the late stage of 17 (21-22 hours AEL) just before hatching when the trachea of wild type embryos fill with air. In comparison to the zygotic *DmManf*$^{\Delta 96}$ mutants the changes in gene expression were more severe (II). In *DmManf*$^{mz\Delta 96}$ mutants 1191 genes were significantly downregulated and 1243 genes upregulated when compared to the wild type (II). Among downregulated genes the most significantly enriched clusters of GO terms were related to membrane transporters (25 genes) and transmembrane proteins (146 genes) (II). There were several enriched clusters referring to different metabolic processes such as amine, amino acid and carboxylic acid catabolic processes (11 genes), DNA metabolic processes (26 genes), and genes related to pyrimidine metabolism (15 genes). In *DmManf*$^{mz\Delta 96}$ mutants, the cluster of mitochondria-related transcripts was highly enriched (28 genes). The lack of DmManf causes downregulation of several components

in all mitochondrial compartments: the lumen, the inner and outer membranes (II). The downregulation genes involved in membrane transport and membranes could be related to both VNC and cuticular phenotype. During the stage 17 the neural activity starts to appear and in synaptic transmission the membrane transporters and vesicle transport are of high significance for proper functioning. The secretion of cuticle components proceeds from stage 16 onward. Coordinated secretion of cuticular components together with chitin synthesis and crosslinking of cuticular layers are most important for the exoskeleton establishment. When the membrane proteins and membrane transport are downregulated, it will definitely have an impact on cuticle secretion as well. Similarly to *DmManf*$^{\Delta 96}$ mutants the genes involved in metabolic processes, though slightly different ones were downregulated. Interestingly, mitochondria related gene expression was among the highly enriched GO clusters – could the mitochondrial failure be associated with the death of these *DmManf*$^{mz\Delta 96}$ mutants?

