Funktionelle Charakterisierung der Metalloprotease Neprilysin 4 aus *Drosophila melanogaster*

Dissertation

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1. Zusammenfassung

Im Menschen regulieren extrazelluläre Metalloproteasen eine Vielzahl von physiologischen Prozessen, wobei deren exakte Funktionen bei der Ausbildung humaner Krankheiten wie beispielsweise Krebs, der Alzheimerschen Krankheit oder Störungen des Herz-Kreislaufsystems vielfach noch unbekannt sind. Insbesondere die Proteinfamilie der Neprilysine wird seit einigen Jahren vermehrt in Bezug auf eine mögliche Anwendung als Therapeutikum gegen die genannten Erkrankungen hin diskutiert.

In dieser Arbeit wurde erstmals die M13-Metalloprotease Neprilysin 4 (Nep4) aus dem Modellorganismus *Drosophila melanogaster* charakterisiert. Zusätzlich wurde zu Beginn dieser Arbeit das ADAM (A Disintegrin And Metalloprotease)-Protein Meltrin analysiert.

Innerhalb der Neprilysin-Familie kommt Nep4 eine Sonderrolle zu, da es im Gegensatz zu den meisten Neprilysinen nicht ausschließlich als membrangebundenes Protein, sondern isoformspezifisch auch in löslicher Form exprimiert wird. In diesem Zusammenhang deuten RT-PCRs, *in situ* Hybridisierungen und Antikörperfärbungen auf ein breites Funktionsspektrum beider Isoformen hin, das in den zahlreichen Geweben, in denen Nep4 exprimiert wird, hauptsächlich der Etablierung und Aufrechterhaltung der Homöostase verschiedener bioaktiver Peptide dienen dürfte. Über den gesamten Lebenszyklus der Fruchtfliege kann Nep4 in Gliazellen des ZNS und in den männlichen Geschlechtszellen nachgewiesen werden, während es im Verlauf der Embryogenese zusätzlich in Herz- und Muskelzellen exprimiert wird. Die regulatorischen Elemente zur Steuerung der neuronalen (ZNS) und mesodermalen (Herz und Muskel) Nep4 Expression konnten in dieser Arbeit identifiziert und für die Erzeugung transgener Fliegenlinien genutzt werden. Mittels semi-quantitativer PCR und durch Untersuchungen von Fliegen, die GFP unter der Kontrolle des mesodermalen Enhancers exprimieren, wurde die endogene Expression von Nep4 im Muskel von Larven des dritten Stadiums nachgewiesen. Da alle nachfolgenden Stadien Reporteraktivität im Herzen und Muskel zeigen, wird die Peptidase vermutlich durchgängig in diesen Geweben benötigt.

Die katalytische Aktivität von Nep4 konnte anhand der Peptide Substanz P und Angiotensin I demonstriert werden. Dabei ist die Enzymaktivität, wie für die Neutralen Endopeptidasen (Nep) typisch, von einem neutralen pH-Wert abhängig und wird durch bekannte Inhibitoren der humanen Neprilysine, Nep und Nep2 reduziert.

Bei einer künstlich erhöhten Expression von Nep4 in der Muskulatur der Fruchtfliege ist, entgegen der Erwartung, nicht die katalytische Aktivität, sondern ausschließlich die nicht katalytische, intrazelluläre Domäne ursächlich für eine nekrotische Gewebedegeneration. Um

eine mögliche Funktion der intrazellulären Domäne des Nep4 Proteins im Muskel genauer zu erforschen, wurden Proteininteraktionsstudien durchgeführt, erste Interaktionspartner identifiziert und deren Interaktion auf Proteinebene nachgewiesen.

2. Einleitung

2.1. Die Klassifizierung der Metalloproteasen

Bei den Metalloproteasen handelt es sich um eine sehr diverse Gruppe von Enzymen, die zurzeit mehr als 60 Familien aus den unterschiedlichsten Organismen umfasst (Rawlings und Barrett 1995; Merops Database, http://merops.sanger.ac.uk/cgi-bin/family_index?type=P#M). Die Mehrheit ihrer Vertreter gehören zu den Zinkinen, die über eine hochkonservierte Konsensussequenz (HExxH) Zink in ihrem aktiven Zentrum binden (Hooper 1994; Bode et al. 1993). Da Zink tetrakoordiniert vorliegt, wird es neben den zwei Histidinen und einem Wassermolekül durch einen weiteren Liganden gebunden, der C-terminal vom eigentlichen Bindemotiv liegt.

Die Zinkine teilen sich in drei Subfamilien auf: die Metzincine, die Gluzincine sowie die Aspzincine (Hooper 1994; Gomis-Rüth 2003), wobei in der vorliegenden Arbeit lediglich Vertreter der beiden erstgenannten Gruppen analysiert wurden.

Namensgebend für die Familie der Metzincine (Bode et al. 1993; 1996; Gomis-Rüth 2009) ist ein konserviertes Methionin im sogenannten „Met-turn“, das wesentlich zur Stabilisierung des aktiven Zentrums beiträgt und somit für die Funktion bzw. Faltung der Proteine unerlässlich ist (Bode et al. 1993; Stöcker et al. 1995; Tallant et al. 2010). Bei den Metzincinen handelt es sich zumeist um Multidomänenproteine. Durch die proteolytische Abspaltung der N-terminalen Prodomäne werden die meist als Zymogene vorliegenden Metzincinvertreter posttranslational aktiviert. Stromabwärts der Prodomäne befinden sich die katalytische Domäne sowie weitere Domänen, die den Zell-Zell Kontakt, die Protein-Protein Interaktion oder auch die subzelluläre Lokalisation der Proteasen bedingen (Abb. 1). Eine den Metzincinen angehörende Proteingruppe stellt die Familie der ADAMs (A Disintegrin And Metalloprotease) dar, zu der das im Verlauf der vorliegenden Arbeit u.a. bearbeitete *Drosophila melanogaster* (*Dm*) Protein Meltrin (CG7649) zählt. Weitere in *Drosophila* vorkommende Vertreter der Metzincine sind beispielsweise die ADAM-TS-Proteine (ADAM Proteasen mit Thrombospondinmotiv) sowie die Matrixmetalloproteasen (MMPs) (Meyer et al. 2011).

2.2. Vertreter der ADAM Metalloproteasen in *Drosophila*

Beim Menschen konnten bislang 23 Mitglieder der ADAM-Familie identifiziert werden (Zolkiewska 2008), während in *Drosophila (Dm)* nur 5 ADAMs existieren. Diesen 5 ADAMs

können drei Säugetier-Homologe gegenübergestellt werden: ADAM10 ist homolog zu *Dm*Kuzbanian und *Dm*Kuzbanian-like, ADAM17 zu *Dm*TACE und für ADAM12 (meltrin-α) liegen zwei Homologe, Meltrin und Mind-meld in *Drosophila* vor (Sapir et al. 2005; Meyer et al. 2011). Für alle drei genannten ADAM Proteine aus Vertebraten konnte proteolytische Aktivität in Form des sogenannten *ectodomain sheddings* nachgewiesen werden (Lunn et al. 1997; Black et al. 1997; Loechel et al. 1998). Da die ADAM Proteine meist membrangebunden sind, wobei die aktive Domäne extrazellulär liegt, beschränkt sich ihre katalytische Aktivität im Allgemeinen auf Zelloberflächenproteine. Durch die Abspaltung der Ektodomänen dieser Substrate werden die entsprechenden Domänen freigesetzt und können als lösliche Signalmoleküle dienen, die auch auf weit entfernte Zellen regulativen Einfluss ausüben. Zusätzlich kann die Abspaltung auch zur Inaktivierung oder Aktivierung des jeweiligen Proteins führen, was verschiedene Signalkaskaden auslösen kann (Blobel 2005).

Eine der am besten verstandenen Funktionen des *ectodomain sheddings* ist die Spaltung verschiedenster Substrate durch ADAM17 (TACE: TNF- α converting enzyme; Review: Gooz 2010), welches hierdurch in humanen Krankheiten wie Krebs, Diabetes, Arthritis, Herz-Kreislauferkrankungen und Alzheimer eine Rolle spielt. In *Drosophila* sind TACE wie auch Kuzbanian beispielsweise an der Spaltung von Delta und Notch, einem Rezeptor-Liganden System mit zentraler Bedeutung für die Spezifizierung von benachbarten Zellen verschiedener Gewebe, beteilig (Delwig und Rand 2008). So ist im Verlauf der Herzentwicklung von *Drosophila* die Kuzbanian-katalysierte Spaltung von Notch sowohl im frühen kardialen Mesoderm als auch im späteren Herzgewebe essentiell: Kuzbanian prozessiert den Rezeptor Notch und bedingt hierüber, mittels lateraler Inhibition, die frühe Determination bestimmter Mesodermzellen zu Herzzellen. Darüber hinaus reguliert Kuzbanian durch den Prozess der asymmetrischen Zellteilung die Ausbildung der finalen Herzmorphologie (Albrecht et al. 2006). Bislang konnte für keines der anderen ADAMs aus *Drosophila* eine Beteiligung an der Herzentwicklung nachgewiesen werden. Im Vertebratenherzen hingegen spielen eine Vielzahl von ADAMs eine Rolle (Asakura et al. 2002; Shi et al. 2003; Zhou et al. 2004; Kurohara et al. 2004; Horiuchi et al. 2005), wobei nach neuesten Erkenntnissen auch das Kuzbanian Homolog ADAM10 beteiligt ist. Die ADAM10 vermittelte Spaltung der in der Kardiomyozytenmembran lokalisierten Peptidase Corin führt zur Aktivierung des atrialen natriuretischen Peptides und somit zur Regulation des Blutdrucks (Jiang et al. 2011).

Zusätzlich zur Herzentwicklung ist Kuzbanian auch im ZNS für das Axonwachstum und die korrekte Wegfindung erforderlich (Fambrough et al. 1996; Schimmelpfeng et al. 2001). Kuzbanian Mutanten zeigen hierbei ähnliche Phänotypen wie Mutationen von Komponenten

des Notch Signalweges. Aus diesem Grund vermuteten Jarriault und Greenwald (2005), dass der Großteil der Notch-vermittelten Zellspezifikationsprozesse durch Kuzbanian und nicht durch andere *Drosophila* Proteasen wie TACE kontrolliert wird. Auch wenn die hydrolytische Aktivität von TACE in Hinblick auf Substrate wie Notch, Delta oder auch Serrat bereits mehrfach gezeigt wurde (Sapir et al. 2005; Delwig und Rand 2008), konnte *Dm*TACE im Gegensatz zum menschlichen Homolog bislang nicht mit entwicklungsbiologischen Prozessen in Verbindung gebracht werden. Vielmehr konnte ausgeschlossen werden, dass das TGF-α Homolog Spitz in *Drosophila* durch TACE prozessiert wird (Lee et al. 2001). Die Expression von *tace*-spezifischen RNAi Konstrukten im Flügel (Sapir et al. 2005) sowie im Embryo (Lee et al. 2001) führte darüber hinaus zu keinem nennenswerten Phänotyp, so dass fragwürdig ist, ob TACE in *Drosophila* eine ebenso essentielle Rolle spielt wie sein Homolog im Menschen.
Kuzbanian-like steht Kuzbanian phylogenetisch am nächsten und zeigt ein ähnliches Substratspektrum (Sapir et al. 2005). Wie Kuzbanian ist es an Notch-abhängigen Signalkaskaden beteiligt und scheint maßgeblich in die Differenzierung der Flügel (Sapir et al. 2005) und in die Morphogenese des Follikelepithels während der Oogenese (Assa-Kunik et al. 2007) involviert zu sein.

Die beiden noch verbliebenen *Drosophila* ADAM Proteine sind Meltrin und Mind-meld. Im Falle von Meltrin konnte das Transkript im Verlauf dieser Arbeit in Zellen des embryonalen bzw. larvalen ZNS detektiert werden (Meyer et al. 2010). Insbesondere die spezifische und durch den Faktor „Ventral nervous system defective“ (VND) lokal begrenzte Expression von Meltrin im Embryo deuten auf eine Funktion des Proteins in der Entwicklung des embryonalen Nervensystems hin. Die Expression der vermutlich drei Transkriptvarianten beschränkt sich jedoch nicht auf die genannten Entwicklungsstadien, sondern erstreckt sich auf konstantem Niveau über den gesamten Lebenszyklus der Fruchtfliege (diese Arbeit, Meyer et al. 2010). Aus diesem Grund spielt Meltrin vermutlich nicht nur in der Neurogenese, sondern auch im ausdifferenzierten Nervensystem eine Rolle. Die proteolytische Aktivität der Metalloprotease konnte durch die Spaltung des Notch Liganden Serrat *in vitro* gezeigt werden (Sapir et al. 2005). Dabei wird die Aktivität von Meltrin möglicherweise durch Oligomerisierungsprozesse reguliert, die durch die ACR-Domäne (A Cysteine Rich) des Proteins realisiert werden (Abb. 1) (Meyer et al. 2010). Für Meltrinmutanten, in denen Meltrin aufgrund einer P-Element Insertion nicht transkribiert wird und für sogenannte

dominant-negative Mutanten, die zusätzlich katalytisch inaktives Meltrin im Tier exprimieren, konnte kein nennenswerter Phänotyp identifiziert werden (diese Arbeit).
Bezüglich Mind-meld liegen zurzeit kaum Informationen vor. Bislang wurde lediglich die Expression im ZNS nachgewiesen (Chase et al. 1987); katalytische Aktivitäten oder physiologische Prozesse, in die das Protein involviert ist, konnten hingegen noch nicht gezeigt werden.

Im Gegensatz zu den Metzincinen zeichnet sich die Familie der Gluzincine (Hooper 1994) durch einen vom katalytischen Zentrum stromabwärts liegenden Glutaminsäurerest aus, der zusammen mit den beiden Histidinen des HExxH-Motivs das katalytisch aktive Zinkion koordiniert. Die im HExxH-Motiv befindliche Glutaminsäure führt hingegen den nukleophilen Angriff auf die zu spaltende Peptidbindung aus. Ein weiterer Unterschied zu den Metzincinen liegt darin, dass Gluzincine nicht als Zymogene, sondern als aktive Enzyme synthetisiert werden und sich durch ihre Spezialisierung auf die Spaltung relativ kleiner Peptidsubstrate auszeichnen (Rawlings und Barrett 1995). Zu den bekanntesten Vertretern dieser Peptidasefamilie gehören beispielsweise die Aminopeptidase-N, Angiotensin konvertierende Enzyme (ACE), Thermolysine, Neprilysine sowie Endothelin konvertierende Enzyme (ECE) (Marchler-Bauer et al. 2011). Das im Verlauf dieser Arbeit vorrangig untersuchte Protein Neprilysin 4 aus *Drosophila melanogaster* ist innerhalb der Gluzincine der Gruppe der M13-Peptidasen angegliedert. Die M13-Familie der Neprilysine wird im Folgenden genauer charakterisiert.

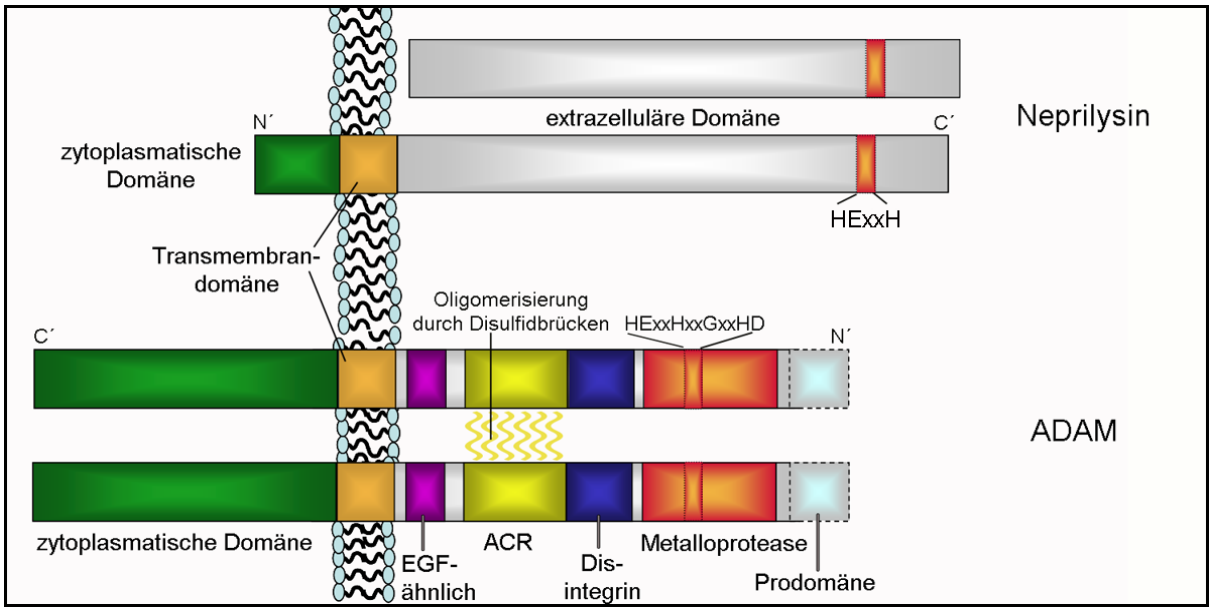

Abb. 1: Domänenaufbau der ADAM Proteine und Neprilysine. Die Familie der ADAM Proteasen setzt sich in der Regel aus einer N-terminalen Prodomäne, einer Metalloproteasedomäne, die ein hochkonserviertes katalytisch aktives Motiv enthält, einer Disintegrin-, einer Cystein-reichen- (ACR), einer EGF-ähnlichen-, einer Transmembran- und einer großen zytoplasmatischen Domäne zusammen. Die Prodomäne interagiert mit der katalytischen Domäne, so dass sie die Substratkatalyse verhindert. Entsprechend wird die Prodomäne in Folge der Proteinreifung abgespalten. Die ADAM Metalloprotease *Dm*Meltrin bildet, vermittelt durch Disulfidbrücken der ACR Domäne, Oligomere aus. Bei den Neprilysinen handelt es sich im Gegensatz zu den ADAMs um membranständige Proteine des Typs II, wobei die zytoplasmatische Domäne häufig kleiner ist als die der ADAMs. Die extrazelluläre Domäne umfasst etwa 700 AS und zeichnet sich durch ein katalytisches Motiv aus, welches den ADAM Proteasen ähnlich ist. Zusätzlich werden einige Neprilysine posttranskriptional als lösliche Enzyme in den Extrazellularraum entlassen. Neprilysin 4 hingegen liegt in Form von zwei Isoformen vor: einer membranständigen Isoform A und einer löslichen Isoform B, die jedoch bereits als lösliches Enzym exprimiert wird.

2.3. Struktureller Aufbau und katalytische Aktivität der Neprilysine

Mitglieder der M13-Familie der Neprilysine werden als Multidomänenproteine bezeichnet und sind zumeist membranständige Proteine des Typs II, bestehend aus einem kleinen intrazellulären N-Terminus (etwa 30 AS), einer einzelnen Transmembrandomäne und einer großen extrazellulären C-terminalen Domäne ($\geq$ 700 AS), die das aktive Zentrum des Enzyms trägt. Die katalytische Domäne der Neprilysine zeichnet sich durch die konservierten Sequenzmotive HExxH und ExxA/GD aus (Hooper 1994; Roques et al. 1993). Dabei koordinieren die zwei Histidine des ersten Motivs, zusammen mit einem Wassermolekül sowie dem Glutamat aus dem zweiten Motiv das divalente Kation Zink. Das zentrale Glutamat aus dem HExxH-Motiv fungiert als Ligand für das Wassermolekül, welches durch das Zinkion stabilisiert wird. Das Wassermolekül wird durch das Glutamat polarisiert, wodurch dieses im Anschluss die Peptidbindung angreift und spaltet. Die Spaltung erfolgt dabei bevorzugt am Aminoterminus großer hydrophober oder aromatischer Reste (Hersh und Morihara 1986).

Die katalytische Funktionalität der HExxH-Domäne hängt dabei sowohl von den beiden Histidinen als auch vom zentralen Glutamat ab (Klimpel et al. 1994; Fushimi et al. 1999; Rioli et al. 2003). Insbesondere die Mutagenese des zentralen Glutamats führt zu einem vollständigen Aktivitätsverlust, wobei die Konformation und Substratbindung des Proteins dadurch vermutlich nicht verändert werden (Rioli et al. 2003). Entsprechend konnte dieses Verfahren bereits zur Identifizierung neuer endogener Peptidasesubstrate genutzt werden (Rioli et al. 2003).

Die extrazelluläre Domäne der Neprilysine bildet zwei alpha-Helices aus, wobei die sich an die Membrandomäne anschließende Helix die beiden Motive zur Substratbindung und Spaltung (HExxH und ExxA/GD) enthält. Zusammen bilden beide Helices eine Substrattasche aus, deren Größe etwa 20 Ångström umfasst und somit nur für Substrate einer molekularen Masse von $\leq$ 3-5 kDa zugänglich ist (Oefner et al. 2000; 2004). Das 40 bzw. 42 Aminosäuren umfassende Amyloid-ß Peptid gehört zu den größten Substraten, die noch durch Vertreter der Neprilysin Proteinfamilie gespalten werden können (Shirotani et al. 2001), wobei nur ein kleiner Teil des Oligopeptids tatsächlich in die katalytische Tasche gelangt, während ein Großteil außerhalb des Neprilysins verbleibt. Weitere physiologisch relevante Substrate der Neprilysine sind Enkephaline, Endotheline, Substanz P oder auch das atriale natriuretische Peptid, deren Größe zwischen 0,5 kDa (5 AS) und 3 kDa (28 AS) liegt.

Zusätzlich zu den beiden genannten konservierten Motiven (HExxH und ExxA/GD) steht auch ein weiteres Motiv (VNAFY) des humanen Neprilysins mit dem jeweiligen Substrat in Kontakt (Oefner 2000). Dabei kommt die VNAFY Sequenz häufig in Neprilysinen vor, während ein modifiziertes Motiv (VNAYY) eher typisch für die ECE-ähnlichen Vertreter ist. Auch *Dm*Nep2 und *Dm*Nep1 weisen das Neprilysin typische VNAFY Motiv auf, während sich in *Dm*Nep3 und *Dm*Nep4 die Sequenz der ECE-ähnlichen Enzyme findet (Bland et al. 2008). Diese Differenz in den Motiven könnte die Substratspezifitäten der Neprilysine aus *Drosophila* signifikant beeinflussen.

Darüber hinaus bedingt das Vorhandensein von konservierten Cysteinresten in der extrazellulären Domäne der Neprilysine eine ähnliche Tertiärstruktur der Proteine und somit vermutlich auch ähnliche katalytische Mechanismen. So sind beispielsweise alle 10 in humanem Neprilysin vorkommenden extrazellulären Cysteine an der Ausbildung von Disulfidbrücken beteiligt (Oefner 2000). Eine entsprechende Anzahl an Cysteinen findet sich auch in *Dm*Nep2 und *Dm*Nep4, während *Dm*Nep1 11 und *Dm*Nep3 14 Cysteine in der entsprechenden Domäne aufweisen.

