Characterization of the Structural and Functional Determinants of MANF/CDNF in Drosophila In Vivo Model

Lindström, Riitta

2013


http://hdl.handle.net/10138/164256
https://doi.org/10.1371/journal.pone.0073928

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.
Characterization of the Structural and Functional Determinants of MANF/CDNF in *Drosophila In Vivo* Model

Riitta Lindström¹, Päivi Lindholm², Jukka Kallijärvi², Li-ying Yu², T. Petteri Piepponen³, Urmas Arumäe², Mart Saarma², Tapio I. Heino¹*

†Department of Biosciences, University of Helsinki, Helsinki, Finland, ²Institute of Biotechnology, University of Helsinki, Helsinki, Finland, ³Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Abstract

Mammalian MANF and CDNF proteins are evolutionarily conserved neurotrophic factors that can protect and repair mammalian dopaminergic neurons in vivo. In *Drosophila*, the sole MANF protein (DmManf) is needed for the maintenance of dopaminergic neurites and dopamine levels. Although both secreted and intracellular roles for MANF and CDNF have been demonstrated, very little is known about the molecular mechanism of their action. Here, by using a transgenic rescue approach in the *DmManf* mutant background we show that only full-length MANF containing both the amino-terminal saposin-like and carboxy-terminal SAP-domains can rescue the larval lethality of the *DmManf* mutant. Independent N- or C-terminal domains of MANF, even when co-expressed together, fail to rescue. Deleting the signal peptide or mutating the CXXC motif in the C-terminal domain destroys the activity of full-length DmManf. Positively charged surface amino acids and the C-terminal endoplasmic reticulum retention signal are necessary for rescue of DmManf mutant lethality when DmManf is expressed in a restricted pattern. Furthermore, rescue experiments with non-ubiquitous expression reveals functional differences between the C-terminal domain of mammalian and CDNF. Finally, DmManf and its C-terminal domain rescue mammalian sympathetic neurons from toxin-induced apoptosis in vitro demonstrating functional similarity of the mammalian and fly proteins. Our study offers further insights into the functional conservation between invertebrate and mammalian MANF/CDNF proteins and reveals the importance of the C-terminal domain for MANF activity in vivo.

Introduction

Neurotrophic factors (NTFs) protect neurons from apoptotic death and promote their regeneration. During development, NTFs regulate neuronal migration, differentiation, maturation, and survival, but also have roles in non-neuronal tissues [reviewed in (1)]. The recently discovered MANF/CDNF family of NTFs consists of two paralogues in mammals, MANF (Mesencephalic Astrocyte-derived Neurotrophic Factor; ARMET) [2] and CDNF (Cerebral Dopamine Neurotrophic Factor) [3]. The sole homologue found in invertebrates is more closely related to mammalian MANF than CDNF [4]. Recombinant human MANF (HsMANF) and CDNF (HsCDNF) protect and repair midbrain dopaminergic (DA) neurons in rodent models of Parkinson’s disease in vivo [3,5,6]. Mammalian MANF can also rescue cortical neurons and cardiomyocytes from ischemia in vivo [7,8]. Importantly, the fly homologue (DmManf) protects DA neurites and maintains DA levels during *Drosophila* development in vivo [4]. Based on their DA neuron survival-promoting and neuro-restorative effects, the MANF/CDNF family of proteins has therapeutic potential for treatment of Parkinson’s disease.

The molecular mechanisms behind the protective properties of MANF/CDNF proteins are still unknown. In addition to the role as a secreted extracellular trophic factor, MANF localizes to the endoplasmic reticulum (ER) and provides a protective function against ER stress in vitro [9–12]. Consistent with the ER function, both HsMANF and DmManf contain putative ER retention signal sequences (RTDL and RSEL, respectively; Figure 1A–B) in the C-terminal end which resemble the canonical KDEL ER retention signal. The expression of mammalian MANF is up-regulated by chemically induced ER stress in vitro [9,10]. During ER stress, the ER homeostasis is disturbed by accumulation of unfolded proteins leading to activation of the unfolded protein response (UPR; reviewed in [13,14]). MANF is also shown to bind to a UPR-related ER-resident protein, Glucose-regulated protein 78 (GRP78) in a Ca²⁺-dependent manner in vitro [8].

Structurally MANF and CDNF proteins show no amino acid sequence homology to other known families of NTFs, e.g. neurotrophins and glial-cell-line-derived neurotrophic factor (GDNF) family ligands. Human MANF and CDNF consist of two α-helical domains connected by a short flexible linker region (Figure 1A) [15–17]. DmManf is expected to adopt a very similar
structure because of the high similarity of amino acid sequence and the strict conservation of the spacing between the eight cysteine residues (Figure 1B). The amino (N)-terminal domain (N-MANF and N-CDNF) is structurally homologous to saposin-like proteins (SAPLIPs), a family of lipid-interacting molecules [15–19]. The C-terminal domain (C-MANF and C-CDNF) shares the highest structural homology with the SAF-A/B, Acinus and PIAS (SAP) domain of Ku70 protein [17]. Ku70, via the SAP-domain, interacts with a pro-apoptotic protein BCL-2 associated X (Bax) in the cytoplasm and inhibits Bax-mediated apoptotic death of mammalian cells \textit{in vitro} [19]. Similar to Ku70, MANF and cytoplasmic C-MANF protect superior cervical ganglion (SCG) neurons from apoptosis \textit{in vitro} [17]. The C-terminal domain also contains a CXXC motif (127CKGC130 and 126CDGC129) in HsMANF and DmManf, respectively, Figure 1A–B) which forms a disulphide bridge [15,17] and may participate in oxidation/reduction reactions as a similar CXXC sequence is found in thiol/disulphide oxidoreductases [20].