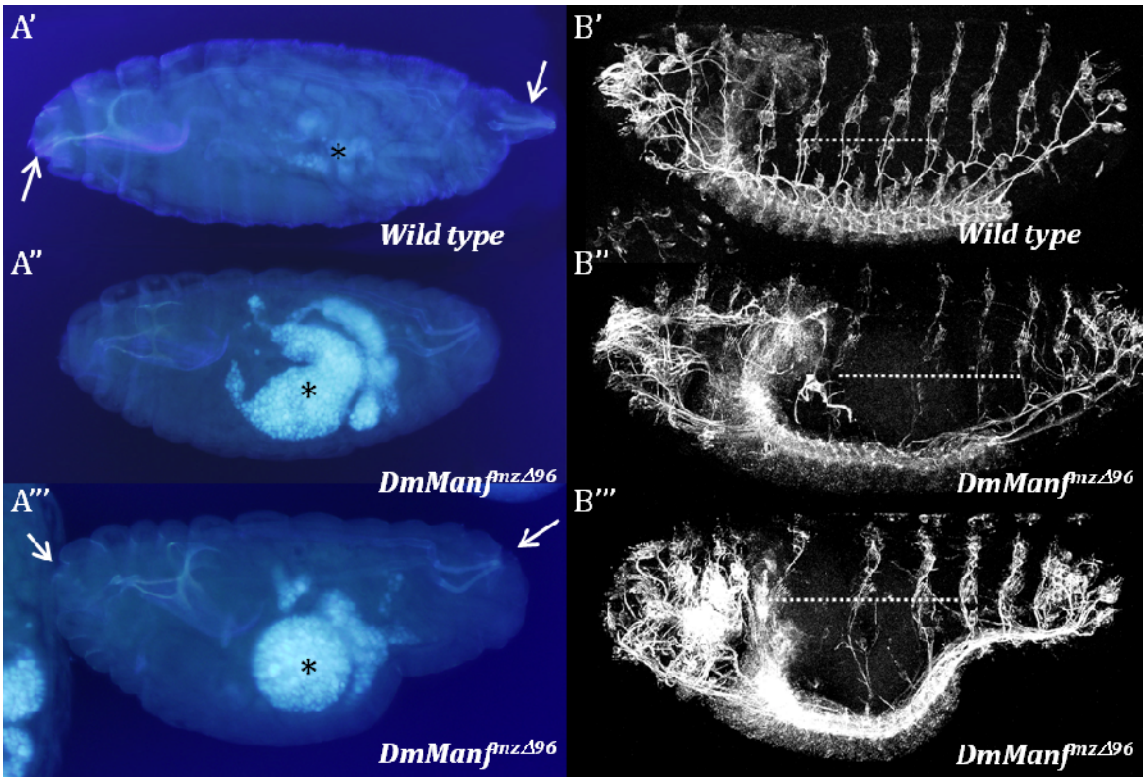

Figure 9. *DmManf* $^{mz\Delta 96}$embryos show severe phenotype at the end of embryogenesis. From A'-A''' filipin staining visualising the persistence of yolk (asterisks) in *DmManf* $^{mz\Delta 96}$ mutant (A''-A'''), yolk is adsorbed in the wild type (A') gut. From B' – B''' anti-futsch staining of the nervous system. Wild type embryos (A') before hatching have well defined anterior and posterior structures missing from the *DmManf* $^{mz\Delta 96}$ mutants. To the end of stage 16 (A'', B' and B'') *DmManf* $^{mz\Delta 96}$ mutant phenotype starts to develop – the abdominal segments broaden (dotted line marking the distance between four peripheral nerves) and the anti-futsch positive peripheral nerves avoid that area. At the end of stage 17 (A', A''' and B''') the ventral cord of *DmManf*$^{mz\Delta 96}$ embryos protrudes out.

Among upregulated genes in *DmManf*$^{mz\Delta 96}$ mutant embryos, the most prominent group of significantly enriched GO terms was immune and defense response (69 genes), followed by the groups related to proteolysis, hydrolases and peptidases (197 genes) (II). The upregulated gene set was also enriched in genes related to actin cytoskeleton organization and actin filament-based process (28 genes). Moreover, genes involved in cell death (28 genes) were prominently enriched as well (II). The later is in contradiction with our data obtained with cleaved Caspase-3 staining for apoptosis in stage 16-17 VNC. On the other hand, there are other cell death pathways than Caspase-3 dependent although it is the central apoptotic caspase. Strikingly, in *DmManf*$^{mz\Delta 96}$ mutants the immune and defense response genes showed almost 10 times enrichment. These genes were enriched in larval *DmManf*$^{\Delta 96}$ mutants as well, confirming that abolishment of DmManf triggers immune and defense response. The UPR and inflammation are closely related processes (Hotamisligil, 2010). In mammals, all branches of UPR can lead to activation of NF-κB-IKK pathway with critical role in the induction of multiple inflammatory mediators.

The maternal lack of DmManf gene products is completely rescued by paternal gene expression when females with homozygous *DmManf*$^{m\Delta 96}$ mutant germline were crossed to wild type males. In this case similar minor amount (less than 5%) of early malformed embryos as in *DmManf*$^{mz\Delta 96}$ was detected and all the rest developed undistinguishable from the wild type to the perfectly normal adults. When the transcriptome of paternally rescued embryos was evaluated 21 hours AEL the changes were evident but restricted to a smaller number of genes than changes caused by the complete lack of DmManf. We found 98 genes were significantly upregulated and only 34 genes downregulated by paternal rescue (II). The clustering by gene ontology terms revealed cluster related to response to stimulus and neurological process (8 genes), including genes like transcription factor *pros*, *pumilio* (*pum*; encoding a mRNA binding protein involved in nervous system development), *pastrel* (*pst*; with unknown molecular function involved in memory and learning), *Ras opposite* (*Rop*; involved in exocytosis and synaptic transmission), *rolled* (*rl*; *Drosophila* ERK) and *small optic lobes* (*sol*; calpain family peptidase) (II). Transcripts of several genes coding membrane proteins also showed enrichment like *klumpfuss* (*klu*) and *odd skipped* (*odd*; DNA binding Zn-finger proteins important for embryonic nervous system development). Other enriched GO term clusters were cell division, cell cycle and cytoplasm (9 genes) (II).

Here we observe the GO clusters important to recover the lack of maternal *DmManf*. The animals were collected 21 hours AEL, much later than the initiation of paternal gene expression (4-6 hours AEL). Still, the expression changes among genes involved in neuronal processes are detectable. Again the genes coding membrane proteins and cell cycle components are enriched similarly to the profile seen in *DmManf* mutants.