2.4. Struktur und Funktion der intrazellulären Domäne der Neprilysine

Die N-terminale, intrazelluläre Domäne der M13-Metalloproteasen ist im Gegensatz zur katalytischen Region hoch variabel (Bland et al. 2008), wobei diese Variabilität innerhalb der Neprilysine der Mammalia weniger stark ausgeprägt ist als bei anderen Mitgliedern der M13-Familie (Sumitomo et al. 2004; Terawaki et al. 2007). Bezüglich der Funktionen dieser Domäne ist bislang nur wenig bekannt. Allerdings konnte in humanem Neprilysin ein Motiv

aus positiv geladenen Aminosäuren (in der Hauptsache Lysine und Arginine) gefunden werden, welches das Tumorsupressorprotein PTEN bindet (Sumitomo et al. 2004) (Abb. 2 A, unterstrichenes Motiv). Des Weiteren konnte auch die Interaktion konservierter Reste der intrazellulären Domäne mit Ezrin/Radixin/Moesin (ERM) Proteinen gezeigt werden (Iwase et al. 2004; Terawaki et al. 2007). Die ERM Proteine können sowohl mit Aktin als auch mit der zytoplasmatischen Domäne einiger Membranproteine interagieren und wirken so als Bindeglied zwischen Zytoskelett und Membran. Terawaki et al. (2007) unterteilten die intrazelluläre Domäne von humanem Neprilysin nochmals in drei Regionen: eine N-terminale polare Region, gefolgt von einer amphipatischen Region mit vorrangig neutralen Aminosäureresten und im Anschluss eine von basischen Resten gekennzeichnete Region. Dabei zeigen Strukturdaten, dass innerhalb der amphipatischen Region ein ß-Strang und eine Haarnadelstruktur ausgebildet werden; diese enthalten konservierte Reste, welche mit einer der drei typischen ERM Domänen „Four point one and ERM“ (FERM) interagieren (Abb. 2 A, überstrichenes Motiv).

Im Gegensatz zu der zytoplasmatischen Domäne der in Säugetieren vorkommenden Neprilysine, sind die entsprechenden Domänen anderer Vertreter der M13-Metalloproteasen (bspw. ECE-1, KELL, PHEX) sehr variabel aufgebaut (Abb. 2 A). Auch in *Drosophila melanogaster* weichen die intrazellulären Domänen der fünf Neprilysine sowohl in ihrer Länge als auch in ihrer Sequenz deutlich voneinander ab (Abb. 2 B, C). Während die intrazelluläre Domäne des Neprilysins im Menschen, in der Ratte oder auch im Kaninchen etwa 30 Aminosäuren (AS) umfasst, so variiert sie in der Fliege zwischen 20 AS bei Nep2 und 112 AS bei Nep1. Die N-terminale Domäne bei Nep4 und Nep3 setzt sich aus 56 bzw. 52 AS zusammen (www.cbs.dtu.dk/services/TMHMM/). Dabei weisen lediglich Nep2 und und Nep3 im Anschluss an die Transmembrandomäne ein ähnliches Motiv auf, das sich vornehmlich aus polaren Resten zusammensetzt (Abb. 2 C). Die intrazelluläre Domäne von Nep1 hebt sich zum einen stark von den anderen Neprilysinen aus *Drosophila*, zum anderen aber auch von Vertebraten-Neprilysinen ab: Sie ist mit 112 AS und damit etwa 12 kDa wesentlich größer als die N-terminalen Domänen der anderen Vertreter dieser Familie (*Drosophila* Neprilysine zwischen 20 und 112 AS; HsNep: 28 AS; HsPHEX: 20 AS; HsECE-1: 68 AS; HsKELL: 47 AS). Zusätzlich enthält sie mehrere, sich wiederholende Glutaminreste, die innerhalb der Familie der *Drosophilidae* relativ konserviert vorliegen (http://blast.ncbi.nlm.nih.gov/Blast.cgi) und somit vermutlich für die Funktion des Proteins relevant sind. Die 28 AS umfassende Sequenz, die unmittelbar auf die Transmembrandomäne

von Nep1 folgt, weist, basierend auf Sequenzanalysen (http://www.pantherdb.org/), darüber hinaus ein ECE-2 ähnliches Motiv auf (Abb. 2 B, unterstrichene Sequenz von Nep1). Zusätzlich zu den genannten konnten keine weiteren Muster im N-Terminus der *Drosophila* Neprilysine identifiziert werden.

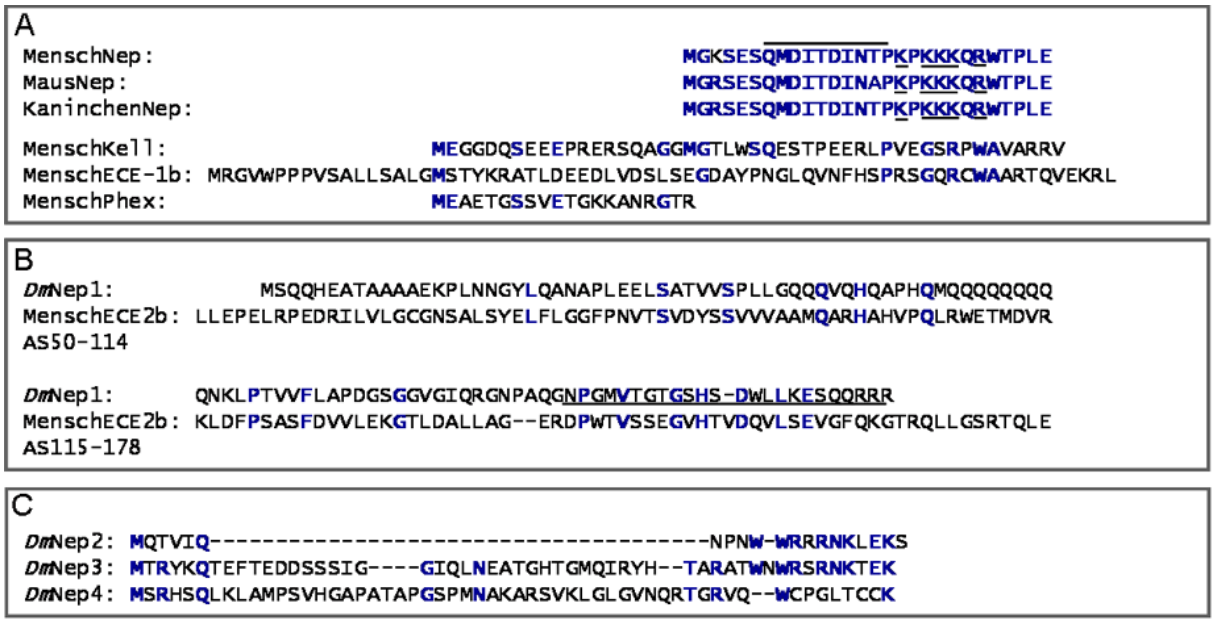

Abb. 2: Die intrazelluläre Domäne der Mitglieder der Neprilysin-Familie. Die Größe der intrazellulären Domänen wurde mit Hilfe von Hydrophobizitätsanalysen (http://www.cbs.dtu.dk/services/TMHMM/) ermittelt und die Aminosäuresequenzen gegeneinander abgeglichen. Fett blau markierte Aminosäuren (AS) zeigen Bereiche auf, die sich auch in anderen der gegenübergestellten Sequenzen wiederfinden. **A**: Die intrazelluläre Domäne der Neprilysine von Mensch, Maus und Kaninchen liegt fast vollständig konserviert vor. Sie enthält ein Motiv aus positiv geladenen Aminosäuren (unterstrichene Sequenz), welches die Bindung der Phosphatase PTEN ermöglicht (Sumitomo et al. 2004). Die amphipatische Region dieser Domänen (fett überstrichen) interagiert mit der FERM Domäne von ERM Proteinen (Terawaki et al. 2007). Der Vergleich der intrazellulären Domänen anderer Vertreter der M13-Familie mit der von Neprilysin zeigt nur einzelne konservierte Reste auf. Als Vertreter aus der Gruppe der ECE Proteine wurde beispielhaft die Isoform B von ECE-1 gewählt, da es in der Plasmamembran von Muskelzellen lokalisiert ist (Kawanabe und Nauli 2011). **B:** Die intrazelluläre Domäne von *Dm*Nep1 wurde ebenfalls mit den anderen Neprilysinen abgeglichen, es konnten jedoch keine nennenswerten Übereinstimmungen mit den anderen Sequenzen der *Drosophila* Neprilysine ermittelt werden, so dass die Sequenz unabhängig von diesen dargestellt wurde. Sie enthält mehrere sich wiederholende Glutaminreste. Da die intrazelluläre Domäne von *Dm*Nep1 eine ECE-2 ähnliche Sequenz (http://www.pantherdb.org/; unterstrichene Sequenz) aufweist, wurde die intrazelluläre Domäne von humanem ECE-2 (Isoform B, größte der existierenden Isoformen, Darstellung ab AS 50 bis zur Transmembrandomäne) mit dieser abgeglichen. **C:** Die N-terminalen Domänen von *Dm*Nep2 und 3 scheinen stärker untereinander konserviert zu sein, als es für die intrazelluläre Domäne von *Dm*Nep4 der Fall ist. Da für *Dm*Nep5 nachweislich kein Transkript existiert (Meyer et al. 2011), wurde es von der Analyse ausgeschlossen.

Zusätzlich zu den membrangebundenen Neprilysinen existieren in seltenen Fällen auch Vertreter dieser Proteinfamilie, die zwar als membranständiges Protein exprimiert, im Anschluss jedoch mittels proteolytischer Spaltung zu löslichen Enzymen prozessiert werden und als sezernierte Isoformen in den extrazellulären Raum gelangen. So wird beispielsweise murines Nep2 (auch SEP = Secreted Endopeptidase genannt) durch alternatives Splicen entweder als membrangebundene oder als sekretierte Isoform exprimiert. Die letztgenannte Transkriptvariante enthält zusätzlich ein 69 Nukleotide umfassendes, in der Ektodomäne liegendes Exon, das für eine Prohormon-Konvertase Erkennungssequenz kodiert. In diesem Fall wird die zunächst noch membrangebundene Isoform durch proteolytische Spaltung im Endoplasmatischen Retikulum (ER) in die lösliche Form überführt und von dort in den Extrazellularraum transportiert (Raharjo et al. 2001).

Humanes Nep2 (auch MMEL2 genannt) weist eine 77,5 %ige Sequenzidentität mit der löslichen murinen Nep2 Isoform auf (Bonvouloir et al. 2001) und wird ebenfalls mittels proteolytischer Spaltung von einem zunächst noch membranständigen in ein lösliches Enzym überführt. Im Gegensatz zu murinem Nep2 ist das lösliche humane Homolog in der Lage das ß-Amyloid Peptid (40 und 42) zu spalten, während lösliches Nep2 aus Maus keine Aktivität gegenüber diesem Molekül aufweist (Spencer und Marr 2010). Somit scheinen sich beide Enzyme trotz hoher Sequenz- bzw. Strukturähnlichkeiten in ihrer Substratspezifität signifikant zu unterscheiden. Die Frage, ob den zurückbleibenden Transmembran- bzw. zytoplasmatischen Domänen eine zusätzliche Funktion nach Abspaltung der Ektodomäne zukommt oder ob diese im Anschluss abgebaut werden, wurde bislang noch nicht untersucht.

Auch in *Drosophila* sind bisher zwei lösliche Neprilysine bekannt. So wird *Dm*Nep2 als lösliches Enzym in den Testes exprimiert (Thomas et al. 2005). Die Primärsequenz kodiert hierbei erneut für ein membranständiges Protein bestehend aus 20 AS auf der zytoplasmatischen Seite, 19-transmembranen AS und 722 extrazellulär gelegenen AS. Ähnlich wie bei den erwähnten humanen bzw. murinen Homologen wird das Protein vermutlich direkt nach der Membrandomäne (AS-Position 41) durch eine Signalpeptidase gespalten (Thomas et al. 2005). Das im Verlauf dieser Arbeit intensiv untersuchte *Dm*Nep4 hingegen nimmt eine Sonderrolle innerhalb der Neprilysine ein, da es der bislang einzige bekannte Vertreter der Neprilysin-Familie ist, der bereits als lösliches Protein exprimiert wird und keiner posttranslationalen Proteolyse unterliegt (Meyer et al. 2009). Dies gilt allerdings nur für eine der beiden Transkriptvarianten des Proteins. Während die Isoform B in löslicher Form exprimiert wird, besitzt Isoform A die für Neprilysine typische Struktur, bestehend aus

einem N-terminalen intrazellulären Teil, einer einzelnen Transmembrandomäne sowie einem großen extrazellulären Bereich, der das katalytische Zentrum trägt.

2.5. Regulation der physiologischen Peptidhomöostase durch Neprilysine

Neben den Peptidtoxinen (z.B. Bienengift-Peptide) oder auch den antimikrobiellen Peptiden stehen vor allem die Neuropeptide sowie die Peptidhormone, die in sekretierter Form als Signalmoleküle fungieren, im Mittelpunkt des wissenschaftlichen Interesses. Neben den genannten finden sich im Zytoplasma weitere Peptide, die aus der proteolytischen Spaltung anderer Proteine hervorgehen und beispielsweise in der Regulation der Genexpression eine entscheidende Rolle spielen.
Neuropeptide und Hormone sind strukturell wie auch funktionell die mannigfaltigsten der Signalmoleküle in Organismen. Sie sind in eine Vielzahl von physiologischen Prozessen wie Neurotransmission (Nässel 2009), Regulation des Metabolismus (Bird et al. 1996), der Herzaktivität (Clerico et al. 2006), des Verhaltens (Donaldson und Young 2008), des Immunsystems (Souza-Moreira et al. 2011) und an verschiedenen Entwicklungsprozessen (Cameron und Elmers 2003; Kondo et al. 2010) beteiligt. Dabei reicht die Diversität dieser Peptide von kleinen Molekülen, wie beispielsweise das Pentapeptid Enkephalin, bis hin zu komplexen Peptidhormonen wie Insulin, welches aus zwei über Disulfidbrücken verknüpften Polypeptidketten besteht und 51 AS umfasst. Vergrößert wird das Spektrum ihrer Wirkungsweisen noch dadurch, dass Peptide aus großen Vorläuferproteinen entstehen, die die Basis für multiple Isoformen darstellen. Diese können dann über Interaktion mit verschiedenen Rezeptoren, meist G-Protein gekoppelt (GPC-Rezeptoren), in die unterschiedlichsten Signalkaskaden eingreifen (Abb. 3; Nässel 2002; Altstein und Nässel 2010).

Im Genom von *Drosophila* werden etwa 30-40 Gene für Neuropeptidvorläuferproteine annotiert, während die Zahl der bisher identifizierten GPC-Rezeptoren bei etwa 40 liegt (Review, Altstein und Nässel 2010). Nach der Translation des Peptidvorläuferproteins an den Ribosomen wird dieses meist im Golgi-Apparat durch spezifische Endo- und/oder Exopeptidasen zu den reifen Peptiden gespalten. Dabei findet die Proteolyse zumeist an basischen Resten statt (Veenstra et al. 2000). Aus einem Vorläuferprotein können sowohl unterschiedliche Peptide, als auch mehrere Kopien eines bestimmten Peptids hervorgehen. Beispielsweise entstehen aus einem FMRF-Vorläuferprotein der Schabe gleichzeitig 23

identische FMRF-Peptide (Predel et al. 2004). Nach der Spaltung werden die Peptide gegebenenfalls zusätzlich durch Glykosylierung, Sulfatierung, Phosporylierung, Amidierung, Acetylierung oder auch der Ausbildung von Disulfidbrücken posttranslational modifiziert (Taghert und Veenstra 2003). Insbesondere die Amidierung bzw. die Acetylierung führen dabei häufig zu einer Erhöhung der Peptidstabilität und somit der Halbwertszeit des entsprechenden Peptids. Die reifen Peptide werden dann in Form von sekretorischen Vesikeln gespeichert oder über den Golgi zur Membran transportiert und dort kontrolliert in den Extrazellularraum entlassen (Abb. 3). Die meisten Neuropeptide aus *Drosophila* werden von neurosekretorischen Zellen und Interneuronen im ZNS, zum Teil aber auch von Motorneuronen oder sensorischen Neuronen sowie von endokrinen Zellen des Verdauungsapparates sekretiert (Nässel, 2002). Die freigesetzten Peptide können dann direkt an benachbarte Rezeptoren binden (parakrine Wirkung) oder über die Hämolymphe weiter entfernte Gewebe erreichen (endokrine Wirkung).

Die Inaktivierung bzw. Regulation der Peptidsignalwege erfolgt dabei entweder durch Signaladaptation des jeweiligen Rezeptors oder durch Peptiddegradation, vermittelt durch extrazellulär aktive Peptidasen (Nässel, 2002), wie z.B. Neprilysine oder Neprilysin-ähnliche Proteine. Zwar sind in Bakterien, Hefen, Pflanzen und eukaryotischen Primärzelllinien bereits einige Di-, Tri- und Oligopepidtransporter identifiziert worden, diese dienen aber vermutlich nicht der Signalinaktivierung, sondern vielmehr der Signalweiterleitung in die Zelle oder der Nährstoffgewinnung (Goodell und Higgins 1987; Daniel et al. 2006; Chothe et al. 2010; Lubkowitz 2011), so dass die Signalinaktivierung in eukaryotischen Organismen in der Hauptsache in der Spaltung durch Peptidasen liegt.

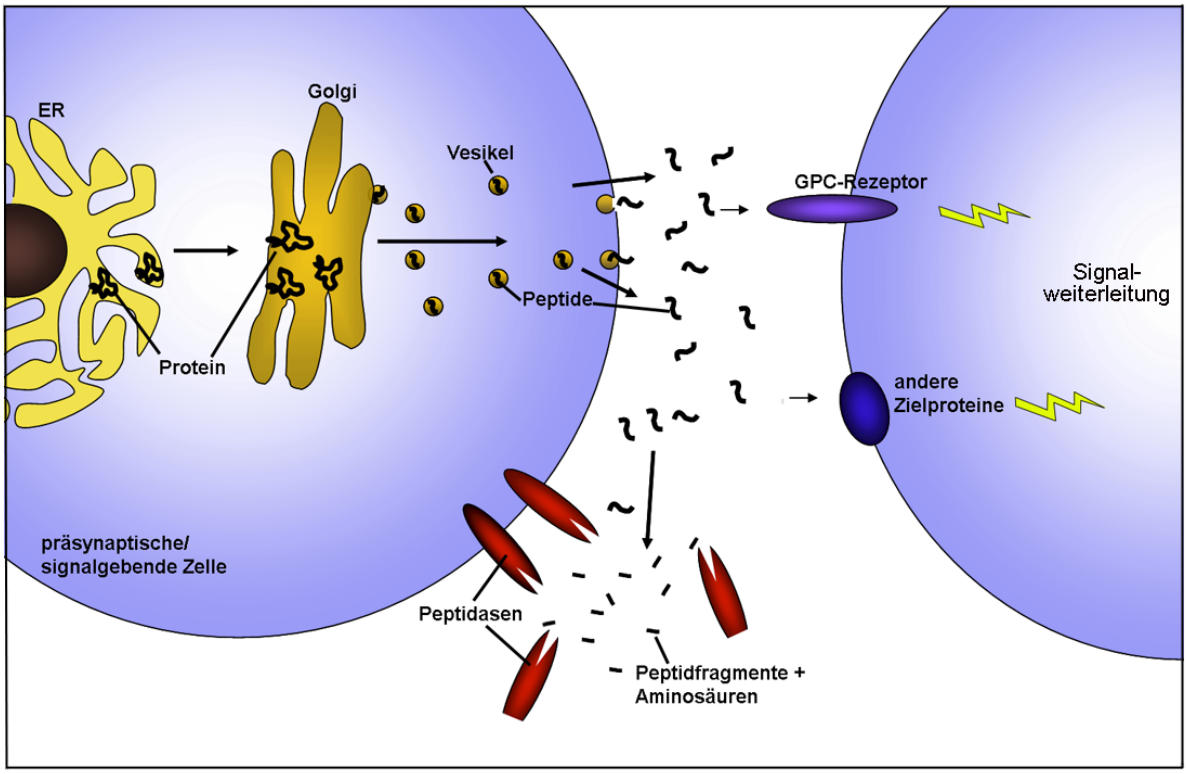

Abb. 3: Schema des Signalpeptid-Metabolismus. Die unreifen Vorläuferpeptide werden vom ER zum Golgi transportiert, dort posttranslational modifiziert und von Proteasen in aktive Peptide prozessiert. Der Transport aus der signalgebenden Zelle erfolgt in der Regel über sekretorische Vesikel in den Extrazellularraum. In diesem können lösliche oder membranständige Metalloproteasen, in der Hauptsache Vertreter der M13-Familie, die Peptide spalten und somit inaktivieren. Intakte Peptide binden an spezifische Rezeptoren von benachbarten oder entfernten Zellen und lösen in der Empfängerzelle eine intrazelluläre Signalkaskaskade aus, die oft G-Protein vermittelt ist (modifiziert nach Fricker 2010).

Neprilysine und Neprilysin-ähnliche Proteine werden, ihrer Funktion entsprechend, hauptsächlich als membranständige Proteine des Typs II synthetisiert und finden sich zumeist in der Plasmamembran, wo sie als Ektoenzyme extrazelluläre Oligopeptide spalten. Aufgrund der Struktur der katalytischen Tasche sind sie jedoch, wie bereits erwähnt, auf Peptide einer molekularen Masse von nicht mehr als 5 kDa beschränkt.

Das wohl bekannteste *in vivo* Substrat von humanem Neprilysin ist das neurotoxische Amyloid-ß Peptid, welches ursächlich für die Ausbildung der Alzheimerschen Erkrankung ist (Iwata et al. 2000). Dementsprechend kommt Neprilysin vorrangig in Bereichen des Gehirns vor, wie dem Hippocampus, in denen auch Amyloid-ß und im erkrankten Gehirn die Ablagerungen dieses Peptids, in Form der sogenannten „Plaques“, zu finden sind. Das Fehlen von Neprilysin oder dessen enzymatischer Aktivität in diesen Geweben führt zu einer vermehrten Ausbildung dieser toxischen Plaques (Iwata et al. 2002). Neben Neprilysin konnte

im Tiermodell gezeigt werden, dass auch noch andere Peptidasen wie ECE-1 oder ACE in der Lage sind, das Amyloid-ß Peptid zu spalten. Welchem dieser Enzyme *in vivo* eine Schlüsselrolle bei dessen Katalyse bzw. der Aufrechterhaltung des Peptidgleichgewichtes im Menschen und damit in der Alzheimerschen Erkrankung zukommt, ist noch unklar. Auch in *Drosophila* ist das Vorläuferprotein für ein ß-Amyloid ähnliches Peptid im Genom kodiert. Es wird sowohl im Nervensystem als auch an der neuromuskulären Endplatte exprimiert und Veränderungen der Expression des Vorläuferproteins führen zu Verhaltensstörungen und einer veränderten Synapsenmorphologie (Luo et al. 1992; Torroja et al. 1999; Chan und Bonini 2000). Darüber hinaus führt die Überexpression von humanem Amyloid-ß Peptid im ZNS der Fliege zu einem signifikanten Neurodegenerationsphänotyp (Finelli et al. 2004).