We have shown in a previous study that zygotic \textit{DmManf} mutant flies die as late first instar larvae and are rescued by ubiquitous expression of transgenic \textit{Drosophila} and human MANF [4]. Here, we used a transgenic approach in the homozygous \textit{DmManf}-deficient mutant fly background to characterize the structural features essential for \textit{in vivo} functioning of the DmManf, HsMANF and HsCDNF proteins. Mutations in \textit{Drosophila} and human MANF and CDNF (Figure 1C–D) were designed based on their known three-dimensional structures [15,17] and amino acid sequence predictions (Figure 1A–B). Mutations were introduced to \textit{Drosophila} as UAS (upstream activation sequence) -transgenes and expressed ubiquitously by the \textit{da-GAL4} driver in the homozygous \textit{DmManf} mutant background (Figure 1E). We also studied the conserved role of DmManf in protection of mammalian sympathetic neurons from apoptotic death \textit{in vitro}.

**Results**

**Separate N- and C-terminal Domains Fail to Rescue \textit{DmManf} Mutant Lethality**

To explore the function of the two domains of MANF (Figure 1A) we asked whether either of the domains, as an independent unit, could rescue \textit{DmManf} mutant lethality \textit{in vivo}. First, the N-terminal (residues 1–94) or C-terminal domain (residues 95–151) of mature DmManf was expressed ubiquitously by \textit{da-GAL4} either with (N-DmManf, C-DmManf) or without a signal peptide (residues ss2–ss22; N-DmManf-Δss, C-DmManf-Δss; Figure 1B–C) in the \textit{DmManf} mutant background. In contrast to full-length DmManf, none of these constructs could rescue the early larval lethality (Table 1). The expression of N- and C-terminal UAS-transgenes was verified by overexpressing the constructs by ubiquitous \textit{da-GAL4} in wild type background. Expression of N-DmManf and C-DmManf with a signal peptide was detected from larval lysates by Western blotting (Figure 2A). Unfortunately, the protein expression level of N-DmManf-Δss and C-DmManf-Δss constructs which lack the signal peptide was below the detection limit. Reverse transcription polymerase chain reaction (RT-PCR) from 1st instar larvae indicated that the mRNAs were expressed \textit{in vivo} (Figure 2B) suggesting that either the translation or the stability of N-DmManf-Δss and C-DmManf-Δss proteins were compromised.

Next we studied whether the \textit{DmManf} mutant lethality can be rescued by the N- and C-terminal domains of DmManf expressed together as two separate transgenes by ubiquitous \textit{da-GAL4} driver (Figure S1A). Interestingly, the two co-expressed independent domains also failed to complement the loss of endogenous DmManf (Table 1) suggesting that intact DmManf protein containing both domains is needed for \textit{in vivo} activity.

Interestingly, when wild type DmManf was overexpressed, a second very faint band corresponding to the size of C-DmManf was detected in addition to the expected band of approximately 18 kDa (Figure 2A). This suggests that abundantly expressed DmManf is partially degraded \textit{in vivo} releasing the C-terminal domain.

**ER Entry but not ER Retrieval is Essential for DmManf Function \textit{in vivo}**

Both mammalian and \textit{Drosophila} MANF localize to the ER and are also secreted [4,9–12,21]. MANF has a signal peptide in the N-terminus that directs newly synthesized protein into the ER (Figure 1B). Since MANF has both extracellular neuro-protective [4,5] and intracellular cyto-protective functions [17], we wanted to test whether entry into the ER and subsequent secretion of DmManf is crucial for its functionality. Therefore, we designed a DmManf transgene with a deletion of the signal sequence (aa ss2–ss22; DmManf-Δss, Figure 1C). Ubiquitous expression of DmManf-Δss could not rescue the early larval lethality of \textit{DmManf} mutants (Figure 2C) suggesting that DmManf entry into the secretory pathway via the ER is necessary for its function during development. Protein expression from DmManf-Δss construct was confirmed from larvae by Western blot analysis (Figure 2D).

Interestingly, the DmManf-Δss showed lower protein expression levels than wild type DmManf although the mRNA was expressed \textit{in vivo} (Figure 2B). This suggests that DmManf-Δss which is not targeted to ER and, instead, processed in the cytoplasm is either unstable or its translation is compromised. Use of two independent insertions of the DmManf-Δss transgene and two copies of \textit{da-GAL4} driver did not notably increase the expression level (Figure S1B) and failed to rescue \textit{DmManf} mutant lethality (Table 2).

We studied the secretion of DmManf-Δss \textit{in vitro} by transiently transfecting \textit{Drosophila} Schneider 2 (S2) and mammalian Chinese hamster ovary (CHO) cells with V5-DmManf-Δss-pMT and DmManf-Δss-pCR3.1 constructs encoding V5-DmManf-Δss and DmManf-Δss, respectively. In S2 cell transfections V5-tagged
Figure 2. Expression analysis of transgenic DmManf constructs and observed rescue of larval lethality. A) Protein expression of DmManf constructs was verified by Western blotting from 3rd instar larvae. Constructs were ubiquitously expressed by da-GAL4 driver in wild type or heterozygous DmManf mutant (red typing) background. da-GAL4/+ flies were used as control for endogenous DmManf expression. Schematic presentation of each construct coloured according to Figures 1B–C is shown under the blot. Calculated molecular weights of full length proteins, N- and C-terminal domains are presented. L1, L2 etc. correspond to independent transformant lines of each construct. B) In vivo transcription from constructs encoding DmManf N- and C-terminal domain was verified by RT-PCR from 1st instar larvae. Constructs were expressed by da-GAL4 in homozygous DmManf mutant background. DmManf and DmManf-Dss were used as positive controls, homozygous DmManf mutant larvae as a negative control. Schematic presentations of each construct are coloured according to Figures 1B–C. C) Rescue of DmManf D96 mutant larval lethality by transgenic wild type or mutated constructs of DmManf ubiquitously expressed by da-GAL4. L1, L2 etc. correspond to independent transformant lines of each construct. Average ± SD. D) Western blot analysis of protein expression of DmManf constructs with similar to size to endogenous DmManf (DmManf, DmManf-Dss, DmManf-D-RSEL, DmManf-NMG1, and DmManf-NMG2). The constructs were ubiquitously expressed by da-GAL4 in wild type background. Expression from a transgenic construct is detected as upregulated protein levels in comparison to endogenous DmManf levels in control flies (da-GAL4/+). L1, L2 etc. correspond to independent transformant lines of constructs. E) Expression analysis of the DmManf-C129S construct in embryos by Western blotting. Constructs were expressed by da-GAL4 in wild type background. Transgenic expression is detected as upregulation of DmManf expression compared to da-GAL4 flies (control). In Western blot analyses, alpha-tubulin was used as loading control.

doi:10.1371/journal.pone.0073928.g002
Table 1. Number of heterozygous pupae in rescue experiments with N- and C-terminal constructs of DmManf.