4.2.4 *DmManf* mutants show diminished volumes of dopamine neurites

The orthologues of *Drosophila* Manf, MANF and CDNF have been shown to support the DA neurons in mammals (Petrova et al., 2003; Lindholm et al., 2007; Voutilainen et al., 2009). To find out is the gene function as well conserved in evolution among the MANF/CDNF family between the invertebrates and vertebrates, we studied the DA neurons in *DmManf*$^{mz\Delta 96}$ and *DmManf*$^{\Delta 96}$ mutants. The common way to visualise

DA neurons is by using TH, the first rate limiting enzyme for DA synthesis. In wild type embryos the expression of TH in neuronal somas is first detectable at late stage 16 and before the hatching of the larvae the neurites also become TH-positive (I). According to anti-TH immunostaining the number of TH positive neurons in both *DmManf* mutants is indistinguishable from the wild type (I, unpublished data).

Next we visualized the neurites of DA neurons by a membrane-targeted GFP construct expressed under the *TH* promoter (Friggi-Grelin et al., 2003) (*TH*>mCD8-GFP) (I). In $DmManf^{\Delta 96mz}$ mutants the TH positive neuronal soma were visible but in comparison to the wild type almost no TH-positive neurites were detected (I). The absence of TH-positive neurites and the embryonic lethality was rescued by ectopic overexpression of DmManf under 69B-GAL4 driver in $DmManf^{\Delta 96}$ background (I) showing that this phenotype is caused by the lack of DmManf only. In order to compare the neurites of 1st instar larvae we mapped the DA neurites according to fas II landmarks (I). When DA neurite network of the wild type larvae was compared to the $DmManf^{\Delta 96}$ zygotic mutant with maternal contribution the volume of DA neurites was also significantly diminished (I). The neuritic degeneration is most prominent in the abdominal area of VNC. We observed similar but more severe degeneration of DA neurites when apoptosis was induced in the DA neurons by activation of pro-apoptotic genes *reaper, hid*, and *grim* (SMAC/Diablo homologues in *Drosophila*) (I). Surprisingly, even despite the massive loss of GFP-positive neurites several DA neuronal somas were still visible in these larvae (I). When apoptosis was induced in DA cells, the larvae died as late first instars at the same developmental time window as $DmManf^{\Delta 96}$. In $DmManf^{\Delta 96}$ VNC there were no significant changes in neurite volume of serotonergic neurons or in the volume of even-skipped positive motoneuron subpopulation verifying that neurite loss occurred specifically in the DA neurons(I). The results from microarray analysis reveal in both *DmManf* mutants among downregulated genes three candidate genes involved in neurite development - *Abelson tyrosine kinase* (*Abl*), *Guanine nucleotide exchange factor GEF64C* (*Gef64C*) and the transcription factor *longitudinals lacking* (*lola*) (II).

Ultrastructural studies of $DmManf^{mz\Delta 96}$ mutant revealed changes in morphology of the cell body glia, decomposition of neuropile from the glia-neuropile border, and the appearance of nonapoptotically dying cells in the VNC. Altogether these results indicate that in *Drosophila* the cell death of DA neurons start from the degeneration of neurites. Since dopamine derived quinones are important for insect cuticle development, the induced cell death in DA producing cells is lethal to the animal before the DA neuronal death commences to the end. The observed non-apoptotic cell death in the VNC of $DmManf^{mz\Delta 96}$ mutants is in line with the results from microarray analysis indicating the upregulation of 28 genes involved in cell death. The localization of DmManf in the cell body glia surrounding the DA neurons raise the possibility that glial-derived DmManf in addition to neuritogenic effect have also survival promoting trophic function for these neurons.