Weitere Substrate des humanem Neprilysins sind beispielsweise Enkephaline, Substanz P, das atriale natiuretische Peptid (ANP) und das Neuropeptid Y. Viele dieser Peptide wurden sowohl im ZNS als auch in weiteren Geweben, wie dem Verdauungsapparat, dem Gefäßsystem oder den männlichen Geschlechtsorganen nachgewiesen. Zusätzlich spielen sie in erheblichem Maße eine Rolle in der Immunantwort (Jonsdottir 2000; Souza-Moreira et al. 2011). Im Gehirn steuern sie als Neurotransmitter u.a. das Empfinden von Schmerz, Angst und Hunger. In *Drosophila* konnten lediglich Neprilysin 3 und Neprilysin 4 im Gehirn nachgewiesen werden; eine eindeutige *in vivo* Funktion oder ein spezifisches Substrat ist aber für keines der genannten Enzyme bekannt (Meyer et al. 2011).
In Säugern konnten neben Neprilysinen sechs weitere M13-Metalloproteasen identifiziert werden: Die beiden Endothelin konvertierenden Enzyme 1 und 2, sowie ein ECE-like 1 (ECEL-1, XCE oder DINE genannt), Kell, PEX und MMEL-1 (SEP/NL-1 oder NEP2 genannt). Die Endothelin konvertierenden Enzyme spalten extrazellulär das zunächst noch inaktive, „große Endothelin“ zum aktiven Endothelin, welches maßgeblich an der Vasokonstriktion und damit an der Regulation des Blutdrucks beteiligt ist. Mutationen in einem oder beiden ECE Enzymen führen unter anderem zu Missbildungen von Kopf- und Herzstrukturen (Yanagisawa et al. 2000). Das katalytisch aktive PEX Protein (auch Phex genannt) ist an der Phosphathomoöstase und damit am Knochenbau beteiligt (Guo und Quarles 1997). Ein physiologisches Substrat des Enzyms ist bislang nicht bekannt, was auch für das Kell Protein gilt. In dem letztgenannten Fall sind allerdings zumindest einige potentielle *in vitro* Substrate identifiziert worden (Clapéron et al. 2005); das Protein selbst konnte als zentrales Antigen auf menschlichen Erythrozyten nachgewiesen werden, ist jedoch auch in anderen Geweben wie Herz oder Testes exprimiert (Russo et al. 2000).

Neben dem Kell Protein werden auch alle weiteren der sechs genannten Vertreter der M13-Familie in den männlichen Geschlechtsorganen exprimiert (Erdös et al. 1985; Korth et al. 1999; Valdenaire et al. 1999; Ghaddar et al. 2000; Meyer und Meyer 2000; Ouimet et al. 2000; Russo et al. 2000; Bonvouloir et al. 2001; Carpentier et al. 2004). In Mäusen bedingt beispielsweise der Verlust des löslichen Nep2 Proteins keinen sichtbaren Phänotyp während der Entwicklung; allerdings deuten kleinere Nachkommen auf eine Rolle der Metalloprotease bei der Befruchtungseffizienz hin (Carpentier et al. 2004). Einen ähnlichen Phänotyp zeigen auch transgene Mäuse, die mutant für das Angiotensin konvertierende Enzym (ACE) sind (Hagaman et al. 1998). Da beide Enzyme im Hoden exprimiert werden und sie ein ähnliches Substratspektrum aufweisen, sind sie möglicherweise in Teilen ihrer Funktion redundant (Carpentier et al. 2004).

Auch in *Drosophila* wird die lösliche Isoform eines Neprilysins im Hoden exprimiert. Das *Dm*Nep2 Protein lokalisiert dabei in Zellen, die die reifenden Spermien umgeben und könnte deshalb an der Elongation der Spermien beteiligt sein (Thomas et al. 2005). Die phänotypischen Auswirkungen die ein Verlust des Proteins im Keimgewebe oder im gesamten Organismus hat, wurden bisher noch nicht untersucht.

Das einzig weitere zurzeit bekannte Neprilysin aus *Drosophila*, das eine geschlechtsspezifische Expression aufweist, ist das im Verlauf dieser Arbeit untersuchte Neprilysin 4 (McIntyre et al. 2006; Meyer et al. 2009). Das Protein ist während der gesamten Entwicklung, angefangen von den embryonalen Gonaden in Stadium 14 bis hin zum adulten Hodengewebe, in den männlichen Keimzellen exprimiert. Die ACE-ähnlichen Enzyme Ance (Angiotensin Converting Enzyme) und Acer (ACE-related) der Fruchtfliege werden ebenfalls, wie ihre Säugerhomologe, in den männlichen Geschlechtsorganen exprimiert. Ance scheint eine essentielle Rolle in der Spermatogenese zu spielen, da in mutanten Allelen unfruchtbaren Männchen auftreten (Tatei et al. 1995). Acer kommt in den Keimzellen beider Geschlechter vor und ist vermutlich am Peptidmetabolismus grundlegender Prozesse der Reproduktion beteiligt (Siviter et al. 2002). Die Zielsubstrate der beiden Enzyme müssen jedoch außerhalb der typischen Substrate für ACE Enzyme liegen, da in *Drosophila* bisher weder Angiotensin I- noch Bradykinin-ähnliche Substrate gefunden werden konnten (Isaac et al. 2000).

Zu möglichen Substraten, die die Reproduktion und das Sexualverhalten in Insekten beeinflussen und durch Neprilysine oder ähnliche Metalloproteasen gespalten werden könnten, gehören beispielsweise die Neuroparsine, verschiedene Insulin-ähnliche Peptide, das Short Neuropeptid F, das Pheromon-Biosynthese aktivierende Neuropeptid (PBAN), SIFamide oder das Sex-Peptid (Altstein und Nässel 2010).

Zusätzlich zu dem bereits genannten, sind Neprilysine auch in myogenen Geweben, wie dem Herzen oder der somatischen Muskulatur, exprimiert. So findet sich humanes Neprilysin nicht nur im ZNS und in den Nieren, sondern auch im Darm, im Herzen und in den peripheren Blutgefäßen (Turner und Tanzawa 1997). In *Drosophila* sind die Metalloproteasen Neprilysin 4 und beide ACE Homologe (Acer und Ance) im Herzen lokalisiert (Taylor et al. 1996; Meyer et al. 2009), wobei Acer erst in späten Stadien der Kardiogenese (Stadium 14/15) exprimiert wird. Neprilysin 4 hingegen konnte bereits ab Stadium 12 in spezifischen Zellen des lateralen Mesoderms und später auch in bestimmten Herzvorläuferzellen detektiert werden. Im Stadium 14 kolokalisiert es mit allen Perikardzellen, die segmental den Transkriptionsfaktor Even-skipped exprimieren (diese Arbeit, Meyer et al. 2009). Zwar lässt sich Neprilysin 4 mittels Immunodetektion im Anschluss an die Embryogenese nur noch in den männlichen Geschlechtsorganen und im Nervensystem nachweisen, es kann aber davon ausgegangen werden, dass das Enzym in geringem Maße auch in der somatischen Muskulatur und im Herz späterer Stadien exprimiert wird. Sowohl Ergebnisse aus quantitativen PCR Ansätzen wie auch *nep4*-Reporterlinien, die das GFP-Reporterprotein in adulter Muskulatur und im Herzen exprimieren, deuten darauf hin, dass das Nep4 auch nach Abschluss der Embryogenese für die Peptidhomöostase und die Integrität myogener Gewebe essentiell ist (diese Arbeit). Dies belegen u.a. auch RNA Interferenz Experimente, die im Verlauf der vorliegenden Arbeit durchgeführt wurden, da eine Reduktion der Nep4 Expression in Muskeln zu einer erheblich verminderten Bewegungsfähigkeit der Larven und spätestens im Puppenstadium zum Tod der Tiere führt. Basierend auf der Expression der Peptidase in Muskelgewebe sollte im Verlauf der vorliegenden Arbeit die Funktion von Neprilysin 4 in diesem Gewebe näher untersucht werden.

2.6. Muskelentwicklung und Physiologie in *Drosophila melanogaster*

Im Verlauf der Embryogenese von *Drosophila* entstehen aus dem Mesoderm zahlreiche unterschiedliche Gewebe. Hierzu zählen sowohl die viszerale als auch die somatische Muskulatur, das Herz, das somatische Gewebe der Gonaden oder auch der Fettkörper.

Im Stadium 6/7 der Embryogenese invaginiert das zukünftige Mesoderm in Form eines ventralen Streifens und dehnt sich lateral sowie nach dorsal aus. Im Zuge dieses Prozesses bildet sich das Keimband aus. Im Stadium 9 hat das Keimband seine volle Länge erreicht, wobei das Mesoderm nun einschichtig auf einer ektodermalen Schicht von Neuroblasten und Gliazellen aufliegt (Bate und Arias 1993). Durch die dorsolaterale Ausbreitung des

Keimstreifens kommen die am weitesten dorsal liegenden Mesodermzellen mit einem Faktor aus dem darüber liegenden Ektoderm in Kontakt und werden als dorsales Mesoderm determiniert (Abb. 4 C, Staehling-Hampton et al. 1994; Frasch 1995). Aus diesem resultieren letztendlich die Gewebe des Herzens (Perikardzellen und Kardioblasten), ein Teil der somatischen Muskulatur (dorsale somatische Muskulatur) sowie die viszerale Muskulatur, die die kontraktile Zellschicht des Darms ausbildet.

Während des frühen elften Stadiums der Embryogenese werden bereits die Vorläuferzellen des Herzens und der somatischen Muskulatur ausgebildet. Dazu erfährt das Mesoderm entlang der anterior-posterior Achse eine sich segmental wiederholende Unterteilung, die durch den Einfluss von ebenfalls segmental angelegten Domänen aus dem Ektoderm vermittelt wird (Abb. 4 D, Azpiazu et al. 1996). Hierdurch entstehen im dorsalen Mesoderm hauptsächlich die Anlagen für die Gewebe des viszeralen Mesoderms und des Herzens, während im ventralen Mesoderm die Primordien des Fettkörpers und des größten Teils der somatischen Muskulatur angelegt werden (Azpiazu et al. 1996; Riechmann et al. 1997).
Im Stadium 11/12 kann die Metalloendopeptidase Neprilysin 4 erstmalig im *Drosophila* Embryo detektiert werden. Das Protein lokalisiert zu diesem Zeitpunkt ausschließlich mesodermal in einzelnen Zellen des dorsalen Mesoderms (Meyer et al. 2009). Im weiteren Verlauf (ab Stadium 13) beschränkt sich die Expression des Proteins auf das kardiale Mesoderm und lokalisiert in spezifischen dorsalen Muskelvorläuferzellen, die am Ende der Embryogenese die Muskeln DA1 (Dorsal Acute 1), DO2 (Dorsal Oblique 2) sowie einen weiteren Muskel unbekannter Herkunft ausbilden.
Die Vorläuferzellen der Muskulatur, aber auch die des Herzens entstehen auf Basis fortwährender Selektionsprozesse wie asymmetrischen Zellteilungen und lateraler Inhibition. Auf diese Weise werden pro Hemisegment 30 spezifische Zellen selektioniert, die sich alle durch jeweils unterschiedliche sogenannte Identitätsfaktoren von einander abgrenzen und durch Fusion zu den einzelnen Muskeln der zukünftigen Körpermuskulatur heranwachsen. Auch die beiden Even-skipped positiven Perikardzellen (zwei pro Hemisegment) gehen aus einer Vorläuferzelle der somatischen Muskulatur hervor. Diese Vorläuferzelle, die zum einen durch den Transkriptionsfaktor Even-skipped, zum anderen aber auch durch Neprilysin 4 markiert wird, teilt sich anschließend asymmetrisch und bildet somit zwei unterschiedliche Zelllinien aus: eine Linie aus der die Muskelzellen des DO2 Muskels und ein Linie aus der beide Even-skipped positiven Perikardzellen entstehen (Carmena et al. 2002; Meyer et al. 2009).

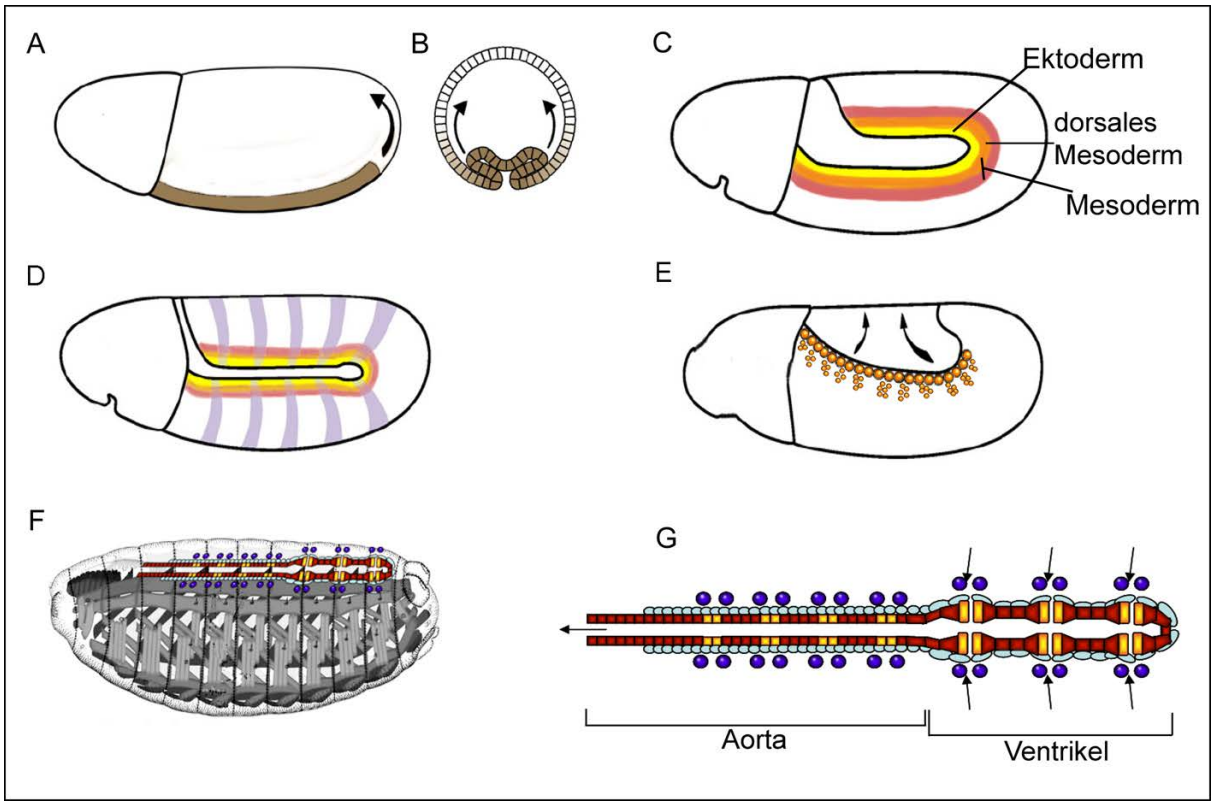

Abb. 4: Mesodermentwicklung im *Drosophila* Embryo. **A:** Schematische Darstellung der Invagination im frühen Embryo (Lateralansicht, Stadium 5-7); der Keimstreifen verlängert sich (Pfeil) und dehnt sich nach dorsolateral aus. **B:** Schematischer Querschnitt durch den Embryo (wie in A), dorsolaterale Migration des Keimbandes durch Pfeile gekennzeichnet. **C:** Embryo des Stadiums 9 mit vollständig ausgestrecktem Keimband; im Ektoderm wird ein spezifischer Faktor produziert, der das am weitesten nach dorsal migrierte Mesoderm als dorsales Mesoderm determiniert. **D:** Gesteuert durch Signale aus dem Ektoderm erfährt das Mesoderm eine Unterteilung in Segmente, wodurch Kompetenzfelder entstehen aus denen später die Herzzellen selektioniert werden. **E:** Im Stadium 14 wandern die Kardioblasten als bilaterale Zellreihe Richtung dorsaler Mittellinie. Die Muskelvorläuferzellen befinden sich im Differenzierungsprozess. **F:** Schematische Darstellung eines Embryos nach Abschluss der Embryogenese (Stadium 17). Sowohl die somatische Muskulatur (grau) als auch das Herz liegen vollständig differenziert vor und verändern sich in den folgenden Larvalstadien nur noch bezüglich ihrer Größe. **G:** Herz im Stadium 17: Die Hämolymphe umströmt das Herz und kommt so in Kontakt mit den Perikardzellen (hellblaue Zellen, dunkelblaue Zellen: Even-skipped/Nep4 positive Zellen) die als Nephrozyten fungieren. Sie gelangt posterior durch Einstromöffnungen (große gelbe Zellen) in den Ventrikel und wird durch die kontraktilen Kardiomyozyten (rote Zellen) nach anterior Richtung Kopf gepumpt (modifiziert nach Hartenstein *Drosophila* Atlas und Bryantsev und Cripps 2009).

Innerhalb des Differenzierungsprozesses der Herzens wandern die bilateralen Herzzellreihen von lateral zur dorsalen Mittellinie, wo sie sich zum funktionstüchtigen Herzrohr zusammen lagern (Abb. 4 E). Der innerste Teil des Herzens umfasst exakt 104 kontraktile

Kardiomyozyten, welche posterior ein größeres Lumen (Ventrikel) ausbilden. In dieses strömt die Hämolymphe durch die sogenannten Ostienzellen ein und wird dann entlang des Rohres Richtung anterior (Aorta) gepumpt. Nach außen werden die kontraktilen Zellen von verschiedenen Perikardzellen flankiert (Abb. 4 G). Ihre genaue Funktion ist unklar, jedoch wird vermutet, dass sie in der Larve als Nephrozyten agieren und Giftstoffe aus der Hämolymphe herausfiltern (Das et al. 2008). Des Weiteren vermuten Buechling et al. (2009), dass die Herzaktivität adulter Tiere durch parakrine Signale, möglicherweise erzeugt von den Even-skipped positiven Perikardzellen, moduliert wird. Diesbezüglich könnte auch Nep4 eine Funktion in der Inaktivierung von Signalen, die in Form von herzmodulierenden Peptiden auftreten, zukommen (Meyer et al. 2009). Dabei wird die Metalloprotease nicht nur in embryonalen Perikardzellen exprimiert, sondern offenbar auch später im funktionellen Herzen. Innerhalb dieser Arbeit erstellte Reporterlinien deuten in diesem Zusammenhang auf eine Rolle von Nep4 in spezifischen, differenzierten Perikardzellen und Kardiomyozyten in Larven, Puppen und adulten Tieren hin.

Nach Abschluss der Embryogenese liegen das Herz und die Muskulatur vollständig differenziert vor (Abb. 4 F) und verändern sich während der folgenden drei Larvalstadien nur noch bezüglich ihrer Zellgröße. Da die Muskeln der Körperwand um das 100-fache ihrer vorherigen, embryonalen Größe anwachsen, müssen auch die neuronalen Netzwerke, die die Muskulatur innervieren und aktivieren angeglichen werden. Dabei werden die etwa 30 synzytialen Muskelzellen pro Hemisegment von etwa 40 Motoneuronen innerviert (Koh et al. 2000). Diese bilden im Kontakt mit der Muskelplasmamembran die sogenannte motorische Endplatte oder die neuromuskuläre Verbindung (neuromuscular junction- nmj) aus. In der motorischen Endplatte verzweigt sich der Nerv kurz vor dem Muskel baumartig, wobei sich die dünnen Nervenfasern am Ende wieder zu den synaptischen Boutons knöpfchenartig verdicken. An jedem Bouton finden sich mehrere Synapsen, die den Kontakt zwischen Nerv und Muskel vermitteln. Die Form der Signalweiterleitung hängt von der Art der Boutons ab, wobei in *Drosophila* drei verschiedene Typen vorliegen (Gramates und Budnik 1999; Rheuben et al. 1999): alle Muskelzellen der Körperwand werden von Boutons des Typs I innerviert. Diese geben Glutamat in den synaptischen Spalt ab und zeichnen sich ultrastrukturell dadurch aus, dass sie mehrschichtig von der direkt benachbarten Muskelmembran, dem subsarkoplasmatischen Retikulum, umgeben sind (Atwood et al. 1993; Jia et al. 1993). Einige Muskelzellen weisen zusätzlich Boutons des Typs II und III auf, welche neben Glutamat auch Octopamine oder Neuropeptide sezernieren können (Koh et al. 2000). Die motorische Endplatte in *Drosophila* Larven wird im Gegensatz zur Situation in

Vertebraten nicht durch Glia bzw. Schwannsche Zellen isoliert; statt dessen tauchen die Boutons stark in oder unter die Muskeloberfläche ein, so dass ein direkter Kontakt mit der sie umgebenden Hämolymphe minimiert ist (Prokop 1999). Zusätzlich bleibt der synaptische Spalt, in den die Transmitterstoffe diffundieren, mit etwa 15 nm Breite relativ klein (Prokop 1999).

In der postsynaptischen Muskelzellmembran sind im Bereich der Synapse vermehrt Rezeptoren, wie z.B. Glutamatrezeptoren, spannungsabhängige Ionenkanäle oder auch Adhäsionsproteine zu finden (siehe dazu Liste Prokop 1999), die das Signal des innervierenden Nervs weiterleiten um letztendlich eine Kontraktion des Muskels auszulösen. Bezüglich der Glutamatrezeptoren sind bis heute fünf unterschiedliche Untereinheiten bekannt, wobei die Ausbildung eines funktionstüchtigen Glutamatkanals durch verschiedene Kombinationen der Untereinheiten erreicht werden kann. Interessanterweise zeigt die Glutamatrezeptor-Untereinheit GluRIID im Embryo ein ähnliches mesodermales Expressionsmuster, wie das Nep4 Protein und liegt zu diesem unmittelbar benachbart im Genom (Qin et al. 2005). Obwohl beide Proteine darüber hinaus auch in der larvalen Muskulatur nachgewiesen wurden (diese Arbeit, Qin et al. 2005), konnte bislang noch kein funktioneller Zusammenhang zwischen ihnen hergestellt werden.

Die kontraktile Regulation des *Drosophila* Herzens erfolgt über mindestens zwei symmetrisch angelegte, kardiale Schrittmacher, die vermutlich im posterioren Teil des Herzens liegen. *In vitro* Experimente zeigen jedoch, dass zusätzlich Neurotransmitter und Peptide die Herzaktivität über die gesamte Entwicklung beeinflussen (Dulcis und Levine 2003). Das erste charakterisierte Neuropeptid, Proctolin (Brown 1975) kommt in *Drosophila* sowohl im ZNS als auch in jenen motorischen Endplatten vor, die die somatische Muskulatur und den Hinterdarm von dritten Larven innervieren (Anderson et al. 1988). Zusätzlich konnte Miller (1979) jedoch zeigen, dass das Neuropeptid auch einen Effekt auf das Insektenherz hat. Weitere myoaktive oder myotrophe Peptide, die sowohl das Herz als auch die viszerale und somatische Muskulatur beeinflussen können, stellen beispielsweise CCAP (Crustacean Cardioactive Peptide) (Nichols et al. 1999; Dulcis et al. 2005), Dromyosuppressin (Johnson et al. 2000), Allatotrophine (Elekonich und Horodyski 2003) oder auch Leukokinine (Terhzaz et al. 1999) dar. Da diese Peptide in *Drosophila* häufig auch im ZNS exprimiert werden, ist es jedoch wahrscheinlich, dass ihre Hauptfunktionen nicht oder nicht nur in ihren myoaktiven Eigenschaften liegen (Nässel 2002).