<table>
<thead>
<tr>
<th>Construct and insertion</th>
<th>Number of balanced pupae counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-DmManf L7</td>
<td>1314</td>
</tr>
<tr>
<td>N-DmManf L1</td>
<td>1213</td>
</tr>
<tr>
<td>N-DmManf-Δss L1</td>
<td>983</td>
</tr>
<tr>
<td>N-DmManf-Δss L3.2</td>
<td>1172</td>
</tr>
<tr>
<td>N-DmManf-Δss L4</td>
<td>1409</td>
</tr>
<tr>
<td>C-DmManf L3</td>
<td>1799</td>
</tr>
<tr>
<td>C-DmManf L4</td>
<td>1614</td>
</tr>
<tr>
<td>C-DmManf-Δss L3</td>
<td>2051</td>
</tr>
<tr>
<td>C-DmManf-Δss L5</td>
<td>1894</td>
</tr>
<tr>
<td>N-DmManf L7+C-DmManf L3</td>
<td>661</td>
</tr>
<tr>
<td>N-DmManf L7+C-DmManf L4</td>
<td>910</td>
</tr>
<tr>
<td>N-DmManf L7+C-DmManf-Δss L3</td>
<td>597</td>
</tr>
<tr>
<td>N-DmManf L7+C-DmManf-Δss L5</td>
<td>977</td>
</tr>
<tr>
<td>N-DmManf-Δss L1+C-DmManf L3</td>
<td>1152</td>
</tr>
<tr>
<td>N-DmManf-Δss L1+C-DmManf L4</td>
<td>910</td>
</tr>
<tr>
<td>N-DmManf-Δss L1+C-DmManf-Δss L3</td>
<td>781</td>
</tr>
<tr>
<td>N-DmManf-Δss L1+C-DmManf-Δss L5</td>
<td>508</td>
</tr>
</tbody>
</table>

No homozygous DmManf<sup>ΔN</sup> mutant pupae were found in any of the rescue experiments listed here. Ls correspond to independent insertions. doi:10.1371/journal.pone.0073928.t001

 Constructs were used to distinguish endogenously produced DmManf protein and the protein expressed from transfected plasmids. After incubation for 72 h, V5-DmManf-Δss and DmManf-Δss were detected in cell extracts but not in the medium of S2 or CHO cells by Western blotting (Figure 3A and Figure S1C) showing that DmManf-Δss was not secreted. We also used salm-GAL4 to express DmManf and DmManf-Δss in 3rd instar larval wing discs and detected up-regulation of DmManf immunoreactivity specifically in the GAL4 expression pattern (indicated by nuclear GFP; Figure S1D). This in vivo analysis further indicated a lack of secreted DmManf-Δss since it showed more strict expression in the GFP-expressing cells whereas wild type DmManf also localized next to them (white arrows in Figure S1Dh–i).

We also created a DmManf mutant transgene with deletion of the putative ER retention signal RSEL (DmManf-ΔRSEL; Figure 1B-C) to study whether ER retention of DmManf is crucial for functionality. Ubiquitously expressed DmManf-ΔRSEL rescued the larval lethality of DmManf mutants to adulthood (Figure 2C and Figure S1E). The localization of DmManf inside the ER (Figure 3B) in 3rd instar larval garland cells of homozygous DmManf mutants was significantly decreased when the putative ER retention signal was deleted (Figure 3B–C). Thus, the retention of abundantly expressed DmManf in the ER is not essential for fly viability.

CXXC Motif in the C-terminal Domain is Crucial for in vivo Functionality of DmManf

The crystal and solution NMR structures of mature HsMANF and HsCDNF revealed that the C-terminal domain contains a CXXC motif (residues 127–130) forming a disulphide bridge (Figure 1A–B) [15–17]. We created a DmManf transgene with a point mutation that replaces cysteine-129 with serine (C129S; Figure 1C), a mutation that destroys the CXXC motif and prevents the formation of the C-terminal disulphide bridge (Figure 1A). Interestingly, this full-length DmManf transgene with a mutant C-terminal motif (DmManf-C129S) did not rescue the DmManf mutant lethality (Figure 2C). Only one of the three tested insertions resulted in very few rescued pupae (DmManf-C129S L1 in Figure 2C). The expression of DmManf-C129S in Drosophila under ubiquitous da-GAL4 driver was verified by Western blotting (Figure 2E). The secretion of V5-DmManf-C129S was not compromised in comparison to V5-DmManf in transiently transfected S2 cells analyzed by Western blotting (Figure 3A).

Positive Charge on the Surface of the N-terminal Domain of DmManf is not Essential for Fly Viability

The N-terminal domain of MANF is characterized by a globular saposin-fold of five α-helices stabilized by three disulphide bridges [15–17]. Two groups of positively charged surface amino acids were identified in the N-terminal domain of HsMANF which could interact with negatively charged phospholipids in the cell membrane [15]. To disrupt the putative lipid interaction, we designed two constructs in which selected positively charged lysines and arginines of one of the surface amino acid groups were replaced with neutral alanines: DmManf-NMG1 (N-terminal Mutant Group 1) containing amino acid changes K79A, K83A and K86A, and DmManf-NMG2 with mutations K43A, K45A and R95A (Figure 1C and Figure 2D). In the DmManf mutant background, neither of these mutation groups abolished the functionality of DmManf when ubiquitously expressed, i.e. both of the constructs rescued larval lethality of the DmManf mutants (Figure 2C).