4.2.5 *DmManf*$^{mz\Delta 96}$ embryos show extremely low dopamine levels and disturbed expression of DA pathway enzymes

Because of the diminished volume of DA neurites and cuticular defects we measured the DA content of *DmManf*$^{mz\Delta 96}$ embryos (I). Indeed, the DA levels of *DmManf*$^{mz\Delta 96}$ embryos were extremely low (I). Nonetheless, the transcripts of DA producing enzymes TH and Ddc showed significant upregulation (II). Also Pu, needed for the synthesis of the cofactor of TH, was upregulated in *DmManf*$^{mz\Delta 96}$ embryos (II). One possible explanation for these alterations is the lack of substrate, tyrosine, for DA synthesis. Tyrosine, the essential amino acid for DA synthesis, is transported into the cell from hemolymph. In *DmManf* mutants several amino acid membrane transporters were downregulated. The lack of tyrosine, together with low amounts of the end product, DA, probably leads to the upregulation of the transcripts for the enzymes in DA synthesis pathway and their cofactors.

4.2.6 Cuticular defects of *DmManf* mutants

DmManf is strongly expressed in epidermis and required for viability. In *DmManf*$^{mz\Delta 96}$ embryos we observed severe cuticular defects. Ultrastructural analysis revealed that all layers of the *DmManf*$^{\Delta 96mz}$ mutant cuticle were disorganized (I, II). At the embryonic stage 17, the mutant epithelial cells responsible for cuticle secretion showed disrupted ER morphology and excessive accumulation of vesicles in the apical part (II). In *DmManf*$^{\Delta 96}$ mutant larvae with gradually fading maternal contribution, the cuticle showed no disruption and the chitin layers were deposited and organised normally (II). Instead, the shedding of the old cuticle failed and often the 1st instar cuticle remained attached to the body (II, my observations). These observations imply that the maternal loading of *DmManf* gene products in larval *DmManf*$^{\Delta 96}$ mutant is sufficient to overcome defects in early cuticle development, secretion and layering, but insufficient to complete the first molt.

Among the downregulated genes in *DmManf*$^{mz\Delta 96}$ embryos, 14 genes were coding the structural components of insect cuticle (II). Several other genes responsible for cuticle development were upregulated, such as the genes encoding enzymes involved in chitin synthesis like chitin synthase *kkv* (Moussian et al., 2005), membrane-bound extracellular protein involved in chitin microfibril formation *knk* (Moussian et al., 2006), and *Syx1A* involved in secretion of cuticle components (II). Additionally, several genes involved in epithelial development and morphogenesis were upregulated and significantly enriched among the GO terms (35 genes) (II).

It is likely that disturbances in membrane transporter expression and exocytosis, together with misbalance in cuticular components, lead to disorganised and disrupted cuticle in *DmManf*$^{mz\Delta 96}$ mutant embryos. Alternatively the cuticular disorganization may be a consequence of extremely low DA levels needed for the synthesis of cuticle crosslinkers — quinones.

4.2.7 Gene expression profiling of *DmManf* mutant and overexpression conditions

4.2.7.1 Commonly regulated genes in both DmManf *mutants*

The commonly downregulated 208 genes between the *DmManf*$^{\Delta 96}$ and *DmManf*$^{mz\Delta 96}$ mutants make roughly 30% of potential overlap (II). The highest enrichment of GO terms fell into RNA metabolism and ribosome biogenesis (19 genes). Around 10% of all known ATP binding genes were downregulated (28 genes) together with 14 genes of the purine and pyrimidine metabolism (II). Additionally, the transcription of sugar transporters and genes involved in transmembrane transport highly represented in *DmManf*$^{mz\Delta 96}$ mutant embryos was repressed. GO terms related to DNA replication were also enriched among the commonly downregulated genes in both *DmManf* mutants. The downregulation of genes related to RNA, ribosomes and purine/pyrimidine metabolism could be a consequence of eIF2α phosphorylation and subsequent translational inhibition (DuRose et al., 2009). We showed that in *DmManf*$^{mz\Delta 96}$ mutants the amount of Ser51 phosphorylated eIF2α is two-fold upregulated (II).

A third of all upregulated genes (229) were induced in both mutants (II). Immune and defense response was the most enriched functional cluster (29 genes) along with the group consisting of monooxygenases, oxidoreductases, vesicular fraction, endoplasmic reticulum, Cytochrome P450 and lipid metabolic process (21 genes). One of the known targets of UPR less explored is lipid metabolism (Hotamisligil, 2010). These results implicate that in both mutants the oxidative stress and ER stress could be behind the observed immune and stress response.