Im Verlauf der Metamorphose zum Imago wird ein Großteil der Gewebe histolysiert und im Anschluss neu synthetisiert. Auch die Gewebe der somatischen Körpermuskulatur und Teile des Nervensystems (Tissot und Stocker 2000) und des Herzens werden in der Puppe aufgelöst und für den adulten Organismus *de novo* ausgebildet. Da der größte Teil der Herzzellen jedoch erhalten bleibt, handelt es sich bei den adulten Kardiomyozyten des Herzens um die gleichen Zellen, die auch schon im Embryo vorlagen (Molina und Cripps 2001; Sellin et al. 2006). Sie werden jedoch teilweise „reprogrammiert", so dass beispielsweise einige embryonale Herzzellen (die Seven-up positiven Kardioblasten) zu funktionellen Ostienzellen umgewandelt werden, die im adulten Herzen die Eintrittsöffnungen für die Hämolymphe bilden. Aufgelöst werden nur wenige posteriore Segmente, so dass das Herz im Abdomen nicht mehr wie in der Larve von den Segmenten A1- A7, sondern nur noch von A1-A5 reicht. Hierdurch vermindert sich die Zahl der Kardiomyozyten von 104 im Embryo auf etwa 84 im adulten Tier (Sellin et al. 2006). Während die indirekte Flugmuskulatur und die longitudinale Muskulatur des Darms nicht histolysiert werden (Fernandes et al. 1991; Klapper 2000), bildet sich die zukünftige adulte, somatische Muskulatur in der Puppe neu aus. Grundlage hierfür sind imaginale Zellen, die bereits im Embryo angelegt wurden. Diese Zellen werden als adulte Muskelvorläuferzellen (AMV) bezeichnet. Sie entstehen während der Embryogenese zeitgleich und aus denselben Zellen, aus denen auch die embryonalen Muskelvorläuferzellen gebildet werden. Die AMVs verbleiben aber bis zum zweiten Larvalstadium undifferenziert und inaktiv und liegen in direktem Kontakt mit den embryonalen bzw. larvalen Muskelfasern und peripheren Nerven vor. Die Proliferation dieser Zellen setzt während der larvalen Entwicklung ein, so dass sie während des pupalen Stadiums als Reservoir für die Bildung der Muskelfasern der adulten Fliege dienen (Figeac et al. 2007; 2010).

2.7. *Drosophila* als Modell für Muskeldegeneration

Humane Krankheitsbilder, die durch eine fortschreitende Degeneration von Muskelzellen des Herzens oder der Skelettmuskulatur charakterisiert sind, sind häufig durch Mutationen in Strukturgenen, Proteinen der nukleären Membran oder Proteinen, die das Muskelzytoskelett mit der extrazellulären Matrix verbinden, bedingt. Zwei der häufigsten humanen X-chromosomal vererbten Muskeldystrophien sind die Duchenne und die Becker Muskeldystrophien, die sich auf ein Fehlen bzw. eine Reduktion des Proteins Dystrophin zurückführen lassen (Hoffman et al. 1987).

Bei dem Protein Dystrophin handelt es sich um ein zytoplasmatisches stäbchenförmiges Protein, welches das Zytoskelett mit der Plasmamembran und indirekt auch mit der Extrazellulären Matrix verbindet. Dabei vermitteln vier Proteindomänen des Dystrophins die Verbindung zu den unterschiedlichen Komponenten des Dystrophin-Glykoprotein Komplexes (DGK) und dem Aktin (Campbell 1995) (siehe Abb. 5). Die N-terminale Domäne des Dystrophins bindet das Strukturprotein Aktin, darauf folgt eine stäbchenförmige Domäne, die reich an Spektrin ähnlichen Motiven ist, danach schließt sich eine cysteinreiche Region an, die mit Dystroglykanen und Sarkoglykanen interagiert und als letztes folgt eine C-terminale Domäne, die Syntrophine und Dystrobrevine bindet (Blake et al. 2002).
Alle genannten Komponenten des humanen DGKs sind in *Drosophila* konserviert (Greener und Roberts 2000) und Mutationen dieser Gene erzeugen auch in der Fliege häufig einen Muskelphänotyp. RNA Interferenz Experimente, in denen die Expression von Proteinen wie Dystroglykan oder Dystrophin vermindert wird, führen zu einer fortschreitenden Kletterunfähigkeit und zu einer deutlichen Muskeldegeneration in adulten Tieren (Shcherbata et al. 2007; van der Plas et al. 2007). Einen ähnlichen Phänotyp erzeugt das Fehlen des membranständigen Sarkoglykans, was eine verminderte Beweglichkeit und ein vergrößertes Herz mit reduzierter kontraktiler Funktionalität zur Folge hat (Allikian et al. 2007). Auch Enzyme, die die Glykosylierung von Komponenten des DGKs vermitteln, sind für die Integrität der Muskulatur unerlässlich. Mutationen in O-Mannosyltransferasen führen beim Menschen zum Walker-Warburg-Syndrom und entsprechend auch in der Fruchtfliege zu sichtbar dünneren und beschädigten Muskeln (Haines et al. 2007). Darüber hinaus kommt es zu einer vermehrten Apoptose von Myoblasten (Ueyama et al. 2010) und einer gestörten neuromuskulären Signalweiterleitung gekoppelt mit einer veränderten Zusammensetzung der Glutamatrezeptoren in der motorischen Endplatte (Wairkar et al. 2008).

Neben der Bedeutung des DGKs als Verbindung zwischen Zytoskelett und Plasmalemma scheint der Komplex zusätzliche Funktionen außerhalb der Muskelstabilisierung zu erfüllen: So werden Dystroglykan und Dystrophin nicht nur in der Muskulatur sondern auch in Neuronen und Gliazellen exprimiert und beeinflussen dort die neuronale Transmission und die Ausrichtung von Axonen (Shcherbata et al. 2007). Zudem wurden Untereinheiten des Dystrophin-Glykoprotein Komplexes auch im Zellkern verschiedener Zellen gefunden und fungieren dort möglicherweise als Gerüst der nukleären Matrix (Fuentes-Mera et al. 2006). Durch Experimente mit verschiedenen *Drosophila*-Mutanten konnten Kucherenko et al. (2008) Gene identifizieren, die mit dem DGK interagieren oder seine Regulation und

Signalwirkung beeinflussen. Dabei fanden sich neben den erwarteten Faktoren des Zytoskeletts auch Moleküle des EGFR-, Notch, und TGF-ß Signalweges, was darauf hindeutet, dass der DGK auch in die intrazelluläre Signalweiterleitung involviert ist.

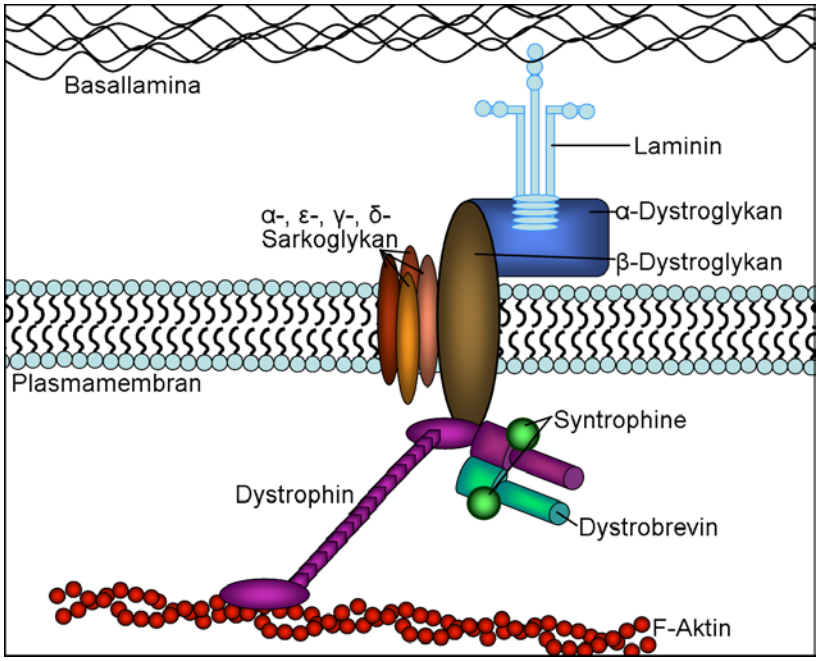

Abb. 5: Schematische Abbildung des Dystrophin-Glykoprotein Komplexes (DGK). Der Dystrophin-Glykoprotein Komplex vermittelt die Verbindung zwischen der extrazellulären Matrix/Basallamina und dem Zytoskelett. Das zytoplasmatische Dystrophin trägt zur Zellintegrität bei, indem es mit seiner N-terminalen Domäne filamentöses Aktin bindet, während es am C-Terminus mit dem membrangebundenen ß-Dystroglykan als auch mit Syntrophin und Dystrobrevin interagiert (modifiziert nach Greener und Roberts 2000).

Veränderungen der neuromuskulären Verbindung oder von Motoneuronen können ebenfalls einen Verfall der Muskulatur hervorrufen. So wird beispielsweise das Protein Survival Motor Neuron (SMN) in allen Zellen ubiquitär exprimiert und hilft bei der Bereitstellung und Proteinkomplexbildung der kleinen nukleären Ribonukleinproteine, die am Spleißen der prä-mRNA beteiligt sind (Burghes und Beattie 2009). Es scheint essentiell für die Lebensfähigkeit jeder Zelle zu sein, so dass homozygote Mutationen dieses Gens letal sind. Dies trifft auf alle Metazoen zu, die nur eine Kopie dieses Gens besitzen (Monani 2005). Im Menschen finden sich hingegen zwei SMN Genkopien, so dass nur das Fehlen beider Kopien embryonal letal ist (Burghes 1997). In Patienten ist fast ausschließlich das SMN1 Gen von Mutationen bzw. Deletionen betroffen (mit 96% der bekannten Fälle) und führt zum Krankheitsbild der Spinalen Muskel Atrophie (SMA). Mutationen des SMN2 Gens verursachen hingegen keinen Phänotyp, da das SMN2 Gen des Menschen Punktmutationen enthält, wodurch nur etwa ein Zehntel der *SMN2*-mRNA korrekt gespleißt und als

vollständiges Protein translatiert werden kann. Dabei hängt der Schweregrad der Erkrankung, welcher sich in Form von Muskelatrophie und den Verlust bestimmter Motoneurone widerspiegelt, von der Anzahl der im Genom vorhandenden Kopien des SMN2 Gens ab (Wirth 2000). Entsprechend zeigen Patienten einen Muskelatrophiephänotyp und den Verlust bestimmter Motoneurone. In Analogie hierzu zeigen auch Fliegen, die mutant für SMN sind, eine verkürzte Lebensspanne, verringerte Mobilität sowie eine Degeneration der adulten Thoraxmuskulatur (Chan et al. 2003; Chang et al. 2008). Immunhistochemische Färbungen in *Drosophila* und Mäusen zeigen in diesem Zusammenhang, dass SMN mit Strukturproteinen der Muskelsarkomere kolokalisiert. Entsprechend könnte die Funktion von SMN in der Muskulatur und nicht die Rolle des Proteins in der Assemblierung von RNA-Protein Komplexen ursächlich für den Muskelphänotyp von SMA Patienten sein (Rajendra et al. 2007).

Zurzeit werden verschiedene Screeningansätze hauptsächlich auf Basis der RNA Interferenz genutzt um in *Drosophila* neue Faktoren zu identifizieren, die einen Einfluss auf die Integrität der Muskulatur haben (Montana und Littleton 2006; Bai et al. 2008; Kucherenko et al. 2011). Dabei zeigen die Ergebnisse, dass für die Entwicklung und den Erhalt der Muskulatur in der Hauptsache Proteine nötig sind, die die Struktur oder die Regulation der Muskeln beeinflussen. Darüber hinaus scheinen jene Proteine relevant zu sein, die die Homoöstase im Muskel (z.B. die Peptid-, Ionen-, Homöostase und die Proteinhomöostase reguliert durch das Ubiquitin-Proteasom-System) aufrechterhalten (Bai et al. 2008). Da viele der mit Hilfe dieser Ansätze ermittelten Proteine phylogenetisch konserviert sind, können in *Drosophila* erhobene Daten bezüglich der Muskelphysiologie bzw. -entwicklung wertvolle Hinweise auf die Zusammenhänge und Ursachen humaner Myopathien geben. Aus diesem Grund war die Analyse der Funktion, die Neprilysin 4 in der Aufrechterhaltung der Muskelintegrität von *Drosophila* einnimmt, ein zentraler Aspekt der vorliegenden Arbeit.

3. Ergebnisse-Publikationen

Die folgenden Artikel wurden in die zur Begutachtung vorgelegte, kumulative Thesis eingebracht und weichen geringfügig von den veröffentlichten Zeitschriftenartikeln ab.

- Meyer H, Von Ohlen T, Panz M, Paululat A (2010).
  The disintegrin and metalloprotease Meltrin from *Drosophila* forms oligomers via its protein binding domain and is regulated by the homeobox protein VND during embryonic development. *Insect Biochemistry and Molecular Biology*, 40(11):814-23.
  http://dx.doi.org/10.1016/j.ibmb.2010.07.010

- Meyer H, Panz M, Zmojdzian M, Jagla K, Paululat A (2009).
  Neprilysin 4, a novel endopeptidase from *Drosophila melanogaster*, displays distinct substrate specificities and exceptional solubility states. *Journal of Experimental Biology*, 212(Pt 22):3673-83.
  http://dx.doi.org/10.1242/jeb.034272

- Panz M, Vitos Falleato J, Jendretzki A, Heinisch J, Paululat A, Meyer H (2012).
  A novel role for the non catalytic intracellular domain of Neprilysins in muscle physiology. *Biology of the Cell*, .104(9):553-568.
  http://dx.doi.org/10.1111/boc.201100069

3.1. The disintegrin and metalloprotease Meltrin from *Drosophila* forms oligomers via its protein binding domain and is regulated by the homeobox protein VND during embryonic development

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Abstract

A Disintegrin And Metalloprotease (ADAM) proteins belong to the metzincin superfamily of metalloproteases that are known to play important roles in several physiological and developmental processes including myoblast fusion, tumor necrosis factor-α release or fertilization. They are characterized by a typical domain structure with a proteolytically active domain and the protein binding domains both facing the extracellular space. Regulatory mechanisms are largely unknown. Here we report on the potential of the *Drosophila* ADAM Meltrin to form oligomers via its substrate binding domain. Significantly, oligomerization occurs apparently in a redox dependent manner. Further analysis revealed that the ACR domain is responsible for aggregation while the disintegrin-like and EGF-like domains are not capable of oligomer formation. Stage dependent transcript analysis revealed a constant expression of three different splice variants, two of which were characterized by sequencing. Like many other ADAM proteins, Meltrin shows a highly restricted expression pattern during embryogenesis with at least two of the respective transcripts being present in a subpopulation of neuronal cells in the embryonic central nervous system. Finally, we report on the identification of the first regulator of *meltrin*: the homeobox protein ventral nervous system defective specifically excludes Meltrin expression from the embryonic ventral neuroectoderm.

Keywords: ADAM / Drosophila melanogaster / metalloprotease / oligomerization / redox

Introduction

ADAM (a disintegrin and metalloprotease) proteins are type 1 transmembrane glycoproteins and belong to the superfamily of metzincins. More than 40 ADAMs have been identified to date and their physiological relevancies include fertilization (ADAMs 1, 2, 3 (Almeida et al., 1995; Evans et al., 1995; Wolfsberg and White, 1996)), muscle fusion (ADAM 12 (Gilpin et al., 1998; Yagami-Hiromasa et al., 1995)), cardiogenesis (ADAM 10 (Albrecht et al., 2006)), release of tumor necrosis factor-α from the plasma membrane (ADAM-17 (Blobel, 1997; Werb and Yan, 1998)), processing of the amyloid-β precursor protein (ADAM 10 and 17 (Buxbaum et al., 1998; Lammich et al., 1999)) or cleavage of the Notch receptor or its ligand delta (ADAM10 (Blobel, 1997; Pan and Rubin, 1997; Qi et al., 1999)) at cell surfaces. The majority of ADAM actions described so far rely on the proteolytic activity of a certain extracellular domain, inducing a process known as ectodomain shedding. This process requires an active metalloprotease domain which most of the ADAM proteins contain. The proteolytically active domain however is only one of several domains characteristic for ADAM proteins. The typical domain architecture consists of a prodomain, the metalloprotease domain, a disintegrin-like domain, a cysteine rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic domain of variable size (Weskamp and Blobel, 1994; Wolfsberg et al., 1995). With respect to physiological relevancies, the metalloprotease domain is attributed to ectodomain shedding; the remaining domains however are still subject to speculations. While the prodomain is believed to be involved in protein folding and regulation of activity (Suzuki et al., 1998), the intracellular domain is discussed to be responsible for proper trafficking of the protein (Cao et al., 2002). The disintegrin-like, cysteine rich and EGF-like domains on the other hand are considered to be the site of protein-protein interaction. In this context especially the disintegrin-like domain is known to interact with integrins and thereby mediates cell adhesion (Zolkiewska, 1999).
In mice, the ADAM protein Meltrin β was shown to be expressed in the peripheral nervous system, skeletal muscles, bones and in the embryonic heart (Kurisaki et al., 1998; Yagami-Hiromasa et al., 1995). In the latter tissue, Meltrin β seems to be required for ventricular septum formation (Komatsu et al., 2007; Kurohara et al., 2004; Zhou et al., 2004), however, physiological substrates have not been determined yet. With respect to *Drosophila*, five ADAM metalloproteases are known: two homologs for mammalian ADAM10, Kuzbanian and Kuzbanian-like, a single homolog for ADAM17, TACE and two homologs for ADAM12, Mind-meld and Meltrin. Co-transfection experiments in S2 cells have established that Kuzbanian, Kuzbanian-like and TACE exhibit similar potencies of cleaving the Notch ligand Delta, whereas Meltrin appears to be inactive. Serrate, the second ligand of Notch in *Drosophila*, is also cleaved by Kuzbanian, Kuzbanian-like and TACE, but not by Meltrin (Sapir et al., 2004). The ability of Mind-meld to process Delta or Serrate has not been tested yet.
In the present study we report on the potential of Meltrin from *Drosophila melanogaster* to form multimers in a redox dependent manner. While oligomerization itself has already been reported for other family members, e.g. ADAM 1 and ADAM 2 (Blobel et al., 1990; Waters and White, 1997), protein domains responsible for the formation of oligomers have rarely been identified. Our data demonstrate that the cysteine rich domain (ACR) is responsible for oligomerization of the Meltrin protein interaction domain while the disintegrin-like and EGF-like domains seem to be incapable of oligomer formation. Furthermore, we have identified distinct cells in the central nervous system that are expressing at least two splice variants of Meltrin during embryonic development and provide first evidence that Meltrin expression is regulated by the transcription factor ventral nervous system defective (VND).

Materials and Methods

Strains and growth conditions
Drosophila melanogaster w1118 strain was grown on standard media and used as wildtype. Kruppel Gal4 was described in (Cowden and Levine, 2003). The IND null mutant fly lines used were *indRR108/TM3 ftzlacZ* and the VND null mutant lines were *vnd6/FM7ftzlacZ* (McDonald et al., 1998; White et al., 1983). UAS*ind* and UAS*vnd* were constructed as described in (McDonald et al., 1998; Von Ohlen et al., 2007).

Sequence analysis, RT-PCR, in situ hybridization and Northern blot
Meltrin isoform predictions (FBgn0051314, http://flybase.org) were verified by RT-PCR. Total-RNA (RNeasy Mini Kit, Qiagen, Hilden, Germany) from embryonic stage was treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and used as a template for cDNA synthesis (AMV First Strand cDNA Synthesis Kit for RT-PCR, Roche, Mannheim, Germany). The coding sequences of the two transcript variants were amplified with the following primer-pairs: Isoform C (4224 bp): atgttgcacttgcatttgtt (forward, FW), ctacttgctctgctcgaatcg (reverse, RV); isoform D (2928 bp): atgttgcacttgcatttgtt (FW), ttagtttttggcacacctcgc (RV). Amplification products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced. To identify sequences being specific to additional splice variants, RT-PCRs with variable combinations of the following primers were performed: ccacctatgaatcacaccgat (FW), gttattgacgttgttgcagga (FW), atcttatctttgggagtgcca (FW), tctagattgatcatttaa (FW), acgtttaaagctacgttt (FW), taagtcacgatcgcaaga (FW), aaatagattgtttaacttgat (RV), actctttgcttganaatgcga (RV), tttccggcttgttcctcaggc (RV), ggttgagtgcgtggagtgcag (RV), ggtgaagatgcatcgttca (RV), ttagtttttggcacacctcgc (RV), accatttggatctgtggctgg (RV).
Templates for riboprobe synthesis were generated with primer-pairs being specific to either *meltrin* isoform C (FW: ctgatcccaagctaaatgtta; RV: catcaattacggagtaaatgt, 537 bp), isoform D (FW: gtatgtatcgacctcctcctt; RV: ttagtttttggcacacctcgc, 522 bp) or both isoforms (FW: aactcggagcattgctacaac; RV: accatttggatctgtggctgg, 503 bp). RNA probes were synthesized by in vitro transcription with "DIG RNA labeling kit" (Roche). Hybridizations on whole mount embryos were performed as described (Duan et al., 2001). Northern blots were generally conducted with total-RNA (15 µg/lane) according to standard protocols at a hybridization temperature of 65 °C. In individual cases, mRNA was isolated from adult Drosophila (Dynabeads mRNA DIRECT Kit, Invitrogen) and used for northern blot analysis as well (1.5 µg/lane).

Immunohistochemistry
Embryos we collected and fixed in 4% formaldehyde in PBS. Rabbit anti IND antibody was used at a concentration of 1:2000 as described in (Von Ohlen and Moses, 2009).

Microscopy
All embryos were imaged with a Leica DM500 digital camera attached to a Leica DM5000 compound microscope. Figures were compiled using Photoshop software (Adobe Systems, San Jose, CA, USA).

Bacterial expression, purification and cleavage
Expression in *E. coli* was done essentially as described in (Meyer et al., 2009) with the modification that the respective Meltrin protein domains were fused to the maltose binding protein (MBP) by cloning them into the pMAL-p2X vector (New England Biolabs, Ipswich, MA, USA). Subsequent to induction of expression (0.5 mM IPTG), cells were incubated at 37 °C (3 h). Proteins were purified with amylose resin according to the manufacturer's

instructions (New England Biolabs). To test for redox dependent oligomerization, the eluted protein fractions were boiled in Laemmli-buffer for 2 min with (reducing conditions) or without (oxidizing conditions) supplementation of 10 % ß-mercaptoethanol and subjected to SDS-PAGE. Factor Xa cleavage was done according to the manufacturer's protocol (New England Biolabs).

Insect cell culture and protein expression
Heterologous expression in a eukaryotic system was done in Sf21 cells using the Bac-to-Bac baculovirus expression system (Invitrogen). The complete extracellular domain of Meltrin (2295 bp) was cloned into the pFastBacDual vector downstream of the polyhedrin promoter with the protein construct being fused to a C-terminal His-tag by appropriate primer design. Primers used were: tactcaggatccatgttgcacttgcatttgttt (FW) and tactcatctagactaatgatgatgatgatg atgatgatgtcgcttgaagcccacgga (RV). To track transfection efficiency, an eGFP reporter gene was inserted into the same vector (p10 promoter). Infected Sf21 cells were grown in 175 cm^2 flasks to 95% confluency and harvested by centrifugation (100 x g, 10 min). Cell disruption was done in PBS with a glass-teflon homogenizer. To test for redox dependent oligomerization, protein extracts were boiled in Laemmli-buffer for 2 min with (reducing conditions) or without (oxidizing conditions) supplementation of 10 % ß-mercaptoethanol and subjected to SDS-PAGE. Detection of the expressed construct was realized by western blot and anti-His antibodies (Qiagen, Hilden, Germany, 1:1000).