Table 2. Number of heterozygous pupae in rescue experiments with different expression levels of the DmManf-Δss construct.

<table>
<thead>
<tr>
<th>Construct and insertion</th>
<th>da-GAL4 dosage</th>
<th>Number of balanced pupae counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmManf-Δss L1</td>
<td>1x</td>
<td>1031</td>
</tr>
<tr>
<td>DmManf-Δss L5</td>
<td>1x</td>
<td>1065</td>
</tr>
<tr>
<td>DmManf-Δss L1+DmManf-Δss L5</td>
<td>1x</td>
<td>1360</td>
</tr>
<tr>
<td>DmManf-Δss L5+DmManf-Δss L1</td>
<td>1x</td>
<td>1455</td>
</tr>
<tr>
<td>DmManf-Δss L1+DmManf-Δss L5</td>
<td>2x</td>
<td>808</td>
</tr>
<tr>
<td>DmManf-Δss L5+DmManf-Δss L1</td>
<td>2x</td>
<td>479</td>
</tr>
</tbody>
</table>

No homozygous DmManf<sup>ΔN</sup> mutant pupae were found in any of the rescue experiments listed here. Ls correspond to independent insertions. doi:10.1371/journal.pone.0073928.t002
Increased Dopamine Levels of DmManf Mutants are Normalized in Rescued Larvae

The lack of endogenous DmManf leads to the loss of dopaminergic neurites in larval central nervous system (CNS) [4]. Abolishment of both maternal and zygotic DmManf shows a substantial decrease in dopamine levels at the end of embryogenesis [4]. We wanted to characterize the dopaminergic system of rescued larvae and compare it with that of the DmManf mutant. First, we tried to analyze the DA neurites of larval CNS by staining with an antibody against tyrosine hydroxylase (TH), an essential enzyme of DA synthesis and a marker for DA neurons. Unfortunately, this immunohistochemical approach was not sensitive enough. Instead, we measured the dopamine levels of DmManf mutants and rescued larvae to address the phenotypes associated with the dopaminergic system. We collected larvae at the latest stage when the lethality of homozygous DmManf mutants occurs. Homozygous DmManf mutants showed a significant increase in dopamine levels in total larval lysates compared to wild type larvae (Figure 3D). All constructs driven by da-GAL4 that were able to rescue larval lethality (DmManf, DmManf-ΔRSEL, DmManf-NMG1 and DmManf-NMG2) also normalized larval dopamine to the wild type level (Figure 3D).

doi:10.1371/journal.pone.0073928.g003
Restricted Expression of Mutated Constructs Reveals their Effects on DmManf Functionality

When expression of DmManf constructs is driven by da-GAL4, proteins encoded by transgenes are available abundantly and ubiquitously in all tissues. This may mask the effects of mutations, especially if they affect the proportion of secreted MANF, intracellular dynamics or extracellular distribution of the protein. To assess this, we expressed DmManf, DmManf-ARSEL, DmManf-NMG1 and DmManf-NMG2 transgenes by a non-ubiquitous 69B-GAL4 which is not expressed in muscles, fat body or gastric caeca. Expression in the CNS and cuticle is clearly decreased compared to da-GAL4. Despite the restricted expression pattern, the wild type DmManf construct driven by 69B-GAL4 resulted in full rescue of the DmManf mutant (Figure 4A and Figure S1E) as previously shown [4]. The overall GAL4 expression level was slightly weaker in 69B-GAL4 than da-GAL4 analyzed by Western blotting (Figure S1F). However, with both drivers the expression of ectopic DmManf was clearly higher than the endogenous level of DmManf (Figure S1F).

Deletion of the ER retention signal (DmManf-ARSEL) clearly reduced the proportion of rescued pupae with 69B-GAL4 driver compared to ubiquitous da-GAL4 driver (Figure 4A). We analyzed whether the ARSEL mutation affected the secretion of DmManf by transiently transfecting S2 cells with V5-DmManf-pMT and V5-DmManf-ARSEL-pMT plasmids. In comparison to V5-DmManf, the level of cellular V5-DmManf-ARSEL protein was clearly decreased while the level of secreted V5-DmManf-ARSEL in the medium was at least as high as the level of secreted V5-DmManf, indicating that the proportion of secreted DmManf was increased (Figure 3A). These data suggest that ER retention is important for correct DmManf function when protein availability is spatially and/or temporally restricted.

When expressed by 69B-GAL4 driver, DmManf-NMG2 fully rescued the larval lethality of DmManf mutants similar to the wild type DmManf construct (Figure 4A). In contrast, restricted expression of the DmManf-NMG1 construct by 69B-GAL4 lead to almost complete loss of rescued DmManf mutant pupae (Figure 4A). Similar amounts of secreted and cellular V5-DmManf were detected from S2 cells transiently transfected with V5-DmManf-pMT, V5-DmManf-NMG1-pMT and V5-DmManf-NMG2-pMT constructs (Figure 3A). These data demonstrate that the neutralization of positively charged surface amino acids K79, K93 and K96 (DmManf-NMG1) but not K43, K45 and R95 (DmManf-NMG2) affect other molecular properties of DmManf than the secretory efficacy.

DmManf is Functionally more Related to Human MANF than CDNF

In our previous studies transgenic human CDNF (HsCDNF-6N9C; Figure S2A) did not rescue the DmManf mutant lethality [4]. Because the HsCDNF-6N9C construct used in the previous rescue experiments encoded HsCDNF with some extra N- and C-terminal amino acid residues (which were introduced into the construct during cloning) we decided to remove these additional residues and generate a new HsCDNF transgene (Figure 1D). To our surprise, this HsCDNF transgene expressed by da-GAL4 could, as efficiently as HsMANF, rescue the DmManf mutant lethality to adulthood (Figure 4B and Figure S1E). The extra N-terminal amino acids were likely responsible for deficient functionality of the HsCDNF-6N9C construct (Figure S2A–C).