Altogether even despite of the difficulties to interpret the expressional changes observed in both *DmManf* mutants, there are several indications that part of these changes could be a consequence of UPR. Another distinct part of changes in the *DmManf* mutants are related to misexpression of genes related to membrane transport.

4.2.7.2 Genes related to UPR are upregulated in DmManf *mutants*

Out of ER stress related *Drosophila* genes, 30% (29 genes) showed altered gene expression in our microarray analysis (II). One of the best validated targets of our microarray expression analysis is *CG10420,* an annotated gene with unknown function in *Drosophila*. In *DmManf*$^{mz\Delta 96}$ mutants *CG10420* was upregulated and in DmManf overexpression downregulated (II). The human homologue of *CG10420* is nucleotide exchange factor *SIL1* (*Saccharomyces cerevisiae* ER chaperone homologue), a BiP binding protein. In humans, several mutations in *SIL1* gene disrupting the protein cause the Marinesco-Sjögren syndrome, a rare disease associated with autosomal recessive cerebellar ataxia complicated by cataracts, developmental delay and myopathy (Senderek et al., 2005). Recently mammalian MANF was also shown to bind to BiP (Tadimalla, 2010). Thus it is possible that DmManf and CG10420 compete in binding to BiP together with unfolded proteins. The ultrastructural changes in *DmManf*$^{mz\Delta 96}$ mutants reveal swollen and dilated ER in epidermal cells, indicating severe disturbances of ER structure (II). In *DmManf*$^{mz\Delta 96}$ mutant embryos the extent of phosphorylated eukaryotic initiation factor eIF2α was more than two fold upregulated when compared to the wild type indicating the activation of translation inhibition (II). It is probable that the UPR PERK pathway is activated in *DmManf*$^{mz\Delta 96}$ mutants. The second UPR sensor,

IRE1, is able to activate several downstream branches. One of the branches leads to the activation of Jun kinase and death pathway (Woehlbier and Hetz, 2011). In both *DmManf* mutants *Drosophila* JNK kinase *hemipterous* showed significant upregulation. The other branch of Ire1 leads to unconventional splicing of transcription factor Xbp1 and consequential upregulation of chaperone genes activated by spliced form of Xbp1. In *DmManf*$^{mz\Delta 96}$ mutants we were unable to detect the spliced form of Xbp1 (my unpublished results). Conclusively, the lack of DmManf results in severe ER stress and upregulation of many genes involved in ER stress and UPR finally leading to the cell death.

4.2.7.3 Disturbances in expression of genes involved in membrane traffic

Ultrastructural study of *DmManf*$^{mz\Delta 96}$ mutants revealed overload of vesicles next to the apical part of epidermal cells (II). Together with the severe defects observed in the cuticle secretion and organisation it suggested an involvement of the genes of exocytosis pathway. In *DmManf* mutants, several genes implied in exocytosis and vesicle transport from the Golgi complex to the plasma membrane were downregulated (*Syx1A, Syx6, SNAP29*), whereas the ER residing syntaxins - *Stx17* and *Stx18* - were upregulated (II). Possible the accumulation of vesicles in *DmManf*$^{mz\Delta 96}$ mutant close to the apical plasma membrane result from inhibition of membrane traffic from Golgi complex to the plasma membrane. There are several common features of *DmManf* mutants and mutants with impaired exocytosis (Table 4.) The discrepancy between *DmManf*, and *Syx1A* and *ROP* mutants is that the last mentioned two mutants the VNC condensation fails but in *DmManf*$^{mz\Delta 96}$ mutants the VNC overcondenses.