Results

Meltrin occurs in at least two splice variants

The *meltrin* gene (synonyms: Neu3, CG7649) is located on the third chromosome at position 88C10-88D1. Sequence analysis using standard software (http://flybase.org, FBgn0051314) predicts the existence of two Meltrin isoforms with isoform D consisting of 975 amino acids (4148 nucleotides) and isoform C of 1407 amino acids (4954 nucleotides), respectively (Figure. 1A). The unusual nomenclature starting with isoform C is due to prior annotations that were recently replaced. Alignments with mammalian databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) demonstrate that the highest level of similarity exists between the *Drosophila* protein (isoform D) and murine Meltrin alpha (ADAM 12, 52% of similar and 35% of identical amino acids), Meltrin beta (ADAM 19, 49% similar, 33% identical) and Meltrin gamma (ADAM 9, 48% similar, 31% identical). Like most of the homologous proteins from mammals, both *Drosophila* splice variants show a domain structure typical among ADAM proteins: an N-terminal prodomain precedes a metalloprotease domain containing a HExxHxxGxxHD zinc-binding motif, a disintegrin-like domain, a cysteine-rich domain, an EGF-like domain, a transmembrane helix and a cytosolic part. The presence of the zinc-binding motif strongly indicates metalloprotease activity. While the majority of the mentioned domains is apparently conserved among the isoforms, the

intracellular domains harbor significant differences with respect to their sizes. In contrast to the rather large cytosolic C-terminus of isoform C (619 amino acids), the intracellular C-terminus of isoform D consists of only 186 amino acids (Figure. 1B). Good evidence for the transcription of the predicted splice variants can be inferred from EST data: while several EST-clones contain sequence fragments shared by both transcripts (e.g. SD04085, SD04095, SD27008, Figure 1A, blue arrows), the clone EC063299 is unique to transcript D (Figure 1A, violet arrow), and the clones SD22443 and EK126102 are specific for transcript C (Figure 1A, yellow arrows, FBgn0051314, http://flybase.org).

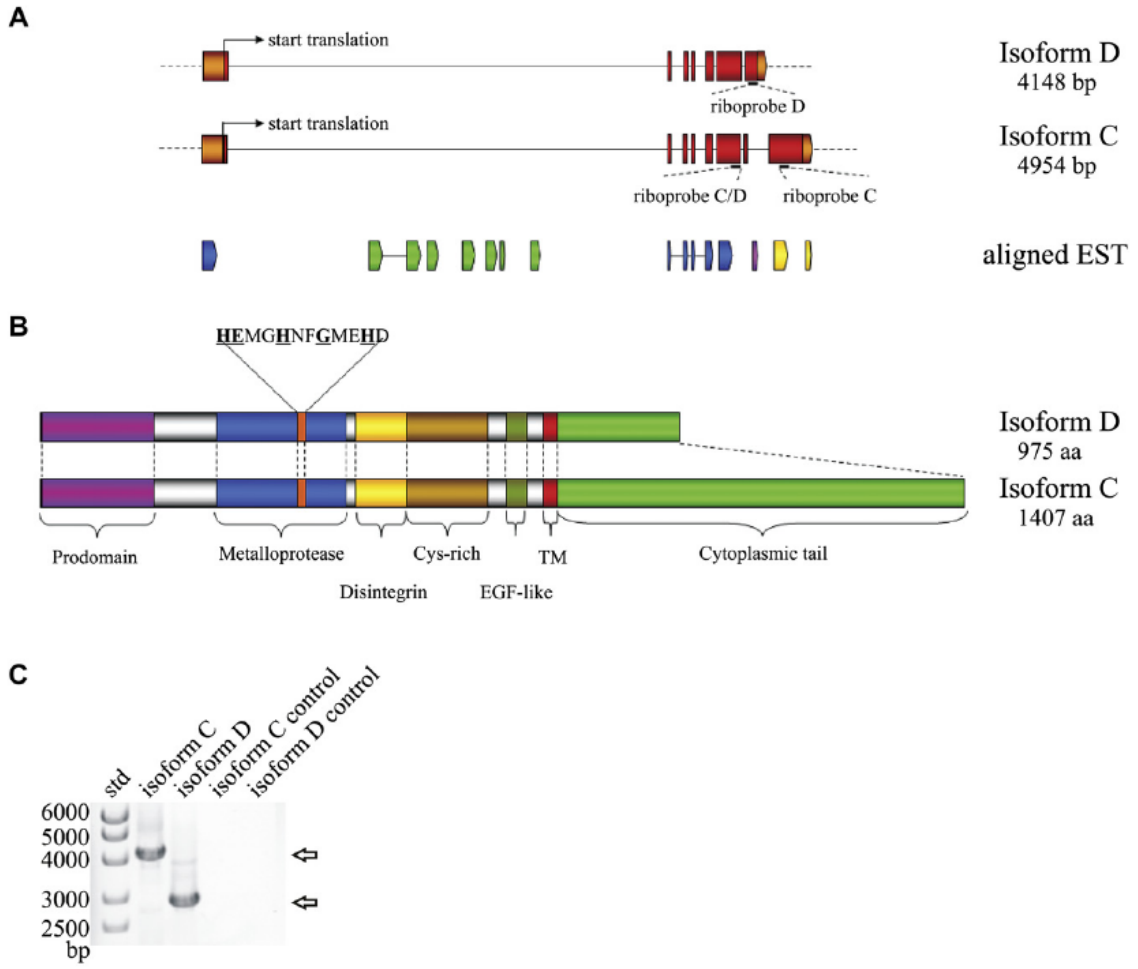


Figure 1. Schematic representation of the *Drosophila meltrin* gene. *A*, *meltrin* is present at position 88C10-88D1 on the *Drosophila* cytogenetic map. Comparison of cDNAs generated by RT-PCR from total RNA preparations (this study) and EST and cDNA sequences of the corresponding genome region (http://flybase.org) confirms the existence of the predicted two *meltrin* mRNAs. The exon/intron structure is illustrated by red boxes (exons), orange boxes (untranslated regions) and lines (introns). EST clones containing sequence data shared by isoforms C and D are depicted as blue arrows while those being unique to isoform C are yellow and the isoform D specific one is violet. EST clones harboring sequences not occurring in any of the two confirmed isoforms are coloured in green. Translational start sites are marked. While antisense riboprobes C and D are specific to the respective isoforms, riboprobe C/D does not distinguish between transcript variants. All probes were used for northern blot analysis and whole mount *in situ* hybridization. *B*, illustration of the overall protein structure of the

Meltrin isoforms C and D. Both isoforms show an almost identical domain structure typical for ADAM proteins; however, with respect to isoform C, the intracellular C-terminus is significantly elongated. The zinc binding motif present in the respective metalloprotease domains is marked. *C*, RT-PCR products of *meltrin* isoforms C and D separated by agarose gel electrophoresis. mRNA expression of both isoforms is verified using reverse transcribed embryonic RNA preparations. In control experiments without cDNA-templates, no amplicon is visible.

Using transcript specific primers and embryonic total RNA, we confirmed the presence of both predicted splice variants by RT-PCR (Figure 1C). By cloning and sequencing the amplicons, we verified that the isolated cDNAs correspond to the predicted transcripts C and D. The absence of introns in the respective sequences excluded the possibility of genomic DNA priming. Noteworthy, by containing sequence data not occurring in the confirmed transcripts C or D, some EST-clones (e.g. GH14307, RH35352, RH30043, EN05318, EN10939, EN09848) indicate the existence of additional splice variants (Figure 1A, green arrows). However, since none of these ESTs includes an accurate open reading frame and since the derived polypeptides do not show any appreciable homology to any protein sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi), we consider at least these clones as non-coding ESTs.

Meltrin is expressed in intermediate neuroblasts

To infer possible physiological functions of Meltrin, we initially analyzed the transcript distribution during embryonic development. Antisense riboprobes corresponding to coding regions shared by both transcripts (riboprobe C/D) or being unique to either transcript C (riboprobe C) or transcript D (riboprobe D, Figure 1A), were generated and used for *in situ* hybridization. Due to the fact that the application of the respective probes showed basically identical *in situ* patterns, yet with reduced signal intensity in the case of transcript D specific probes, only stainings on the basis of riboprobe C/D are shown. As depicted in Figure 2, first *meltrin* mRNA appearance is observed in the neural ectoderm of stage 6 embryos (Figure 2A, B). This tissue gives rise to the neuroblasts of the ventral nerve cord in later embryonic stages. From stage 8 to 10, expression becomes increasingly segmental and restricted (Figure 2C-F) until at stage 11 mRNA can be detected almost exclusively in 2 distinct rows of cells along the dorsoventral axis (Figure 2G, H).

To ascertain the identity of these cells, we did double labeling experiments with certain marker proteins and found that *meltrin* and the homeobox protein IND (intermediate neuroblasts defective) are expressed in the same cells. As shown in Figure 3, the expression patterns of IND (Figure 3A, D) and *meltrin* (Figure 3B, E) are overlapping (Figure 3C, F).

Since IND was reported to be expressed in intermediate neuroectoderm and neuroblasts, where the protein is essential for development of the intermediate column of neuroblasts (Weiss et al., 1998), we conclude that *meltrin* is expressed in these distinct neuroblasts as well and in the neuroectoderm these neuroblasts arise from. In contrast to IND however, *meltrin* expression can also be observed in additional cells arranged in a segmental pattern (Figure 3E, F, arrows). Although the final identification of these cells remains to be done, their position and their proximity to the intermediate neuroblasts makes it very likely that these cells are also neuroblasts. In later stages of embryonic development, *meltrin* expression becomes weaker and diminishes completely after stage 14, except for expression in the tracheal system (Figure 2I, J), indicating a function for Meltrin in the development but not in the physiology of the embryonic nervous system. In accordance with this, previous data reported on Meltrin expression in differentiating tissues e.g. disc proper cells (Firth and Baker, 2007), which again indicates relevance in developmental processes.

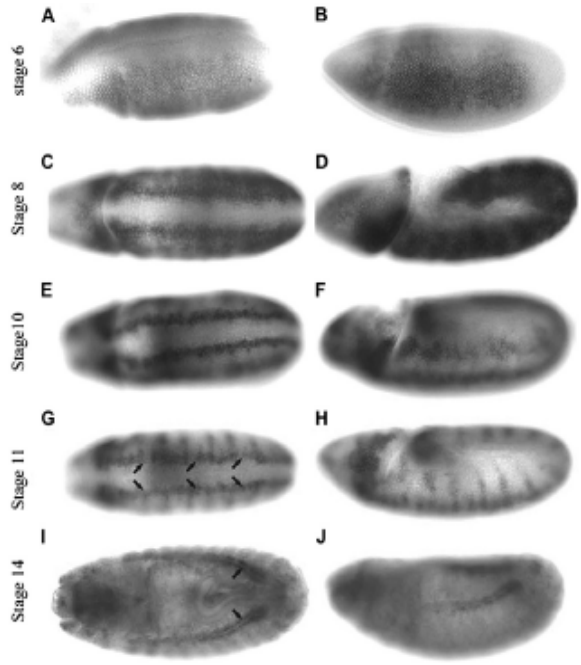


Figure 2. Embryonic expression pattern of *meltrin*. Expression was analyzed by *in situ* hybridization. In stage 6 embryos, *meltrin* mRNA is present in the neural ectoderm (A, B) and becomes increasingly segmental and restricted in stages 8 to 10 (C-F) until in stage 11 mRNA can be detected almost exclusively in 2 distinct rows of cells along the dorsoventral axis (G, H, arrows). In later stages of embryonic development, *meltrin* expression becomes weaker and diminishes completely at stage 14 with the exception of a possible residual expression in trachea (I, J, arrows). Embryos in A, C, E and G are ventral views. Embryos in B, D, F, H and J are lateral views. Embryo in I is a dorsal view. All embryos are positioned anterior to the left.

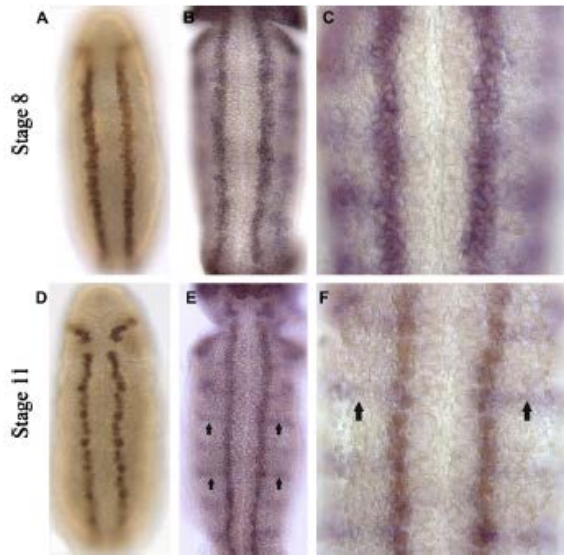


Figure 3. Identification of *meltrin* expressing cells by double labeling experiments with the homeobox protein IND. (A, D) Protein expression of IND. (B, C, E, F) Double labels with mRNA expression of *meltrin* in blue and IND protein in brown. The respective expression patterns are clearly overlapping in the neuroectoderm (A-C) and neuroblasts (D-F). Additional expression of *meltrin* is apparent in cells of yet unknown identity that are arranged in a segmental pattern (E, F, arrows). All embryos are ventral views and anterior is up.

To ascertain the postembryonic expression of *meltrin*, we analyzed corresponding stages for the presence of the respective mRNA by Northern Blot. As shown in Figure 4, *meltrin* mRNA can be detected in embryonic, larval, pupal and adult stages with male and female adults showing comparable transcript amounts. The latter finding indicates a non sex-specific function for the protein. In contrast to our prediction based anticipation of two splice variants (see above), the application of a probe with specificity for both predicted variants (riboprobe C/D, see Figure 1A) resulted in the detection of three transcripts, indicating the existence of a third isoform. According to the respective transcript lengths, it appears conceivable that the smallest transcript represents splice variant D (Figure 4A, closed arrow), while the middle sized one corresponds to variant C (Figure 4A, open arrow). The detection of a third transcript of larger size (Figure 4A, asterisk) could be explained by the existence of a third isoform, E. On the other hand, the possibility of unspecific hybridization can obviously not be ruled out. To ascertain this issue in more detail, we did additional Northern Blots, yet with the transcript variant specific riboprobes C and D. Interestingly, the application of riboprobe C resulted in distinct detection of a single transcript corresponding to the middle sized one detected by the non-isoform-specific probe (Figure 4B, open arrow). This corroborates the assumption that this transcript represents isoform C. By contrast, the application of riboprobe D mimicked the detection of the remaining two transcripts that were also labeled by the non-isoform-specific probe C/D. Since transcript variant D is predicted to be smaller than variant C and since this

prediction was confirmed by RT-PCR (Figure 1C) it is likely that the lower band represents isoform D (Figure 4B, closed arrow). The recurrent detection of the larger transcript (Figure 4B, asterisk) by two different riboprobes (C/D and D), renders an unspecific hybridization rather unlikely and strongly indicates transcription of a third splice variant. To minimize the possibility that the detected third transcript corresponds to a splicing intermediate, we did additional northern blots, yet with mRNA preparations isolated from adult *Drosophila*. Analogous to the detection in total RNA samples, the additional transcript is also present in mRNA preparations as confirmed by hybridization of riboprobe D (Figure 4B, asterisk). This result further supports the existence of a third splice variant. Unfortunately, efforts to obtain detailed sequence data on putative additional isoforms were not successful up to now. Despite performing numerous RT-PCRs with a large variety of primer combinations (see materials and methods section) and cDNA templates from embryonic, larval, pupal and adult total RNA preparations, we could not identify sequences belonging to a novel *meltrin* isoform. Isoforms C and D however could be amplified from all cDNAs which demonstrates an adequate quality of the respective templates.

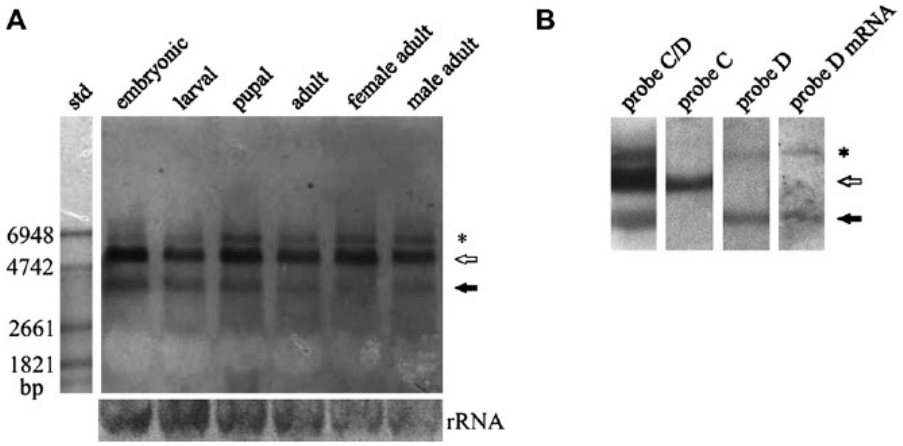


Figure 4. Postembryonic expression of *meltrin* analyzed by Northern Blot and riboprobes that do not distinguish between isoforms. *A, meltrin* mRNA can be detected in embryonic, larval, pupal and adult stages with male and female adults showing comparable transcript amounts. Transcript sizes indicate that the smallest transcript represents splice variant D (closed arrow), while the middle sized one corresponds to variant C (open arrow). The detection of a third transcript of larger size (asterisk) indicates the existence of a third isoform, E. The bottom panel shows part of the radiant red stained gel with ribosomal RNA (rRNA) visible to demonstrate the loading of comparable RNA amounts (15µg/lane).

B, Northern Blot with total RNA isolated from adult stage, mRNA isolated from adult stage and transcript variant specific riboprobes. In total RNA preparations riboprobe C distinctly detects a single transcript corresponding to isoform C (open arrow) while riboprobe D mimics the detection of the remaining two transcripts that were already labeled by the non-isoform-specific probe. The latter detection is also valid in

mRNA preparations (probe D mRNA). Transcript sizes indicate that the lower band represents isoform D (closed arrow) while the upper one (asterisk) may be related to a third transcript variant.

Meltrin expression is regulated by VND

Due to highly similar expression patterns of *meltrin* and the homeobox protein IND, a direct regulation of Meltrin expression by IND was investigated. In this context, we examined the expression of *meltrin* mRNA in embryos mutant for IND and embryos ectopically expressing IND across the dorsoventral axis. For the latter approach, we used a Kruppel Gal4 line that drives expression in a broad domain across the embryo. We found that in IND null mutant embryos the expression pattern of *meltrin* was normal (Figure 5C), while ectopic expression of IND resulted in a slight expansion of *meltrin* expression into more ventral regions (Figure 5B, arrows). However, this subtle expansion could be due to direct repression of the homeobox gene ventral nervous system defective (*vnd*) by IND in the ventral regions of the neuroectoderm (Von Ohlen and Moses, 2009) if VND was a regulator of *meltrin*. To test this we examined the expression of *meltrin* in VND null mutant embryos and found that, similar to IND expression (Weiss et al., 1998), *meltrin* expression is expanded ventrally to the midline in this genetic background (Figure 5D). Furthermore, ectopic expression of VND across the dorsoventral axis resulted in strong repression of *meltrin* expression (Figure 5E). These data demonstrate that IND does not regulate *meltrin* expression directly and indicate that transcription of both proteins, IND and Meltrin, is regulated by VND with Meltrin expression being specifically excluded from the ventral neuroectoderm by VND.

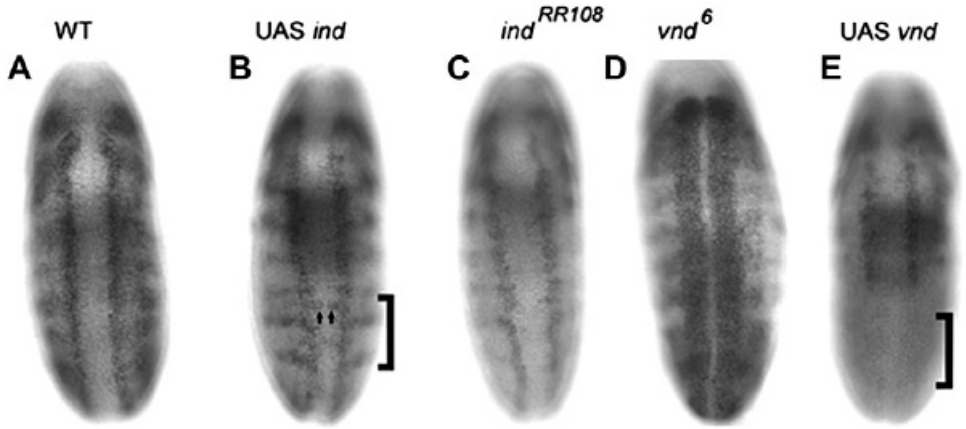


Figure 5. Regulation of Meltrin expression by VND as shown by *meltrin* mRNA staining in variable genetic backgrounds. *A*, wildtype embryo. *B*, Kruppel Gal4 x UAS*ind*, expression expands slightly into more ventral regions (arrows). *C*, *ind* null mutant embryo, expression similar to wildtype. *D*, *vnd* null mutant embryo, expression is expanded ventrally to the midline. *E*, Kruppel Gal4 x UAS*vnd*, expression is strongly repressed in the affected region. All embryos are stage 10 ventral views and anterior is up. Brackets indicate the approximate location of the Kruppel Gal4 driven expression of the VND or IND transgenes.