To study the functionality of the independent N- and C-terminal domains of human MANF and CDNF in the Drosophila in vivo model, we designed mutated human MANF (N-HsMANF, C-HsMANF, C-HsMANF-Δss; Figure S3A) and CDNF (N-HsCDNF, C-HsCDNF, C-HsCDNF-Δss; Figure S2A) constructs. In line with the corresponding DmManf constructs (see above), these constructs could not rescue the larval lethality of DmManf mutants (Table 3). While only N-HsCDNF was detected from larval lysates by Western blotting (Figures S3B–C), mRNA expression was verified by RT-PCR from adult fly lysates of all of these constructs (Figures S3D–E).

We used non-ubiquitous expression of the HsMANF and HsCDNF transgenes to rescue DmManf mutant lethality by 69B-GAL4 driver. With a restricted expression pattern, HsMANF could fully rescue DmManf mutant lethality to adulthood, while HsCDNF showed clearly decreased amount of rescued pupae and no emerging adults (Figure 4A and Figure S1E). This suggests that human MANF and CDNF have diverged during evolution. We also asked whether the C-terminal domain of HsCDNF could complement the function of C-HsMANF by replacing the C-terminal domain of HsMANF (residues 105–158) with that of HsCDNF (residues 101–164; HsMANF-HsCDNF; Figure 1D). Ubiquitous expression of this construct rescued DmManf mutant lethality to adulthood (Figure 4B and Figure S1E). However, the proportion of rescued pupae with restricted expression of HsMANF-HsCDNF was clearly decreased compared to HsMANF, but closely resembled that of HsCDNF (Figure 4A). This demonstrated that functional properties of the C-terminal domain of HsCDNF differ from HsMANF and DmManf. To test this hypothesis we designed a construct with the N-terminal domain of HsCDNF (residues 1–100) and the C-terminal domain of HsMANF (residues 96–158), but failed to obtain transgenic flies.

We found that ubiquitous but not restricted expression of HsMANF-ARTDL, an HsMANF transgene with deletion of the ER retention signal RTDL (Figure 1D), could rescue DmManf mutant lethality (Figure 4B and Figure S1E). Correspondingly, the DmManf construct (DmManf-ARSEL) expressed in a restricted pattern decreased the amount of rescued DmManf mutant pupae. This further supports the importance of ER retained MANF during fly development.

DmManf Rescues Mouse SCG Neurons from Apoptosis in vitro

After studying the functionality of mammalian MANF/CDNF in the Drosophila system we addressed the opposite, i.e. can DmManf rescue the mammalian cells. We used cultured apoptotic SCG neurons overexpressing the MANF constructs as this is currently the only reliable mammalian in vitro bioassay for MANF/CDNF. At neonatal stage the SCG neurons require nerve growth factor (NGF) for survival but are also sensitive to other apoptotic stimuli, thus being an excellent model to test the survival-promoting compounds. Our recent studies have shown that a plasmid encoding HsMANF or purified recombinant HsMANF protein protects mouse SCG neurons from apoptosis when microinjected into the neuronal nucleus or cytoplasm, respectively [17]. Independent cytoplasmic C-HsMANF-Δss also shows survival promoting activity in SCG neurons. Interestingly, overexpressed HsMANF protects the neurons against etoposide treatment, but only marginally against NGF deprivation [17]. We microinjected the NGF-maintained SCG neurons derived from neonatal (P0) mouse with DmManf-pCR3.1 and C-DmManf-Δss-pCR3.1 plasmids and treated neurons with etoposide to induce apoptosis. Importantly, both full-length DmManf with the endogenous fly signal peptide and the intracellular C-DmManf-Δss were able to rescue mouse SCG neurons from apoptosis (Figure 4C), similar to HsMANF in vitro [17]. To eliminate the possibility of intracellular DmManf leakage from apoptotic cells
into the medium, we transfected etoposide-treated mammalian CHO cells with DmManf-pCR3.1 and DmManf-Dss-pCR3.1 constructs. In contrast to DmManf, no DmManf-Dss protein was detected in the medium sample by Western blot analysis (Figure S1C).

### Discussion

A neuro-protective function for MANF and CDNF has been demonstrated both in vitro [2,17] and in vivo [3–6]. Since very little is known about the mechanism of MANF and CDNF action, we used a transgenic approach in the Drosophila Manf mutant model to characterize the structural features of MANF/CDNF proteins.
needed for activity in vivo. We first used ubiquitous expression of transgenes and based on obtained results, used non-ubiquitous expression of selected transgenes. While ubiquitous abundant expression should provide sufficient MANF concentration for all cells it could mask some effects of the designed mutations affecting intracellular dynamics, extracellular distribution, or ratio of intra- and extracellular MANF levels. Therefore restricted expression (in which the transgenic wild type DmManf construct fully rescues) may reveal the effects of mutations more efficiently.

Previous studies have demonstrated that MANF is an ER resident protein upregulated by ER stress [9–12,21]. Loss of the relatively weak ER retention signal RTDL leads to increased secretion of MANF [8,22]. In this study we showed that deletion of the C-terminal RSEL sequence also increased secretion of DmManf and significantly decreased its localization to the ER. We found that ER retention of MANF was not absolutely necessary for MANF function in vivo since ubiquitously expressed DmManf-ARSEL was able to rescue DmManf mutant lethality. However, the restricted expression of DmManf-ARSEL or HsMANF-ARTDL did not fully rescue DmManf mutant lethality. This suggests that limited availability of MANF inside the cell compromises the intracellular functions of MANF.