Table 4. Comparison of *DmManf* mutants and mutants with defective exocytosis

Feature or organ	***DmManf*$^{mz\Delta 96}$**	***Syntaxin 1A*^{D299}**	***ROP*^{G27}**
cuticle	cuticle disorganised	cuticle defects, secretion	cuticle missing or little
lethality	fails to hatch	fails to hatch	fail to hatch
Malpighian tubules	dye in Malpighian tubules	urea secretion normal	no urea secretion
VNC	VNC overshortened	VNC fails to shorten	VNC fails to shorten
VNC	VNC attached to epidermis		VNC attached to epidermis
eye		phenotype in mild alleles	
yolk	yolk in the gut	yolk in the gut, gut abnormal	yolk in the gut, gut abnormal
tracheas	no filling with air, full of debris (TEM)	tracheas full of fluid	no filling with air
autofluor.	no autofluorescence of embryos		no autofluorescence of embryos
function	regulation of membrane traffic	exocytosis impaired, not proceeding	SNARE binding

Ultrastructural analysis of *DmManf*$^{mz\Delta 96}$ mutant secretory tissues revealed that the cells of gastric caeca contain huge vesicles filled with cellular debris resembling multivesicular bodies and autophagosomes and completely missing in wild type cells. Genes coding for components of multivesicular body formation were especially altered (II). It is possible that these vesicles contain the misfolded proteins to be degraded or, alternatively, that the autophagy pathway is activated. The accumulation of vesicles full of debris to be degraded could be also due to the blockage in lysosomal degradation. Our microarray analysis revealed transcriptional change in 45% of lysosome related genes present in the KEGG database. Many of them were downregulated in *DmManf*$^{mz\Delta 96}$ embryos and some in *DmManf*$^{\Delta 96}$ larvae (II). The ATPase V-type H^+ transporting subunit that maintains acidic environment in lysosomes showed downregulation in both mutants and upregulation in DmManf overexpressing larvae. The expression of other lysosomal membrane proteins and several lysosomal hydrolases was also altered (II).

4.2.7.4 Gene expression changes when DmManf is overexpressed

When comparing the gene expression profiles between DmManf overexpressing and wild type larvae we found 614 genes upregulated and 340 genes downregulation. Among upregulated genes the enrichment was highest in cluster of GO terms as related to regulation of gene expression, protein localisation and transport, and cell cycle (*e.g. kokopelli*, an uncharacterized cyclin involved in stem cell maintenance and *Retinoblastoma-family protein*, the human *Rb* homologue). Genes involved in regulation of cell death were also upregulated (*e.g. CG7188*, a putative Bax inhibitor, *rl*, and *klu*) (II).

When comparing the upregulated genes in both paternally rescued embryos and in DmManf overexpressing larvae, the common represented GO term clusters were ion binding (14 genes), membrane fraction (7 genes), oxidation reduction (8 genes) and cell cycle (5 genes) (II). Among the gene set there were well known genes like *Cbl, diaphanous* (*dia*, formin, essential for actin-mediated events involving membrane invagination)*, Kinesin-like protein at 68D* (*Klp68D*), *rl,* and *Rop* (II).

Conclusively, in DmManf overexpression situation the typical mediators of growth factor signalling *rl* and *Cbl* were upregulated. The links upstream of these mediators and downstream of secreted DmManf are still missing. The second cluster of genes was directly linked to membrane modifications and transport. Interestingly, the intracellular protein modification processes in ER were also enhanced by DmManf overexpression. So presumably DmManf has a dual role - one intracellularily in the ER, and the other extracellularly after being secreted.

4.2.7.5 Parkinson's disease related misexpressed genes

Among the known human genes involved in PD, 32 are conserved between mammals and *Drosophila*, and 44% of these were differentially expressed in our microarray assay (II). Importantly, several genes from dopamine uptake (*DAT*), intracellular transport (*VMAT*), and synthesis (*ple*, *Ddc,* and *Pu*) were differentially expressed (II). The expression of many genes involved in mitochondria and ubiquitin proteasome pathways were also altered. Genes encoding mitochondrial oxidative complex I components show upregulation in *DmManf* mutants. The other mitochondrial

proteins encoded by nuclear genes involved in PD such as *Htra2* and *DJ-1* expression is downregulated in *DmManf* mutants. Several members of ubiquitin pathway were altered as well (II).

Oxidative stress and protein misfolding play an important role in the pathology of PD. DA neurons are already sensitised to ROS because of the high oxidative potential of DA itself and its synthesis byproducts. 6-OHDA has been shown to induce ER stress in neuronal cells (Holtz and O'Malley, 2003). Maybe here lay the reasons why MANF family of proteins is protective for dopaminergic neurons: to suppress oxidative stress, enhance protein folding in the ER, and maintain the dopamine synthesis and transport. How exactly they do it still remains an open question. The role of DmManf in mitochondrial processes definitely needs to be further evaluated but clearly the issue is highly intriguing.