The Meltrin protein binding domain oligomerizes in a redox dependent manner

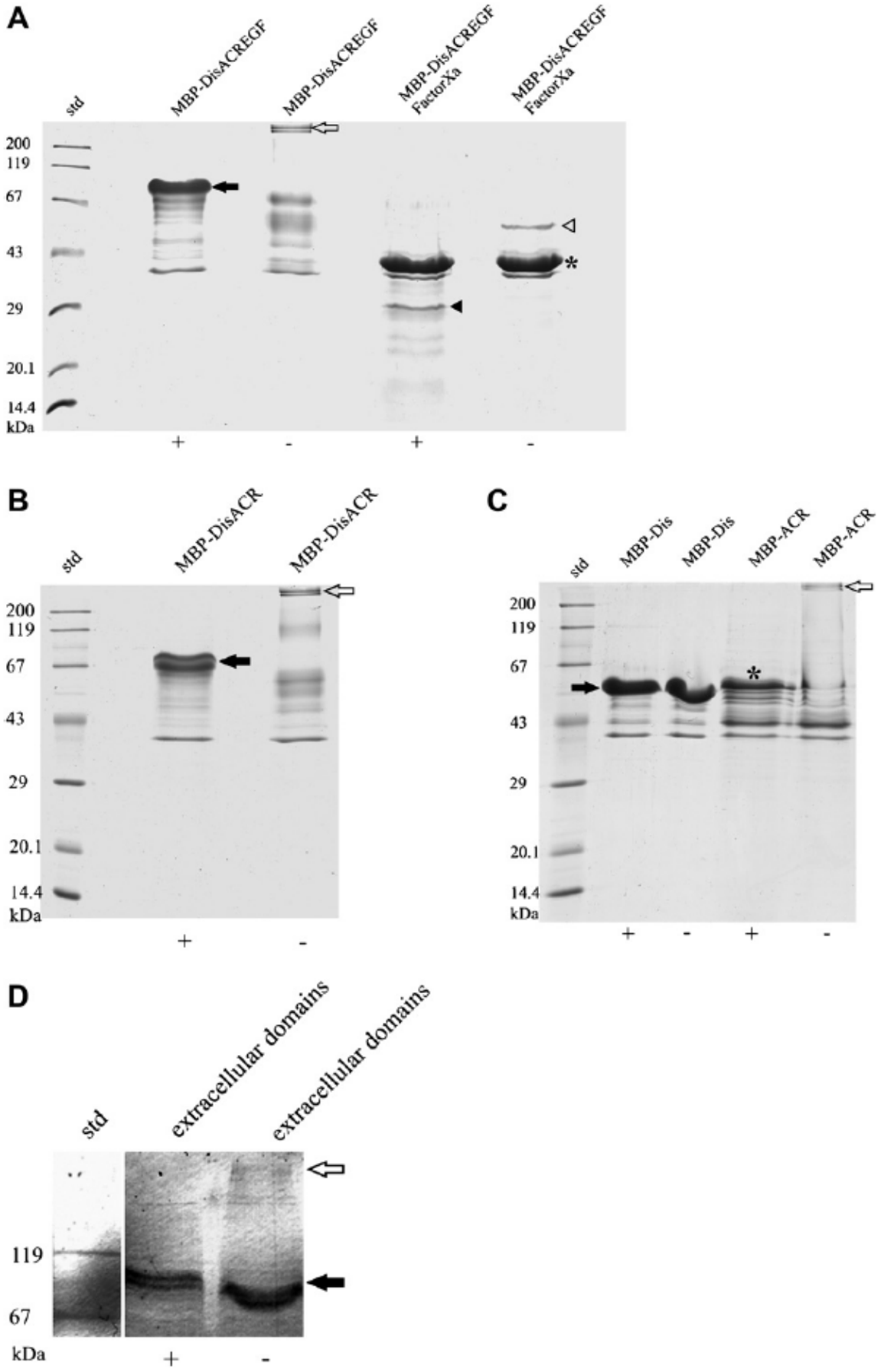
While the potential of ADAM proteins to form oligomers has been shown previously (Blobel et al., 1990; Gan et al., 2007; Waters and White, 1997) direct evidence for responsible protein domains has remained elusive in most cases. In order to analyze the potential of the Meltrin protein interaction domains to oligomerize, we expressed the disintegrin-like (Dis), the ACR and the EGF-like domains in variable combinations. To optimize the amount of natively folded protein, expression was done in the periplasma of *E. coli* cells with the respective constructs fused to the highly soluble maltose binding protein. Especially the oxidizing conditions in the periplasma should be quite beneficial to native folding since they promote the formation of disulfide bonds between the several cysteins present in the interaction domains of ADAM proteins.

Using these conditions, we initially expressed the complete interaction domain (DisACREGF, 31 kDa) and purified it via the fused maltose binding protein (MBP, 43 kDa). Subsequent to purification, we could detect a major protein band migrating at about 75 kDa on SDS-PAGE (Figure 6A, closed arrow), which corresponds quite well to the theoretical size of the construct (74 kDa). In addition to this band we also detected smaller proteins. Since these proteins, as well as the full length protein, were recognized by an antibody against MBP (not shown), they can be considered as degradation products of the full length construct. This

migration appearance however was only valid if the proteins were boiled in Laemmli-buffer containing 10% ß-mercaptoethanol prior to SDS-PAGE. If the reducing agent was omitted, the major band representing the full length protein disappeared almost completely and was replaced by proteins of high molecular weight (Figure 6A, open arrow), indicating oligomerization of the respective protein together with some of its degradation products. To ascertain whether the interaction domain or the MBP-fusion was responsible for oligomerization, we cleaved the two proteins by incubation with factor Xa. It appeared that MBP alone did not form multimers; the migration of the protein remained constant, independent of the presence or absence of ß-mercaptoethanol (Figure 6A, asterisk). By contrast, the DisACREGF domain migrated at its predicted molecular weight (31 kDa) only under reducing conditions (Figure 6A, closed arrowhead). If ß-mercaptoethanol was omitted from the sample buffer, the respective protein band disappeared and was replaced by a band at about 60 kDa (Figure 6A, open arrowhead), a clear indication for the formation of redox-dependent dimers. To ascertain the question which of the Meltrin interaction domains was responsible for the observed dimerization we expressed the same construct, however this time without the EGF-like domain. As shown in Figure 6B, the removal of this domain (6.4 kDa) did not change the migration behaviour significantly. As expected, under reducing conditions the construct migrated at about 68 kDa (Figure 6B, closed arrow), which corresponds almost perfectly to the anticipated molecular mass of 67.6 kDa. The absence of ß-mercaptoethanol however again resulted in the disappearance of this band which again was replaced by high molecular weight bands (Figure 6B, open arrow). From this we conclude that oligomerization occurs independently of the presence or absence of the EGF-like domain and that this domain is therefore not responsible for the apparent oligomerization under oxidizing conditions. To narrow down the responsible domain, we expressed the disintegrin-like and the ACR domain separately in fusion to MBP and analyzed the respective migration behaviours: while the disintegrin-like domain did not show any redox-dependent change in migration (Figure 6C, closed arrow), the ACR domain was clearly susceptible to changes in redox conditions.

As already observed for the larger protein constructs, oxidizing conditions resulted in the formation of high molecular weight proteins (Figure 6C, open arrow). With ß-mercaptoethanol in the sample buffer however, the protein migrated exactly at its theoretical mass of 59 kDa (Figure 6C, asterisk). This result clearly demonstrates that the ACR domain is able to form redox-dependent multimers and indicates that this domain is responsible for multimerization of the complete binding domain and therefore of the full length protein.

To ensure that the observed oligomerization was not based on artefactual disulfide bond formation that may occur after expression of eukaryotic proteins in *E. coli*, we expressed the complete extracellular region of Meltrin in fusion to a C-terminal His-tag in Sf21 insect cells. Analogous to the bacterial expression, protein preparations were boiled in Laemmli-buffer either with or without supplementation of ß-mercaptoethanol. This time however, multimerization was monitored by western blot and detection of the fused His-tag instead of using purified proteins. As shown in Figure 6D, both, reducing and oxidizing conditions, result in the detection of a major band at about 90 kDa (Figure 6D, closed arrow). Oxidizing conditions however, cause the formation of additional bands of high molecular weight that are also recognized by the anti-His antibody (Figure 6D, open arrow). This clearly indicates at least partial oligomerization of the protein construct and thus confirms the data from the expression in *E. coli*. The discrepancy between the apparent molecular mass of the detected band (90 kDa) and the theoretical mass of the expressed construct (85.3 kDa) is presumably due to posttranslational modifications, e.g. glycosylation. Accordingly, the fact that detection of this protein, but also of the high molecular weight oligomers, occurred only in protein preparations from transfected cells but not from untransfected control cells (not shown), clearly confirms the respective identities.


A
std
MBP-DisACREGF
MBP-DisACREGF
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MBP-DisACREGF FactorXa
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MBP-DisACR
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Figure 6. SDS-PAGE based oligomerization analysis of the disintegrin-like (Dis), the ACR and the EGF-like domains in variable combinations. *A*, Expression of the complete interaction domain in fusion to MBP (MBP-DisACREGF). Under reducing conditions (+) the protein construct is migrating at its predicted molecular mass (closed arrow), while oxidizing conditions (-) result in the disappearance of this band which is replaced by proteins of high molecular weight (open arrow). MBP alone is not able to form multimers; subsequent to cleavage from the interaction domain, the migration of MBP remains constant independent of the presence (+) or absence (-) of ß-mercaptoethanol (asterisk). By contrast, the DisACREGF domain migrates at its predicted molecular weight only under reducing conditions (+, closed arrowhead). Without ß-mercaptoethanol the respective protein band disappears and is replaced by a band of higher molecular weight (-, open arrowhead).
B, Expression of the disintegrin-like and the ACR domain in fusion to MBP (MBP-DisACR). Under reducing conditions (+) the protein construct is migrating at its predicted molecular mass (closed arrow), while oxidizing conditions (-) result in the disappearance of this band which is replaced by proteins of high molecular weight (open arrow). *C*, Individual expression of the disintegrin-like and the ACR domains in fusion to MBP (MBP-Dis, MBP-ACR). While the disintegrin-like domain does not show any redox-dependent changes in migration behaviour (closed arrow), the ACR domain is clearly susceptible to changes in redox conditions. Under reducing conditions (+) the protein construct is migrating at its predicted molecular mass (asterisk), while oxidizing conditions (-) result in the disappearance of this band which is replaced by proteins of high molecular weight (open arrow). *D*, Western blot based detection of the complete extracellular region after expression in Sf21 cells. While under reducing conditions (+) only the monomeric form of the protein is detected (closed arrow), oxidizing conditions (-) cause the additional formation of high molecular weight multimers (open arrow).

Discussion

Dimerization or oligomerization is a common physical property of proteins and represents a constantly recurring theme in biological systems. Protein dimers and oligomers contribute to numerous cellular processes, e.g. regulation of enzymatic activities, transfer of signals or solutes across the cell membrane or control of DNA binding and gene expression.

Apart from novel data regarding expression and regulation of the *meltrin* gene, this study demonstrates the potential of the protein's ACR domain to oligomerize in a redox dependent manner. Since the domain structure is highly conserved among ADAM proteins, ACR based oligomerization may emerge to be a general attribute within this protein family. Furthermore, our result that oligomerization is redox dependent and resistant to SDS-PAGE strongly indicates direct involvement of disulfide bridges realized by cysteines that are characteristic for ACR domains. In order to optimize the amount of natively folded protein and to minimize the risk of artefactual oligomer formation, expression was done in the oxidizing periplasm of *E. coli*, a redox environment that should be comparable to the *in vivo* situation in which the

respective protein domains are facing the extracellular space. To further confirm the *in vivo* relevance of protein multimerization, we expressed the complete extracellular part of the protein in the eukaryotic Sf21 insect cell line. Unfortunately, we could detect protein expression only in the cytoplasm of the cells and not, in a secreted form, in the culture medium. This may be due to the fact that the Sf21 cells do not recognize the endogenous signal sequence present in the construct (SignalP 3.0 Server, http://www.cbs.dtu.dk/services/SignalP/). Nevertheless, even after expression in the relatively reducing cytosol, a considerable fraction of the extracellular region formed oligomers (Figure. 6D, open arrow). The observation that, compared to the bacterial expression system, a larger contingent of the expressed constructs is still occurring in a monomeric state (Figure 6D, closed arrow) is presumably due to the mentioned reducing conditions in the cytosol of the insect cells. These conditions apparently impede the oligomerization of the protein that occurs quite efficiently in the oxidizing periplasm of *E. coli*. Since, in *Drosophila*, the protein is acting most likely in the oxidizing extracellular space, we consider the predominantly multimeric appearance, observed after expression in an oxidizing environment, as being close to the *in vivo* situation. The physiological relevance of the observed oligomerization however is currently less clear. In 2005, it was shown that Meltrin has a limited capacity to cleave the Notch ligand serrate. Except for the data from this cell culture based assay though, no developmental or physiological function for the protein was reported, apparently as a consequence of the unavailability of a knock-out strain and the lack of any RNAi knock-down mediated phenotype (Sapir et al., 2005). Nevertheless, the evidence that Meltrin is an active protease may suggest that oligomerization is involved in activity regulation. Such a regulative mechanism has already been shown in the case of other proteases like FtsH (Akiyama and Ito, 2000), the SARS 3C-like protease (Fan et al., 2004) or the HIV-1 protease (Wlodawer et al., 1989). Our data, that *meltrin* mRNA can be detected quite distinctly in intermediate neuroblasts during embryonic development (Figure 3) clearly suggest a role for the protein in the development of the nervous system. In particular, the VND controlled restricted expression and the tight exclusion from adjacent tissues like the ventral or the dorsal neuroectoderm (Figure 3, Figure 5) indicates a function in the proper development of these tissues. However, to ascertain this issue accordingly, clearly the availability of a knock-out strain would be quite beneficial. In addition to these embryonic expression data, the presence of *meltrin* mRNA in every developmental stage in comparable amounts indicates a constant need for Meltrin activity throughout the complete life cycle of the fly.

Isoform C amino acid position	Isoform D amino acid position	Corresponding kinase
835	813	Casein kinase 2
847	817	Casein kinase 2
962	873	Casein kinase 2
1010	893	Casein kinase 2
1037		Casein kinase 2
1044		Casein kinase 2
1134		Casein kinase 2
1148		Casein kinase 2
1154		Casein kinase 2
1216		Casein kinase 2
1235		Casein kinase 2
1265		Casein kinase 2
813	845	Protein kinase C
865	903	Protein kinase C
917	937	Protein kinase C
939		Protein kinase C
990		Protein kinase C
1044		Protein kinase C
1059		Protein kinase C
1064		Protein kinase C
1074		Protein kinase C
1165		Protein kinase C
1202		Protein kinase C
1282		Protein kinase C
1327		Protein kinase C
1354		Protein kinase C
1382		Protein kinase C
1400		Protein kinase C
1129	797	Tyrosine kinase
1146		Tyrosine kinase
1194		Tyrosine kinase
1304		cAMP dependent kinase

Table 1. Prediction of phosphorylation sites in the cytoplasmic tails of Meltrin isoforms C and D, respectively. While the cytoplasmic domain of Meltrin D contains 8 putative phosphorylation sites, the corresponding domain of Meltrin C holds 32 sites. The identification of intracellular domains was done with TMHMM v2.0 (http://www.cbs.dtu.dk/ services/TMHMM-2.0); prediction of phosphorylation sites was done with Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

This need is apparently sex independent since male and female flies show similar rates of mRNA expression (Figure 4A). An interesting observation in this context is the fact that the individual isoforms are expressed concertedly without any obvious stage dependent up or downregulation. Generally, physiological activities of ADAMs are exerted by their extracellular domains, which are identical with respect to Meltrin C and D. This may suggest similar physiological functions for the isoforms. Nevertheless, the fact that both splice variants are, at least in embryos, expressed in an identical time and tissue specific manner (see results section) clearly indicates isoform specific functions. Since the only difference between the isoforms regards the nature of

their cytoplasmic tails (Figure 1), the physiological reason for the simultaneous expression has to be linked to this domain. In recent years, it has become apparent that activity of ADAM proteins can be modulated by phosphorylation of, or binding of interacting proteins to, the respective cytoplasmic tails. Such modifications are discussed to affect expression at the cell surface, localization to specific membrane domains, association with other surface proteins, stability or the ability to cleave distinct substrates in response to specific stimuli (Abram et al., 2003; Cousin et al., 2000; Seals and Courtneidge, 2003). Regarding Meltrin, it is obvious that due to the substantial differences between the particular cytoplasmic tails, isoform specific modifications are likely. In this context, sequence based prediction identified 8 putative phosphorylation sites in the cytoplasmic domain of Meltrin D and 32 sites with respect to the cytoplasmic tail of Meltrin C (Table 1). This considerable difference is a good indication for an isoform specific, phosphorylation based regulation of activities that was also proposed with respect to other ADAMs, e.g. ADAM17 (Diaz-Rodriguez et al., 2002). Since for ADAM proteins, up to now alternative splicing was reported to occur only in the cytoplasmic domains and, in individual cases, in the cysteine rich region (Seals and Courtneidge, 2003), it appears conceivable that also the putative third isoform, that was individually detected by two different *meltrin* specific riboprobes (Fig. 4B), is differing from isoforms C and D mainly in the nature of its cytoplasmic tail. Due to the size of the detected transcript, the cytoplasmic domain of this isoform is presumably the largest of all Meltrin splice variants. To ascertain whether or not the variability in the respective cytoplasmic tails accounts for different physiological relevancies that may, in part, be based on isoform specific phosphorylation states or the localization in different subcelluar compartments, clearly further research and the availability of isoform specific antibodies is necessary. Nevertheless, the data presented in this study distinctly extend the current knowledge on ADAM proteins and imply novel mechanisms to regulate their activities.

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3.2. Neprilysin 4, a novel endopeptidase from Drosophila melanogaster displays distinct substrate specificities and exceptional solubility states

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Summary

Proteins belonging to the family of neprilysins are typically membrane bound M13-endopeptidases responsible for the inactivation and/or activation of peptide signaling events on cell surfaces. Mammalian neprilysins are known to be involved in the metabolism of various regulatory peptides especially in the nervous, immune, cardiovascular and inflammatory systems. Although there is still much to learn about their participation in various diseases, they are potential therapeutic targets. Here we report on the identification and first characterization of neprilysin 4 (NEP4) from *Drosophila melanogaster*. Reporter lines as well as *in situ* hybridization combined with immunolocalization demonstrated NEP4 expression during embryogenesis in pericardial cells, muscle founder cells, glia cells and male gonads. Western blot analysis confirmed the prediction of one membrane bound and one soluble isoform, a finding quite unusual among neprilysins with presumably strong physiological relevance. At least one NEP4 isoform was found in every developmental stage indicating protein activities required throughout the whole life cycle of *Drosophila*. Heterologously expressed NEP4 exhibited substrate preferences comparable to human neprilysin 2 with distinct cleavage of substance P and angiotensin I.

Introduction

Since the identification and characterization of the first neprilysin from rabbit renal brushborder membranes (Kerr and Kenny, 1974), the family of M13 zinc metallopeptidases has become increasingly important. Members of this family in general and neprilysin in particular are subjects of various medical investigations regarding Alzheimer's disease (Iwata et al., 2000), hypertension (Molinaro et al., 2002), analgesia (Whitworth, 2003) and the progression of cancers (Turner et al., 2001). These investigations focus on the enzyme's ability to hydrolyze signaling peptides like enkephalins, bradykinins, tachykinins, the natriuretic atrial factor, substance P or even the neurotoxic amyloid β-peptide (Roques et al., 1993). A disregulation of the extracellular peptide homeostasis may influence the corresponding signaling pathways and account for the mentioned diseases.

In mammals, seven members of the M13 family are known including neprilysin, endothelin-converting enzymes (ECE-1, ECE-2), the KELL blood-group protein and PHEX (Turner and Tanzawa, 1997). However, currently only limited data are available for most of these proteins and a crystal structure is known only in individual cases (Oefner et al., 2000). Among the members of the M13 family, human neprilysin is characterized best. The protein specifically cleaves N-terminal peptide bonds at aromatic and bulky hydrophobic amino acids (Hersh and Morihara, 1986), and is potently inhibited by phosphoramidon from *Streptomyces* (Oefner et al., 2000). Human neprilysin is a type II integral membrane protein of 750 amino acids, structured into a short cytoplasmic domain, a membrane spanning region and a large extracellular domain containing the active site with its characteristic HExxH motif, which typically can be

found in various other zinc peptidases. In contrast to zinc proteases however, two distinct protein domains prevent neprilysin from cleaving larger substrates simply by restricting active site access to oligopeptides (Oefner et al., 2000). With the exception of neprilysin 2, which is expressed in a membrane bound state but becomes soluble due to proteolytic cleavage (Ghaddar et al., 2000; Ikeda et al., 1999), almost every member of the mammalian M13 family analyzed so far shares this membrane bound topology. In the vast majority of cases however, the identification of *in vivo* functions and substrates is the main task for future investigations.

Here we report on the characterization of neprilysin 4 (NEP4) from *Drosophila melanogaster*. In contrast to almost every other neprilysin analyzed so far, NEP4 from *Drosophila* is expressed not only as a membrane bound but also as a soluble protein. The two solubility states are due to two different splice variants with variable N-termini: in contrast to the larger isoform A, isoform B lacks the N-terminal cytosolic and transmembrane regions and is therefore presumably expressed in a soluble state. RT-PCR as well as Northern and Western blot analysis validated protein expression in every developmental stage. Reporter lines and *in situ* hybridizations combined with immunostainings identified NEP4 in a subset of pericardial cells, in three dorsal muscle founder cells and numerous types of glia cells during embryonic development. In larval and adult flies, NEP4 is present in the nervous system and in the testis. Activity assays with heterologously expressed enzyme demonstrate that the peptides substance P and angiotensin I are cleaved with high efficiency while other peptides like bradykinin, tachykinin or PDF have to be considered as poor substrates.

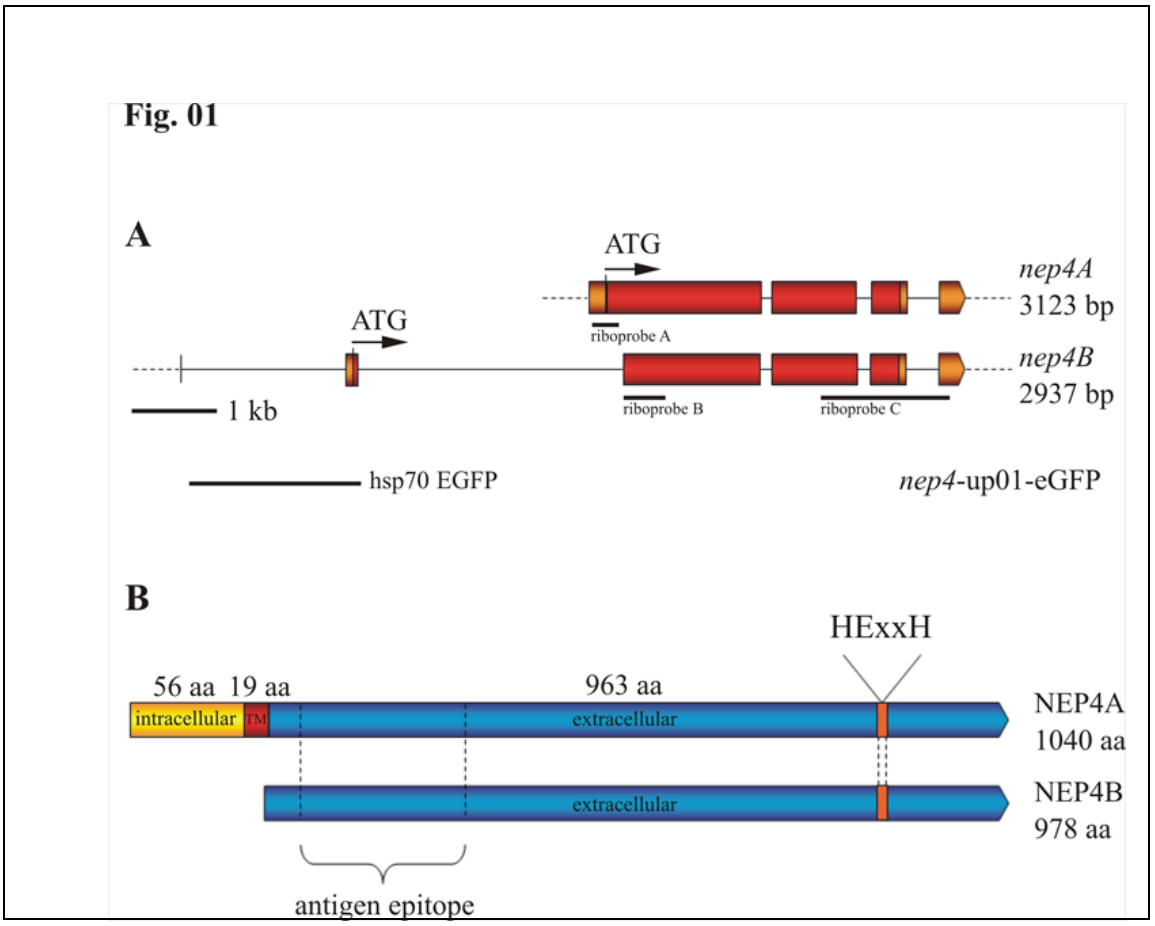

Fig. 1. Schematic representation of the *Drosophila nep4* gene. *A*, *nep4* is present at position 92F4-92F5 on the *Drosophila* cytogenetic map. Comparison of cDNAs generated by RT-PCR from total RNA preparations (this study) and EST and cDNA sequences of the corresponding genome region (http://flybase.org) confirms the existence of the

predicted two *nep4* mRNAs. The exon/intron structure is illustrated by red boxes (exons), orange boxes (untranslated regions) and lines (introns). Translational start sites are marked by an ATG. Antisense riboprobes A and B were used for northern blot analysis and whole mount *in situ* hybridization, probe C was used for fluorescence *in situ* hybridization. The bottom part of the figure shows the location of the DNA fragment capable of driving reporter gene expression in transgenic animals. *nep4*-up01-eGFP drives eGFP expression in a pattern that resembles part of the endogenous NEP4 expression. *B*, illustration of the overall protein structure of the isoforms NEP4A and NEP4B. NEP4B lacks the intracellular as well as the transmembrane (TM) domain. The region used for antigen expression is marked.