Our study showed that signal sequence-mediated entry to the secretory pathway via the ER is crucial for the proper function of MANF since DmManf-As was unable to rescue DmManf mutant lethality. Furthermore, the MANF protein synthesized in the cytoplasm seemed to be unstable. In future studies, it would be important to address whether the loss of the ER resident or secreted pool of MANF was crucial for the functional failure of the DmManf-As mutant.

Interestingly, we found that the CXXC motif in the C-terminal domain is crucial for DmManf function in vivo since the DmManf-C129S mutant could not complement for the loss of the endogenous DmManf protein. One possibility is that MANF could relieve ER stress by helping proteins to fold properly [1] by facilitating intracellular disulphide bond formation via the C-terminal CXXC motif and thus functionally resemble thiol/oxidoreductases [1,20]. However, no oxidoreductase activity of MANF was found in vitro [9]. Mutation of cysteine-129 abolishes the CXXC disulphide bond which may result in an inactive conformation of the protein. The thiol groups could also perform other essential functions, such as redox signaling or metal ion binding that might be defected in the DmManf-C129S mutant. Additionally, the MANF CXXC disulfide bond which may result in an inactive domain is crucial for DmManf function since DmManf-terminal CXXC motif and thus functionally resemble thiol/endogenous DmManf protein. One possibility is that MANF C129S mutant could not complement for the loss of the secreted pool of MANF was crucial for the functional failure of the DmManf mutant. In contrast to zygotic and maternal DmManf mutant embryos [4], the homozygous zygotic DmManf mutant larvae showed increased dopamine levels. Microarray analysis [12] revealed in both embryonic and larval DmManf mutants upregulation of TH transcript expressed specifically in the hypoderm [24]. Hypodermal cells give rise to cuticle where DA derivatives are used as cross-linkers. In zygotic and maternal DmManf mutant embryos with decreased DA levels the total lack of DmManf could lead to deficient DA synthesis despite increased TH transcript levels. While the zygotic mutant larvae still possess a pool of maternal DmManf, increased DA synthesis might remain with an increased rate due to the elevated amounts of TH until the maternal pool of DmManf has been depleted.

DmManf also efficiently protected apoptotic mammalian neurons. Although the in vitro SCG neuron model and the in vivo fly rescue models are not directly comparable, this result shows that the basic neuro-protective function of MANF has persisted during evolution. However, the protective mechanisms in these two models may significantly differ, as C-DmManf-As protected SCG neurons but was ineffective in rescuing the mutant fly phenotype. Moreover, the MANF/CDNF N- or C-terminal domains alone, even if expressed together within the same cells, could not rescue the larval lethality of DmManf mutants demonstrating that an intact full-length protein is crucial for fly viability.

In conclusion, our study provides the first in vivo demonstration of structural and functional determinants of the MANF/CDNF protein family.

Materials and Methods

Fly Stocks and Maintenance

Flies were maintained at 25°C on malt-based media. The fly stocks used were: w+, UAS-DmManf [33] (insertion L3), UAS-DmManf [15] (insertion L5), UAS-HsCDNF-6N9C, and DmManf / TM6 Tb Sb [4]; UAS-lacZ [25]; UAS-GFP.nls #4775 [26], sgl-EYFP-ER #7195 [27], da-GALA #5460 [28], 69B-GALA #1744 [25], tub-GALA #5138 [29], and salb-GALA #5818 [30] from Bloomington Drosophila Stock Center.

Generation of Transgenic Flies

Mutations were generated by PCR mutagenesis using Phusion® Hot Start High-Fidelity DNA polymerase (Finnzymes) and subcloned into the pUAST vector [25] (Table S1). Most mutations were created on templates in pBlueScript SK or pCR3.1 (Invitrogen) vectors (Table S1). The remaining constructs or templates were created according to the following (numbers correspond to Table S1, primers are listed in Table S2): (1) DmMANF-K9SA-Bluescript was created by inverse PCR mutagenesis with primers DmMANF-R9SA.fwd and DmMANF-Nterm.rev, DmMANF-Bluescript [4] was used as a template; (2) HsMANF-pCR3.1 – HsMANF with honeybee melittin secretion signal [4] was subcloned as an EcoRI-XhoI fragment from the pUAST vector into pCR3.1. To create FL-HsMANF without extra C-terminal residues, a stop codon was introduced by PCR; (3) HsMANF-HsCDNF-pCR3.1 – a C-
For quantification of rescued adults, amounts of rescued pupae of 0.33 (homozygous UAS lines) or 0.17 (heterozygous UAS lines) divided by the amount of all pupae and the ratio normalized by 10 days after egg laying (AEL). The amount of normal pupae was 3 days. Normal crosses were done and transferred twice to new vials every 2–14 AEL, respectively. Emerged adults are presented as proportion of rescued pupae.

**Rescue Experiments**

Five GAL4 DmManfF3′/TM6 Tb Sh females were crossed to 2–3 UAS-si/Cyo; DmManfF3′/TM6 Tb Sh males. Five parallel crosses were done and transferred twice to new vials every 2–3 days. Normal (Tb+) and short (Tb−) pupae were counted 9–10 days after egg laying (AEL). The amount of normal pupae was divided by the amount of all pupae and the ratio normalized by 0.33 (homozygous UAS lines) or 0.17 (heterozygous UAS lines). For quantification of rescued adults, amounts of rescued pupae (Tb+) and adults (Sh+) were counted 10 days AEL and daily 10–14 AEL, respectively. Emerged adults are presented as proportion of rescued pupae.