5 Conclusions

Drosophila Manf represents the first highly conserved in evolution invertebrate neurotrophic factor. This study shows that the role of MANF specifically supporting dopaminergic neurons is conserved as well. In the embryonic nervous system of *Drosophila*, *DmManf* is expressed in glial cells. We demonstrated that when *DmManf* is abolished the dopaminergic neurites degenerate and the neuropile degrades leading to neuronal cell death. Our ultrastructural analysis indicate that the glia in the ventral nerve cord become phagocytically active and engulf neuronal debris. Dopamine levels in maternal and zygotic *DmManf* mutant are extremely low and the enzymes and co-factors needed for dopamine synthesis are upregulated indicating the probable lack of substrate for dopamine synthesis, tyrosine. In arthropods dopamine derived metabolites are highly important for cuticle development. The maternal and zygotic null mutant of *DmManf* has severely disorganized cuticle and lacks the well defined cuticular structures as posterior spiracles. Although the embryonic nervous system development of these mutants starts and proceeds initially correctly, at the end of embryogenesis the ventral nerve cord overcondenses and the peripheral nerves become misrouted from anterior abdominal segments. The persistence of yolk in the gut indicates the defects in exo- and endocytosis. The insect cuticle formation is a highly complicated process of coordinated secretion, synthesis and crosslinking of cuticular components. Apparently both the insufficient amount of crosslinking dopamine derived quinones and the impaired exocytosis result in disrupted cuticle of $DmManf^{mz\Delta 96}$ mutants. Ultrastructural studies revealed that the whole intracellular vesicular transport is seriously impaired - epidermal cells are loaded with different type of vesicles and the ER shows dilated and rounded morphology. DmManf is broadly expressed in secretory tissues. In the null mutant gastric caeca, responsible for secretion of digestive enzymes, the multivesicular body-like vesicles full of cellular debris and inclusions are accumulating. Behind this phenomena could be the defects in their further degradation or the over activation of macroautophagy. When evaluating UPR in $DmManf^{mz\Delta 96}$ mutants we found the translation inhibition through the phosphorylation of eIF2α probably via PERK pathway is taking place. We hypothesize that without *DmManf* the persisting UPR finally leads to cell death via Ire1 and JNK kinase pathway but the issue needs further studies. These results together with microarray expression analysis implicate that there are disturbances

in membrane traffic when DmManf is abolished. Based on our results the precise experiments to enlighten this issue can be designed.

We have made an effort to localise DmManf at the subcellular level. Several markers for organelles and certain compartment markers have been used, still, we were unable to find a perfect match. DmManf is localizing around the nucleus overlapping with ER, Golgi complex and few mitochondria at that area. DmManf is at lower level present in several other membraneous structures in cytoplasm as endocytic vesicles, but no colocalisation with lysosomes was detected.

Obvious declines from the wild type transcriptome in *DmManf* mutants occur among genes with functions are important for neurodegenerative diseases and especially PD. Loss of dopamine and accumulation of inclusions named Lewy bodies in the brain tissue are the well established hallmarks for PD. In neurodegenerative diseases several protein inclusions are thought to finally impair the axonal transport and lead to neuronal death. The chronic misfolding of proteins stands behind these inclusions. The way to degrade the misfolded proteins is by proteasome and ubiquitin linked protein degradation or by macroautophagy degrading the whole parts of organelles together with misfolded proteins. Also mitochondrial impairment is associated with PD. In $DmManf^{mz\Delta 96}$ mutant the expression of two known genes coding mitochondrial proteins associated with PD - *DJ-1* and *Htra2* together with several cytochromes were downregulated. Recently the immune response has been implicated to have a role in neurodegeneration as well. Interestingly, according to microarray expression analysis the lack of DmManf initiates strong stress response and upregulation of genes involved in innate immune response of *Drosophila.*

Altogether, DmManf has an important role in *Drosophila* both as non-cell autonomous neurotrophic factor for dopaminergic neurons secreted from glia and as ER residing survival factor induced by UPR in order to relieve cellular stress.

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