Materials and methods

Sequence analysis, RT-PCR, in situ hybridization and Northern blot

nep4 transcript predictions (FBgn0038818, http://flybase.org) were verified by RT-PCR. Total-RNA (RNeasy Mini Kit, Qiagen) from different developmental stages was treated with DNase I (Invitrogen) according to the manufacturer's instructions and used as a template for cDNA synthesis (AMV First Strand cDNA Synthesis Kit for RT-PCR, Roche). The two transcript variants were amplified with the following primer-pairs: Transcript A (3123 bp): atgagtcgccacagccaactg (FW), ctaccaaacgctgcactt ttt (RV); transcript B (2937 bp): tgaagtgtggtgcaa ccataa (FW), ctaccaaacgctgcacttttt (RV). To ensure template specific priming, transcript B was amplified with a forward primer annealing in the 5´-UTR, immediately upstream of the predicted translational start of this splice variant. Amplification products were cloned into the pGEM-T vector (Promega) and sequenced.

Templates for riboprobe synthesis were generated with primer-pairs being specific to either nep4 transcript A (FW: atgagtcgccacagccaactg; RV: ctggaagaagtagaagcattt, 187 bp) or both transcripts (FW: tggtaatgctgccactgaccc; RV: cgcggcgctcccgta ttctga, 489 bp). For reasons of limited specific sequence length, adequate riboprobes against transcript B, suitable for whole mount in situ hybridization or northern analysis, could not be generated. Sense and antisense RNA probes were synthesized with "DIG RNA labeling kit" (Roche). Hybridizations on whole mount embryos, brains of 3rd instar larvae and adult testes were performed as described (Duan et al., 2001). For fluorescence whole mount in situ hybridization an antisense riboprobe was generated using the nep4 DGRC clone LD25753. Triple staining was done as described previously (Jagla et al., 1997). Northern blots were conducted with total-RNA (15 µg/lane) according to standard protocols at a hybridization temperature of 67 °C.

Generation, purification and specificity of antibodies

For the generation of polyclonal antibodies a 630 bp fragment of nep4 (FW: tactcagaattcatgagggat ctgcggaac; RW: tactcaaagcttgaagccggccttatcctt) was cloned into the pET29b vector and transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen). Following protein expression (3 h, 37 °C), cells were harvested by centrifugation (10 minutes, 5000 x g) and sonicated (Branson sonifier 250). The insoluble protein fraction was isolated by centrifugation (10.000 x g, 4 °C, 20 minutes), solubilized in CAPS buffer (50 mM CAPS, 0.3% NLS, 1 mM DTT, pH 11.0) and refolded in dialysis buffer (300 mM NaCl, 50 mM NaH2PO4, 0.1 mM DTT, 2.5 mM imidazole, pH 8.0). Subsequent to Ni-NTA-purification the antigen was used for immunization of two rabbits (Pineda Antibody-Service, Berlin, Germany). The resulting sera were affinity purified and their monospecificity was confirmed by Western blot. Immunohistochemical control stainings were done with preimmune sera, secondary antibody only and antigen blocking of purified sera (50 µl of sera were incubated with 40 µg of purified antigen in 1 ml PBS for 1h at room temperature).

Protein preparation and immunoblotting

Protein extracts from different developmental stages were isolated by three repetitive cycles of homogenization with each cycle including freezing of tissues in liquid nitrogen, thawing on ice and homogenization in a glass-teflon homogenizer. Proteins from larva and pupa were heated at 70 °C for 10 minutes in Laemmli buffer (method B), while embryonic and adult proteins were boiled at 99 °C for 3

minutes in the same buffer (method A). Membrane fractions from homogenized tissues or cells were obtained by differential centrifugation: subsequent to centrifugation at 5000 x g (10 minutes) the supernatant was subjected to ultracentrifugation (100.000 x g, 1 h). The resulting membrane and soluble fractions were further processed according to method A. Subsequent to SDS-PAGE, separated proteins (10 µg/lane) were transferred to nitrocellulose membranes and analyzed by immunodetection. Purified NEP4 antiserum was applied at a dilution of 1:2000 and visualized by anti-rabbit alkaline phosphatase conjugated antibody (Sigma, 1:10.000).

Immunohistochemistry

Whole mount stainings on embryos were performed as described (Sellin et al., 2009). Primary antibodies used were mouse anti-Repo (1:5, Developmental Studies Hybridoma Bank, DSHB), mouse anti-Prospero (1:5, DSHB), rabbit anti-Eve (1:2000, from D. Kosman, (Kosman et al., 1998)), guinea pig anti-Runt (1:2000, from J. Reinitz, (Kosman et al., 1998)), guinea pig anti-Krüppel (1:500, from J. Reinitz, (Kosman et al., 1998)) and mouse anti-GFP (1:500, JL-8; Clontech). Prior to application of NEP4 antiserum in PBS (1:500), fixed tissues were incubated in PBS containing 0.05-0.1% SDS (20 minutes) and blocked with 1% BSA. Brains prepared from 3rd instar larvae were permeabilized as described above before fixation in 1% formaldehyde in PBS for 10 minutes. Secondary antibodies were conjugated with: Alexa Fluor 488 (1:500), Cy2 (1:200), Cy3 (1:100) or Cy5 (1:200, Jackson Immuno Research) and diluted in PBS.

Bacterial expression and purification of NEP4

To express NEP4 isoform B (978 amino acids) in fusion to Glutathion S-transferase (GST), the corresponding sequence was cloned into the pGEX-5X-1 vector (GE healthcare). *E. coli* Rosetta (DE3) were transformed either with the empty vector as a control or with the vector inducing the expression of GST-NEP4B. Cells were grown at 37 °C to $OD_{600}\approx0.5$. To induce expression, 0.2 mM IPTG was added and cells were incubated at 28 °C (5 h). Proteins were purified with glutathione agarose according to the manufacturer's instructions (Machery Nagel). For subsequent activity assays, the eluted protein fractions were dialyzed against 50 mM Tris, 100 mM NaCl (variable pH-values).

Insect cell culture and protein expression

Heterologous expression was done in SF21 cells using the Bac-to-Bac baculovirus expression system (Invitrogen). *nep4* transcript A (3123 bp) was cloned into the pFastBacDual vector downstream of the polyhedrin promoter. To track transfection efficiency, an eGFP reporter gene was inserted into the same vector (p10 promoter). Infected and noninfected SF21 cells were grown in 175 cm^2 flasks to 95% confluency and harvested by centrifugation (100 x g, 10 minutes). Cell disruption was done in PBS with a glass-teflon homogenizer. Membrane fractions were collected by differential centrifugation as explained above and used for activity assays.

Peptidase activity assay

Peptides tested for digestion were substance P (RPKPQQFFGLM-NH_2), angiotensin I (DRVYIH PFHL-NH_2), *Locusta migratoria* tachykinin 1 (GPSGFYGVR-NH_2), bradykinin (RPPGFSPFR-NH_2) and pigment dispersing factor (PDF, NSELINSLLSLPKNMNDA-NH_2). For hydroly-sis assays, 10 µg of SF21-membrane fractions or 1 µg of GST or GST-NEP4 were incubated with 750 ng of peptide in 50 mM Tris, 100 mM NaCl (variable pH values and incubation times, 35 °C). To check for peptidase specific inhibition, phosphoramidon and thiorphan (variable concentrations) were preincubated with the enzyme for 30 minutes at 35 °C prior to addition of peptide. Protein activity was stopped by boiling the samples for 10 minutes. Cleavage was assayed by HPLC as follows: reaction products were loaded onto a C4 column (Vydac 214TP54) in 5% acetonitrile, 0.03% TFA in H_2O and eluted via a linear acetonitrile gradient (final concentration: 80%) over 25 min. UV-spectra (211 nm) were recorded during elution and used for quantifications. MS- and MS/MS-data (collision induced dissociation) were acquired by an ESI-ion trap (Esquire-HCT, Bruker Daltonics) with sequence

information deduced from the obtained MS/MS-data by Mascot search algorithm (Matrix Science) and the MSDB database (ftp://ftp.ncbi.nih.gov/repository/MSDB/msdb.nam).

Laser Scanning Microscopy

Confocal images were captured either with a Zeiss LSM 5 Pascal confocal microscope (Zeiss, Jena, Germany) or a Zeiss LSM 510 Meta. Z-stacks are depicted as maximum projections if not denoted otherwise. High magnification images (Fig. 5G-L) were calculated using the Velocity software (Improvision).

Drosophila stocks and transgenic fly lines

W1118 was used as wild type. P{*eve*-GAL4.eme} was provided by Rolf Bodmer (La Jolla), UAS-eGFP was obtained from the Bloomington Stock Keeping Center (BL6874). The *nep4*-eGFP reporter line was generated by cloning an upstream region of the *nep4* gene into the pH Stinger vector (Barolo et al., 2004). Primers used for the amplification of the regulatory element (1980 bp) were tactcatctagagtgtgagtatttattttggtt (FW) and tactcagctagctggataaggcggtcaaaagga (RV), giving rise to *nep4*-up01-eGFP (Fig. 1A). The construct was subjected to P-element based transformation using commercial services (Best Gene Inc, CA, USA) or the MYORES Network of Excellence, technical platform for fly injection at Clermont-Ferrand (France). Three independent transgenic lines were tested for reporter activity.

Results

NEP4 occurs in two splice variants

Sequence analysis using standard software (http://flybase.org, http://www.bioinformatics.org/sms/index.html) predicts the existence of two NEP4 isoforms. One of them, isoform A, consists of 1040 amino acids (3123 base pairs) and shows a prevalent structure among neprilysin-like proteins: a short N-terminal intracellular domain (56 amino acids) followed by a transmembrane segment (19 amino acids) and a large extracellular part (963 amino acids), which contains the active site with its typical HExxH zinc-binding motif. Isoform B however consists of only 978 amino acids (2937 base pairs). This reduction in size is due to the absence of the intracellular as well as most of the transmembrane domain and leads to the prediction of a soluble protein (TMHMM, v2.0, http://www.cbs.dtu.dk/services/TMHMM-2.0), a state quite unusual among neprilysins in general, which are typically membrane bound enzymes. Apart from these N-terminal differences, the two isoforms are identical (Fig. 1). First evidence for the existence of at least two different splice variants can be inferred from EST data: while several EST-clones indicate the existence of transcript A (e.g. BI483296 or BI580864), the clone AI389953 contains sequence data unique to transcript B (http://flybase.org). Using transcript specific primers, we could confirm the presence of both predicted splice variants in all developmental stages by RT-PCR (Fig. 2A). By cloning and sequencing the amplicons, we verified that the isolated cDNAs correspond to the predicted transcripts A and B (FBgn0038818, http://flybase.org). The absence of introns in the respective sequences excluded the possibility of genomic DNA priming.

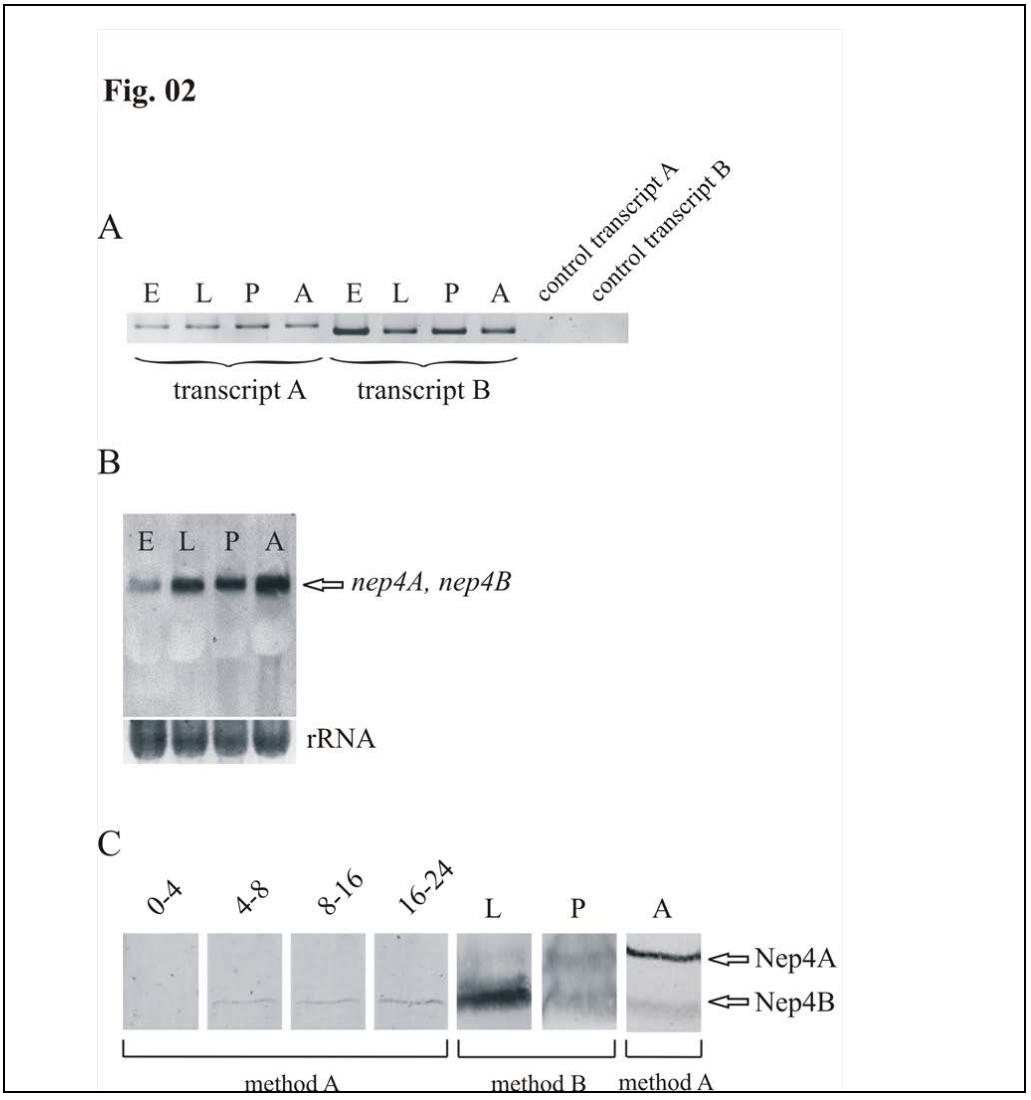

Fig. 2. RT-PCR, Northern and Western blot analysis. A, RT-PCR products of nep4 transcripts A and B separated by agarose gel electrophoresis. mRNA expression of both transcripts is detectable in embryonic (E), 3rd instar larval (L), pupal (P) and adult (A) RNA preparations. In control experiments without cDNA-templates, no amplicon is visible. B, Northern blot probed with antisense riboprobes raised against a coding region shared by both nep4 transcripts. The bottom panel shows part of the radiant red stained gel with ribosomal RNA (rRNA) visible to demonstrate the loading of comparable RNA amounts (15µg / lane). nep4 transcripts are detectable in all developmental stages. C, stage dependence of NEP4 expression. Proteins were isolated from different embryonic time intervals (indicated above each lane in hours), 3rd instar larvae (L), pupae (P) and adults (A). During embryogenesis only isoform B is detectable. A strong signal for NEP4B is also present in 3rd instar larvae. During pupal development, both NEP4 isoforms exhibit similar expression levels whereas adult flies predominantly express NEP4 isoform A.

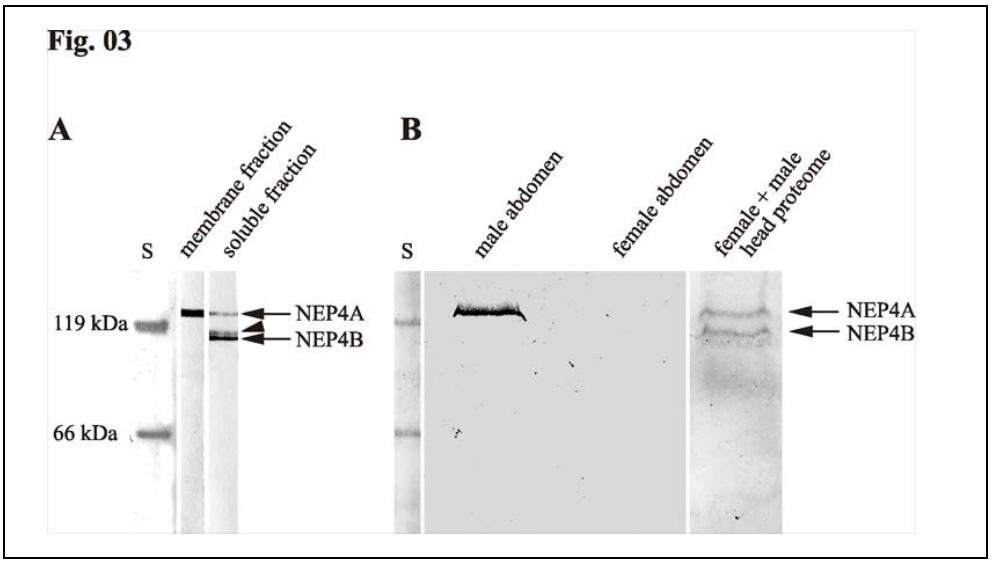

Fig. 3. NEP4 isoforms are expressed in a soluble and a membrane bound state. *A*, membrane and soluble protein fractions from adult flies were probed with NEP4 antiserum. NEP4A is predominantly detectable in the membrane fraction whereas the shorter isoform, NEP4B, appears exclusively in the soluble fraction (arrows). Both signals correspond to the predicted sizes of NEP4A and NEP4B, respectively. Due to the isolation procedure of the soluble protein fraction, a small amount of membrane bound isoform NEP4A is still visible. Notably, a weak band above the NEP4B signal is visible as well and presumably caused by secondary modifications of NEP4B (arrowhead). *B*, protein extracts of male abdomen, female abdomen and adult head preparations were probed with NEP4 antiserum. While in male abdomen, isoform A is detected exclusively, female abdomen do not show any detection. In adult head preparations both isoforms are present (arrows).

NEP4 is expressed in every developmental stage

The expression of *nep4* during development was further analyzed with specific RNA-probes for transcript detection (Fig. 1A) as well as antisera that recognize both NEP4 isoforms (Fig. 1B). In accordance with the results obtained by RT-PCR, *nep4* transcripts are detected in embryonic, larval, pupal and adult RNA preparations, suggesting NEP4 plays a role during the whole life cycle of *Drosophila*. In the course of development, the transcript amounts are gradually increasing with least abundance in embryonic and strongest transcription in adult stages, respectively (Fig. 2B). Since the resolution of the Northern gel was not sufficient to clearly separate the predicted transcripts from each other, we did Northern blots with a transcript A specific RNA-probe. In contrast to transcript B, transcript A harbors a unique 5´ sequence of 186 nucleotides, which allows the generation of a probe with specificity for this splice variant (Fig. 1A). The blots performed with this probe showed distinct signals in all stages (supplemental Fig. 2), however with a considerably reduced intensity compared to blots with probes detecting both transcripts (Fig. 2B). This result demonstrates that transcript A is expressed in all developmental stages. The reduced intensity however indicates, that the stronger signal generated by probes without transcript specificity is based on the combined expression of transcripts A and B. This simultaneous expression of both transcripts was also shown by RT-PCR (Fig. 2A).

To ascertain protein abundance, Western blots were done. Monospecificity of the antisera was validated by preimmune sera and antigen blocking controls (supplemental Fig. 1A). Consistent with the distribution of mRNA, *nep4* protein is present in every developmental stage with expression starting in embryos about 8h after oviposition. While in embryos only the smaller isoform B was detectable, during larval stages isoform A becomes traceable as well, yet with a largely reduced abundance compared to isoform B. At the pupal stage, the two isoforms reach similar expression levels whereas in the adult, NEP4A becomes the dominant isoform (Fig. 2C). Due to variable protein preparation techniques that were necessary to visualize NEP4 in every developmental stage, a significant comparison of protein amounts is only valid for relative isoform abundance within the respective stages. Stage-spanning protein amounts could be distorted by variations in preparation procedures. Nevertheless, the results from Western blot analysis clearly support the data obtained by RT-PCR that already indicated a dynamic and simultaneous expression pattern of both splice variants. The observation that despite the RT-PCR and Northern blot based detection of both transcripts, no NEP4A could be detected in embryonic protein extracts (Fig. 2C) is presumably based on weak embryonic expression levels of the respective isoform and the higher sensitivity of the alternative methods. Thus, in the course of development, in addition to increasing NEP4 expression levels in general, the predominant isoform changes from NEP4B in early developmental stages to NEP4A in later ones.

NEP4 is expressed in a membrane bound but also in a soluble state

Based on the sequence data mentioned above, one membrane bound and one soluble NEP4 isoform can be anticipated. Using a purified antiserum, we were able to confirm this prediction. As expected, in crude lysates of adult flies both isoforms were detected (Fig. 2C). In a membrane fraction however, only the larger isoform A was present and migrated almost exactly at its predicted molecular mass of 120 kDa while the smaller isoform B occurred exclusively in the soluble fraction, again close to its theoretical size of 113 kDa (Fig. 3A). This result, together with the presence of *nep4B* mRNA (Fig. 2A), demonstrates that NEP4B is expressed in a soluble state, which stands in clear contrast to the current opinion that neprilysins are expressed almost exclusively as membrane bound proteins. The presence of a double signal in the soluble fraction (Fig. 3A, arrowhead) remains to be elucidated by additional data. However, posttranslational modifications like glycosylation, which is known to occur in the case of *Drosophila* neprilysin 2 (NEP2, Bland et al., 2006), or protein degradation could account for this observation. Thus, in addition to NEP2, NEP4B may be one of the first members of a new subfamily of soluble neprilysins. In contrast to *Drosophila* NEP2 however, which exists solely in a soluble state (Thomas et al., 2005), or mammalian NEP2, which becomes a soluble peptidase only after cleavage of a membrane bound precursor protein (Ikeda et al., 1999), the isoform specific solubility of NEP4 shown in this study is apparently based on alternative splicing instead of posttranslational modification, a finding that has never been reported for any neprilysin before.

NEP4 expression is restricted to pericardial cells, dorsal muscle founder cells, the central nervous system and the male germline

Expression of NEP4 in embryonic, larval and adult tissues was analyzed by three different approaches. Firstly, we visualized *nep4*-mRNA. Antisense riboprobes, corresponding to coding regions shared by transcript A and B or being unique to transcript A (Fig. 1A), were generated and subsequently used for *in situ* hybridization. Due to the fact that the application of the respective probes showed basically identical *in situ* patterns, yet with reduced signal intensity in the case of transcript A specific probes, only stainings on the basis of probes that do not discriminate between transcripts are shown. Secondly, immunofluorescence microscopy was performed with an anti-NEP4 serum. The specificity of the serum was verified by antigen blocking and control stainings with preimmune sera and without primary antibodies (supplemental Fig. 1B).