**Immunoblotting**

Larvae were homogenized in 300 μl lysis buffer (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) supplemented with Complete protease inhibitor tablets (Roche). Cultured cells were washed once with PBS and lysed in membrane lysis buffer (TBS, 1% Triton X-100, 20 mM NaF, 1 mM EDTA, pH 7.5) containing Complete protease inhibitor tablets (Roche). Insoluble material was sedimented by centrifugation and protein concentration in the cleared lysates was measured using Bradford reagent (Bio-Rad). Samples were run on 14% or 15% SDS-PAGE and transferred onto nitrocellulose filters. The filters were incubated in blocking buffer overnight (5% non-fat dried milk in TBS +0.1% Tween-20) and then incubated in primary antibodies diluted in blocking buffer for 1 hour. Antibodies used were rabbit anti-CDNF [3], rabbit anti-MANF [21], rabbit anti-DmManf [4], anti-V5 (Invitrogen), anti-α-tubulin (DM1A, Sigma) and anti-acetylated α-tubulin (6-11B-1, Sigma; Figures 2A, 2D and S1F). Secondary antibody incubations and enhanced chemiluminescence detection were performed according to standard protocols. Molecular weights were approximated by using the science gateway Protein Molecular Weight calculator (http://www.sciencegateway.org/tools/proteinmw.htm). For infrared imaging, blots were incubated with IRDye secondary antibodies (Li-Cor, Lincoln, NE, USA) according to the manufacturer’s instructions. Blots were visualized by the Odyssey infrared imager (Li-Cor) and band intensities were estimated using the ImageJ program [31].

**Immunohistochemistry, Confocal Microscopy and Image Analysis**

Third instar larvae were fixed and immunostained with rabbit anti-DmManf [4] according to standard protocols. Samples were analyzed with TGS SP5 AOB5 (Leica Microsystems) equipped with HCX PL APO 20x/0.7 Imm Corr (glycerol) or HCX APO 63x/1.30 Corr (glycerol) CS 21 objectives. ImageJ 1.43u was used to create representative images of confocal stacks [31]. AutoQuant X3 (MediaCybernetics) was used in deconvolution and Imaris 7.6.0 (Bitplane AG) in quantification of ER localized and total cellular DmManf.

**Constructs used in Transient Transfection**

DmManf-AsS cDNA was subcloned from the corresponding construct in pBlueScript (Table S1) to the pCR3.1 vector as a Pml–Xhol fragment. V3-DmManf-pMT, V5-DmManf-AsS-pMT, V5-DmManf-ARSEL-pMT, V5-DmManf-C129S-pMT, V5-DmManf-NMG1-pMT, and V5-DmManf-NMG2-pMT were cloned as follows: V5-tag was added to the corresponding constructs in pBlueScript (Table S1) by inverse PCR mutagenesis with forward primer V5Dm.F and reverse primers V5-Dm-5atg.R (DmManf-AsS) or V5-Dm-ss.R (other constructs) and subcloned as EsoRI–Xhol fragments to pMT(A) (Invitrogen).

**Transfection of S2 and CHO Cells**

Schneider 2 (S2) cells were cultured in M3-BPYE medium (Shields and Sang M3, 0.5 g/l KHCO3, 1.0 g/l yeast extract, 2.5 g/l bactopeptone and 10% fetal bovine serum, pH 6.6) at +25°C. Transfections were performed using Fugene HD reagent (Roche). Expression from the metallothionein promoter of pMT was induced with 600 μM CuSO4, Media and cells were collected 3 days post-transfection. Chinese hamster ovary (CHO) cells were cultured in DMEM, 10% fetal bovine serum and antibiotics. The cells were plated in 12-well plates and on the following day transfected with the expression plasmids using Fugene HD reagent (Roche). After 3 days media and cells were collected.

**Dopamine Concentration Measurements**

Larvae were collected from apple juice plates 50–55 h after egg laying in 0.5–3.2 mg samples and homogenized with an ultrasonic processor. Analysis was done with high-performance liquid chromatography (HPLC) using a Gemini C18 3 μm, 4.6×75 mm, column (Phenomenex). A 12-channel ESA CoulArray Electrode Array Detector system and CoulArray for Windows software (ESA Inc.) was used for quantification of dopamine [32]. Dopamine was identified by both exact retention time and characteristic electrochemical properties.

**RT-PCR**

Total RNA was extracted from 50 1st instar larvae (DmManf samples) or 5 adults (HsMANF and HsCDNF samples) by RNeasy Mini Kit (Qiagen) and treated with RNase-Free DNase (Promega), M-MLV Reverse Transcriptase (Promega) was used in cDNA synthesis and GoTag® Hot Start Polymerase in PCR reaction. Primers and expected sizes of PCR products are listed in Table S3.
Microinjection of SCG Neurons

DmManf and C-DmManf-Δss cDNAs were subcloned from corresponding constructs in pBlueScript (Table S1) to the pCR3.1 vector as PstI-XhoI fragments. SCG neurons were prepared from neonatal mice anesthetized by hypothermia and sacrificed by decapitation. All procedures for animal use were approved by the University of Helsinki Laboratory Animal Centre (Protocol number KEK11-020). Neurons were cultured with NGF (Promega) for 3–6 days and microinjected with the plasmids encoding for the respective DmManf constructs or the empty vector (negative control) together with the reporter plasmid of EGFPL. Immediately after microinjection, the neurons were treated with 30 μM of etoposide (Sigma-Aldrich) in the presence of NGF. Living EGFPL-expressing neurons were counted three days later in a “blind” manner and expressed as percent of original injected neurons. The NGF-maintained untreated neurons show the absence of cell death without etoposide. Average ± SEM of 2–5 independent experiments is shown. Data from each experimental group was compared with control plasmid pCR3.1-injected neurons (vector).

Statistical Analysis

The means were compared by one-way ANOVA and post hoc Dunnett’s test (SCG neuron analysis) or by Student’s t-test (dopamine concentration analysis and ER-localised DmManf analysis). The null hypothesis was rejected at p<0.05. Statistical analysis was performed by using GraphPad InStat 3 program (GraphPad Software, Inc.; SCG neuron analysis) or Microsoft® Excel Analysis ToolPak (Microsoft® Office Professional Plus 2010; other analyses).

Supporting Information

Figure S1 Additional information of mutated DmManf constructs. A) Schematic presentation of combinations of DmManf N- and C-terminal domain constructs (with or without secretion signal peptide). Colours are according to Figure 1C. Results of each rescue experiment are presented in Table 1. B) Expression analysis of the DmManf-Δss construct in wild type background by Western blotting from 3rd instar larvae. Expression level of the DmManf-Δss was increased by two copies of construct and by two copies of da-GAL4 driver and compared to that of the DmManf construct and endogenous expression of DmManf in wild type. C) Western blot analysis of transiently transfected Chinese hamster ovary cells reveals that the DmManf-Δss is not secreted while DmManf and DmManf-ARSEL are. Etoposide treatment does not cause leakage of DmManf-Δss from cells into the medium. D) In vivo expression analysis of DmManf (b, c and h) and DmManf-Δss (c, f and i) constructs in wing discs of 3rd instar larvae overexpressed by salm-GAL4. Overexpression of lacZ (a, d and g) was used as a control for endogenous DmManf. Transgene expression was detected as upregulation of DmManf immunoreactivity (red; d-f) in salm-GAL4 expression pattern marked by nuclear GFP (GFPlns in green; a-c). White arrows (h and i) indicate the secreted DmManf. E) Rescue of DmManf Δ96 mutant pupal lethality by wild type or mutated DmManf, HsMANF or HsCDNF. Wild type flies were used as control. Average ± SD. F) Expression level of da-GAL4 and 69B-GAL4 in 3rd instar larvae by Western blot analysis. Endogenous DmManf expression in wild type background was compared to transgenic DmManf (L5) expressed by da-GAL4 or 69B-GAL4 in homozygous DmManf Δ96 mutant background. G) Western blot analysis of cell lysates and medium from Schneider 2 cells transiently transfected with wild type and mutated V5-tagged DmManf-pMT constructs. Band intensities were normalised to DmManf. In Western blotting analyses, alpha-tubulin was used as loading control. (TIF)

Figure S2 Extra N-terminal amino acid residues disrupt HsCDNF functionality in vivo. A) Schematic presentation of HsCDNF, HsCDNF-6N9C, HsCDNF-6N, HsCDNF-9C, N-HsCDNF, C-HsCDNF and C-HsCDNF-Δss constructs, colours according to Figure 1B. Since either the N- or C-terminal extra residues of HsCDNF-6N9C, or both, could be responsible for the loss of its functionality, we designed two HsCDNF transgenes, one with extra N-terminal residues (SLLTQG; HsCDNF-6N) and the other with extra C-terminal residues (LEGTSRSGSL; HsCDNF-9C). Gray boxes indicate the additional N- and C-terminal amino acids in HsCDNF-6N9C, HsCDNF-6N and HsCDNF-9C constructs. Honeybee melittin was used as a secretion signal. B) Similarly to HsCDNF, HsCDNF-6N9C is expressed and secreted from transiently transfected Schneider 2 cells. Thus, the negative rescue result by HsCDNF-6N9C was likely not due to an expression or secretion defect. C) HsCDNF-6N9C fails to rescue DmManf Δ96 mutant lethality while HsCDNF-9C fully rescues, similar to the HsCDNF construct. HsCDNF-6N shows only mild rescue of DmManf Δ96 mutant lethality. This suggested that the extra six N-terminal residues in the original HsCDNF-6N9C construct were responsible for the loss of functionality. Constructs were ubiquitously expressed by da-GAL4. L, independent insertions of the constructs. (TIF)

Figure S3 Independent N- and C-terminal domains of HsMANF and HsCDNF fail to rescue DmManf mutant lethality. A) Schematic presentation of HsMANF, N-HsMANF, C-HsMANF and C-HsMANF-Δss constructs. N- and C-terminal domain constructs failed to rescue DmManf mutant lethality (Table 3). Colours are according to Figure 1B. B-C) Protein expression of HsMANF (B) and HsCDNF (C) constructs was verified by Western blotting from 3rd instar larvae. Constructs were ubiquitously expressed by tub-GAL4 driver in the wild type or heterozygous (red typing) DmManf mutant backgrounds. Coloured boxes under the blot indicate the domains of the construct corresponding to Figure S2A and Figure S3A. Calculated molecular weights of full length proteins, N- and C-terminal domains are presented next to Western blot images. L, independent insertions of the constructs. Alpha-tubulin was used as a loading control. D-E) Transcription from N- and C-terminal domain constructs of HsMANF (D) and HsCDNF (E) was verified by RT-PCR from adult flies. Constructs were expressed by tub-GAL4 in wild type background. Wild type HsMANF and HsCDNF constructs were used as positive controls, wild type flies as negative controls. Coloured boxes indicate the domains of the construct corresponding to Figure S2A and Figure S3A. (TIF)

Table S1 List of constructs used in generation of transgenic flies. Cloning primers are presented in Table S2. Cloning details for 1–8 are presented in Materials and Methods. del, deletion; ins, insertion; ss, secretion signal. (PDF)

Table S2 List of primers used in cloning of constructs for generation of transgenic flies and in RT-PCR. (PDF)

Table S3 Primer pairs used in RT-PCR to detect mRNA expression. (PDF)
Acknowledgments

We are grateful to Bloomingston Drosophila Stock Center for fly stocks and to Genetic Services, Inc. for transgenics services. We thank M. Airavaara, A. Eesmaa, M. Friander, M. Molin, M. Palgi, P. Permi, J. Pera¨nen, K. Sims-Huopaniemi and J. Vuili for invaluable advice and critical comments on the manuscript. A. Ikavaliko, I. Mustonen and K. Virtanen are acknowledged for excellent technical assistance.

References


Author Contributions

Conceived and designed the experiments: RL, PL, UA MS THH. Performed the experiments: RL, PL, JK L-YY TPP THH. Analyzed the data: RL, PL, TPP UA. Wrote the paper: RL, PL, UA MS THH.