First appearance of NEP4 is obvious at stage 12 in the dorsal mesoderm in a segmental pattern (Fig. 4A, G). From stage 13 to stage 17, expression of NEP4 is maintained solely in cells of the cardiac mesoderm, which are located in two one-cell wide rows along the a-p axis of the embryo. In addition, we found NEP4

expression from embryonic stage 14 onwards in cells of the central nervous system (Fig. 4E, K). To elucidate the identity of NEP4 expressing cells precisely, we performed double and triple immunostainings with antibodies that specifically mark individual muscle founder cells in the dorsal mesoderm. Even-skipped (Eve) is expressed in founder cells that give rise to muscle founder DA1, DO2 and a subset of pericardial cells. Initially, Eve is expressed in two cell clusters, in one of which (cluster 2) Eve initiates the expression of Krüppel (Kr). This progenitor, transiently coexpressing Eve and Kr, divides to yield two founder cells that express Runt. One of these founders gives rise to muscle DO2 and reveals continuous Runt expression. The other one gives rise to the so-called even-skipped positive pericardial cells (EPCs). Shortly later, DO2 loses Eve expression, whereas the EPCs remain Eve positive. One cell from the second Eve cluster (cluster 15) divides to yield the DA1 muscle founder (that maintains Eve expression) and a second cell that is assumed to die (Alvarez et al., 2003; Carmena et al., 1998; Fujioka et al., 2005; Han and Bodmer, 2003). Besides DA1, Krüppel additionally labels a second muscle founder within the dorsal mesoderm, which is the DO1 founder (Ruiz-Gómez et al., 1997). During stage 12-14, *nep4* transiently colocalizes with Eve, Krüppel and Runt in some, but not all dorsal muscle founder cells. Thus we conclude that NEP4 is expressed in the progenitor of the even-skipped pericardial cells, muscle founder DA1, muscle founder DO2 and a third muscle founder of a yet unknown identity (Fig. 5A-L). The expression of NEP4 in dorsal muscle founder cells is transient; in contrast, NEP4 expression in the even-skipped positive pericardial cells is maintained until end of embryogenesis (Fig. 5Q).

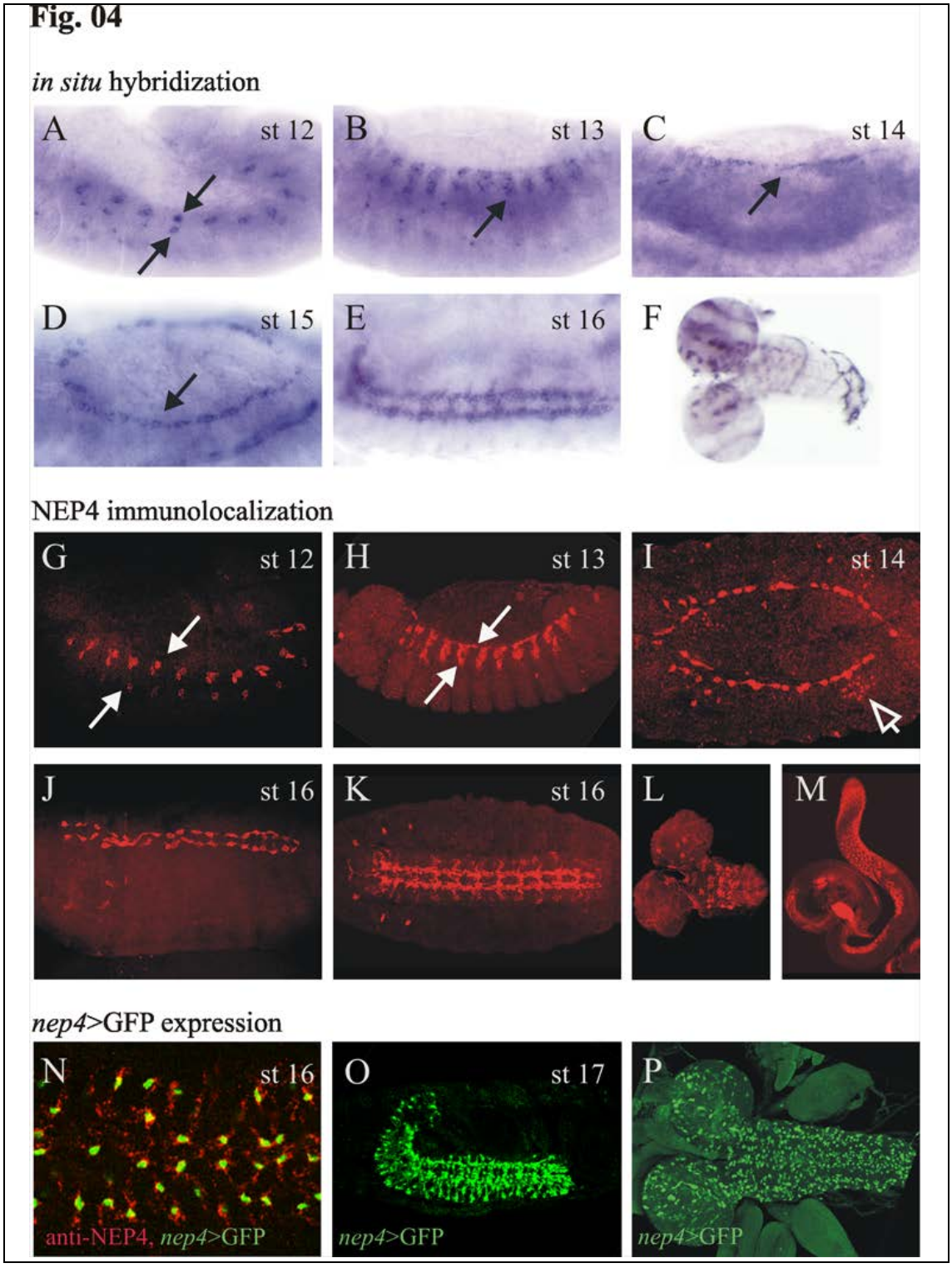
Fig. 04
in situ hybridization
A st 12
B st 13
C st 14
D st 15
E st 16
F
NEP4 immunolocalization
G st 12
H st 13
I st 14
J st 16
K st 16
L
M
nep4>GFP expression
N st 16
anti-NEP4, nep4>GFP
O st 17
nep4>GFP
P
nep4>GFP

Fig. 4. Expression of neprilysin 4 in embryos, larvae and adult flies. *A-F*, show embryos at different developmental stages and a CNS prepared from 3rd instar larvae hybridized with *nep4* antisense-RNA probes that recognize both transcripts. At stage 12, *nep4* mRNA is detectable in two patches per hemisegment in the dorsal mesoderm (*A*, lateral view, arrows). At stage 13 (*B*, lateral view), transcript distribution in the dorsal mesoderm appears broadened (arrow). During further differentiation (*C*, stage 14, lateral view; *D*, stage 15, dorso-lateral view), *nep4* expression becomes restricted to cells of the dorsal vessel. *nep4* is furthermore present in the CNS of the embryo (*E*, stage 16, ventral view) and in cells of the brain hemispheres and ventral ganglion of 3rd instar larvae (*F*). *G-M*, show embryos (*G-K*), a larval brain (*L*) and an adult testis (*M*) stained with affinity-purified NEP4 antiserum. NEP4 distribution resembles the embryonic expression pattern seen with *in situ* hybridization. NEP4 is also detectable in embryonic gonads (*I*, arrowhead) and adult testes from early (tip of testis) to late spermatogenesis (seminal vesicle) (*M*).*N-P*, show stage 16/17 transgenic embryos (*N* and *O*) and a CNS prepared from a transgenic 3rd instar larva (*P*). The transgene carries a 2 kb upstream genomic element that confers expression resembling the endogenous distribution of the *nep4* protein in the central nervous system as shown by colocalization with NEP4-immunostainings (*N*). NEP4 expression starts during embryogenesis (*E*, *K*, *N*, *O*), is maintained throughout larval stages (*P*) and still present in adults (not shown).

From stage 14 to stage 17, expression of NEP4 is seen in cells of the central nervous system (Fig. 4E, K). Double stainings for NEP4 and Repo (Reversed polarity), which is specifically expressed in all glia cells of the nervous system (Halter et al., 1995), demonstrated NEP4 expression in glia cells in the embryonic and larval CNS but not in neuronal cells (Fig. 5S). Further colocalization studies with antibodies labeling individual subsets of glia cells, including anti-Prospero (Doe et al., 1991) (Fig. 5R) revealed that NEP4 is present in three major types of glia cells. These are the cell body glia, the lateral glia cells, which are partially marked by Prospero and the medial intersegmental nerve root glia (Fig. 5R, S (Beckervordersandforth et al., 2008)). A reporter line expressing GFP under the control of the native *nep4* promoter element confirmed the expression of NEP4 in glia cells (Fig. 4N-P) and furthermore revealed persistent expression in the larval, pupal and adult central nervous system (Fig. 4P, pupal and adult stages not shown). Control stainings with anti-NEP4 antibodies showed an overlap between the immunosignal and GFP expression (Fig. 4N). It should be noted, that the identified regulatory region of the *nep4* gene recapitulates the neuronal expression of endogenous NEP4 but lacks the regulatory elements for muscle and pericardial gene expression. Anti-NEP4 staining and *in situ* hybridization on the CNS of 3rd instar larva substantiate the observed reporter expression (Fig. 4F, L). Western blots on protein extracts isolated from adult heads confirmed the expression of both isoforms in the adult brain (Fig. 3B).
In addition to the described tissue specific expression of NEP4 in pericardial, muscle founder and glia cells, NEP4 is also obvious in gonads of late embryonic stages (Fig. 4I, arrow). The NEP4 signal in embryonic gonads is apparent in about 50% of embryos of that stage, indicating sex specificity. Previous data from genome wide microarray approaches already provided evidence for a sex specific NEP4 expression (McIntyre et al., 2006). Additionally, *nep4 in situ* hybridization experiments conducted on adult testes as part of the FlyTED-project (www.fly-ted.org) corroborate a potential role for NEP4 in reproduction. Therefore, we performed NEP4 immunostainings and *in situ*-hybridizations on adult ovaries and testes. While we did not detect NEP4 in ovaries (not shown), we found strong expression in adult testes, where NEP4 is located in early spermatocytes to late stage spermatids. At the apical tip of the testis NEP4 was detected in mitotically amplifying gonial cells and was still present postmeiotically in early spermatids, as well as in mature sperm, as indicated by a strong NEP4 immunosignal in seminal vesicles (Fig. 4M). To ascertain the isoform distribution in this tissue, we did Western blots with abdominal protein preparations isolated from males and females, respectively. Consistent with the absence of immunostainings in ovaries, female abdominal preparations did not harbor any *nep4* protein in detectable amounts. In male preparations however, a strong expression of isoform A was apparent, while the smaller isoform B was not detectable (Fig. 3B). This result demonstrates a negligible function for soluble NEP4 in testes.

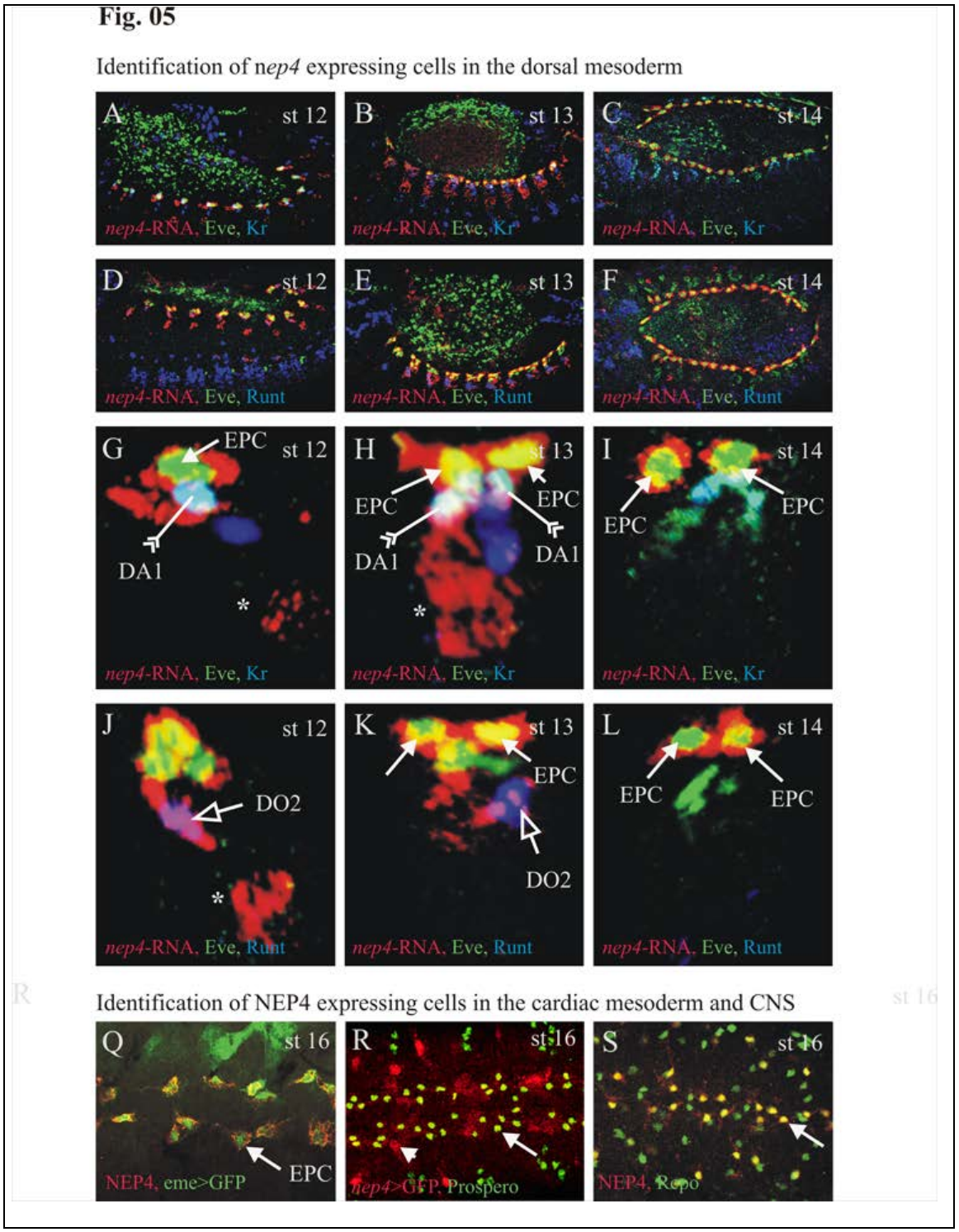

Fig. 5. Colocalization analysis reveals the identity of NEP4 expressing cells. Triple staining either for *nep4*-RNA/anti-Eve/anti-Krüppel (*A* and *G*) or *nep4*-RNA/anti-Eve/anti-Runt (*D* and *J*) reveals *nep4* expression in Eve-positive pericardial cells (arrows), in muscle founder DA1 (double arrows), muscle founder DO2 (black arrows) and an unidentified muscle founder (asterisk). This expression pattern is also seen at stage 13 (*B, E, H* and *K*). Shortly later, at stage 14 (*C, F, I* and *L*), *nep4* expression diminishes in muscle founder cells but is maintained in the Eve-positive pericardial cells (see arrows in *I* and *L*). *Q* shows a late stage 16/17 embryo, dorsal view. Double staining for

eme-driven eGFP and anti-NEP4 reveals that NEP4 is strongly restricted to the Eve-positive pericardial cells population. Colocalization studies with anti-Prospero (*R*) and anti-Repo (*S*) antibodies identify the cell body glia (*S*, arrow), the lateral (*R*, arrow) and the medial intersegmental nerve root glia (*R*, arrowhead) to be NEP4 positive.

Heterologously expressed NEP4 exhibits distinct substrate specificities

In order to assay the enzymatic activities of NEP4, we expressed isoform A in SF21 cells, isolated the membranes and tested them for protein expression. As expected, Western blots confirmed the presence of a considerable amount of *nep4A* protein in the membrane fraction. However, in untransfected control cells a protein of about the same size but considerably less abundance was detected as well (not shown), which might be an indication for the presence of endogenous NEP4 in this cell type. This observation makes it difficult to distinguish between endogenous and heterologous neprilysin activity and clearly renders SF21 cells problematic for heterologous NEP4 expression. For this reason we used *E. coli* as expression system and performed activity assays with purified *nep4* protein and different peptides as putative substrates (Fig. 6). These peptides included tachykinin, substance P, bradykinin, angiotensin I and the pigment dispersing factor (PDF). The first two, both belonging to well-characterized groups of neuropeptides, were chosen because of the strong NEP4 expression in the central nervous system. This expression pattern points to neuropeptides as potential substrates. Bradykinin and angiotensin I were chosen because of their known relevance to act as tissue hormones in several vertebrate species. PDF was previously discussed to be a putative substrate of *Drosophila* neprilysins (Isaac et al., 2007) and was therefore included in the activity assay.

It appeared that especially the peptides substance P (85.8% of degradation) and angiotensin I (51.6%) were cleaved quite efficiently in a time dependent manner (Fig. 6C) while the remaining peptides were either cleaved to a much lesser extent (PDF, 27.8%) or not at all (bradykinin, tachykinin, Fig. 6A). With respect to the main substrate, substance P (RPKPQQFFGLM), the decreasing amount of undegraded peptide exhibited a linear correlation with increasing abundance of two cleavage products: a major one, lacking three amino acids (RPKPQQFF) and a minor one lacking two amino acids (RPKPQQFFG) at the C-terminus. These data demonstrate that hydrolysis occurs predominantly at the Phe^{8}-Gly^{9} and, to a lesser extent, at the Gly^{9}-Leu^{10} bond and therefore adjacent to bulky hydrophobic residues, a preference that is shared by many mammalian peptidases (Turner et al., 2001). The latter site of hydrolysis is also reported for human neprilysin and neprilysin 2 (Rose et al., 2002), while hydrolysis between Phe^{8}-Gly^{9} represents a cleavage site not reported previously. These cleavage characteristics were substantiated by results from hydrolysis assays performed with NEP4A expressing SF21 cells. Identified peptide substrates (substance P and angiotensin I) added to SF21 cell membrane preparations were degraded rapidly in samples from infected cells but also from noninfected control cells. In both cases we detected the cleavage fragments mentioned above. However, relative cleavage activity was enhanced by 30% in membranes from infected cells compared to control cells and the amount of resulting peptide degradation products increased correspondingly (not shown). Thus, the data obtained by SF21 expression confirmed the activities and specificities already measured with the purified protein from *E. coli*.

With respect to the typical inhibitors of neprilysins, phosphoramidon and thiorphan, we could show that these have only limited capacity to inhibit NEP4. Apparently, inhibitor concentrations have to be in high micromolar ranges (100µM) to reduce enzyme activity significantly. However, even at this concentration, inhibition rates did not exceed 20-30% (Fig. 6D). Again in accordance with purified *nep4* protein expressed in *E. coli*, the addition of the respective inhibitors to SF21 membranes containing heterologously expressed NEP4 hampered protein activity at comparable rates (not shown). Noteworthy, this reduced susceptibility to phosphoramidon and thiorphan was also shown for human NEP2.

In terms of pH dependence, NEP4 characteristics are similar to those of other neprilysins. As shown in Fig. 6B, the highest catalytic efficiency can be measured in the neutral range (pH 7) with a dramatic reduction in activity above pH 8 and below pH 6.

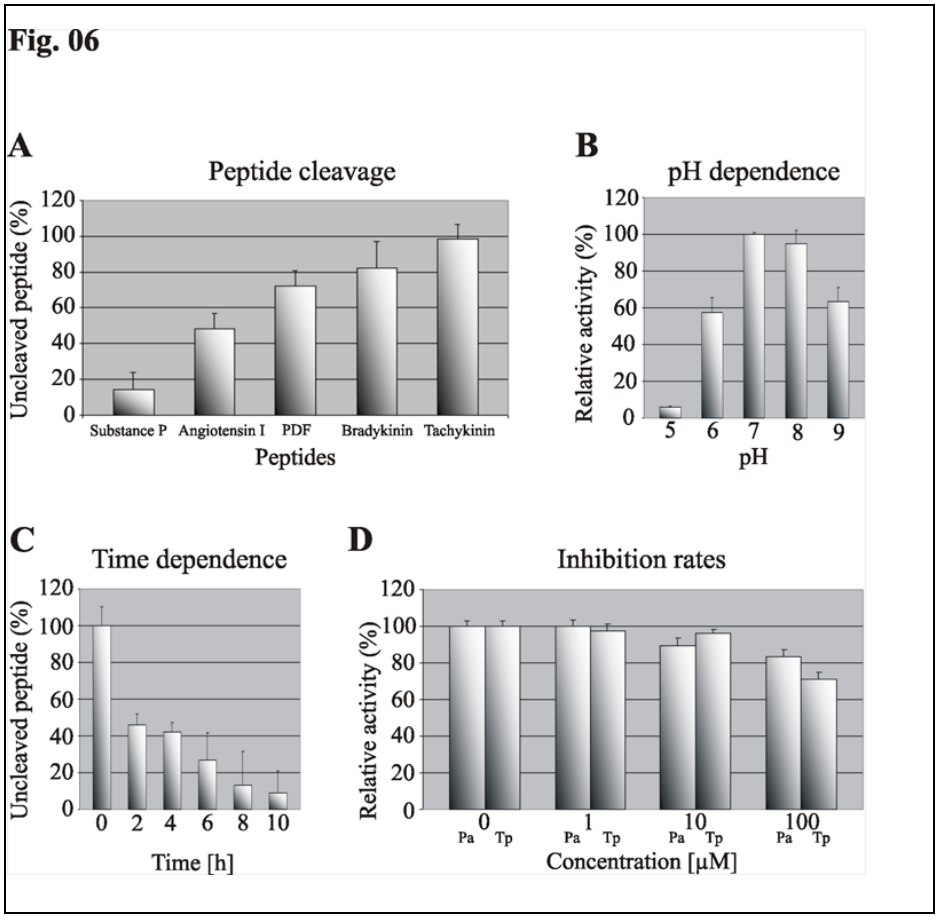

Fig. 6. Cleavage activity and enzymatic properties of NEP4. *A*, hydrolysis of different peptides catalyzed by NEP4. Rates were determined by HPLC to measure the decline in peptide amount after 10 hours of incubation with GST-NEP4 at pH 7. The amounts of uncleaved peptide present after 10 hours of incubation with purified GST as a control are considered as 100%. *B*, pH profile of NEP4 dependent peptide hydrolysis. The relative activity for angiotensin I hydrolysis by NEP4 was determined at different pH-values. The maximum rate of peptide degradation was considered as 100%. *C*, time dependence for peptide hydrolysis catalyzed by NEP4. Rates were determined by HPLC to measure the decline in peptide amount during incubation with NEP4. The amount of uncleaved peptide present prior to addition of NEP4 is considered as 100%. *D*, inhibition of NEP4 dependent peptide hydrolysis by phosphoramidon (Pa) and thiorphan (Tp). Inhibition rates of phosphoramidon and thiorphan were generated by measuring the degradation of angiotensin I in the presence of different concentrations of inhibitors. Data are expressed relative to uninhibited activity. In either case, values represent the means + s. d. of at least three independent determinations.

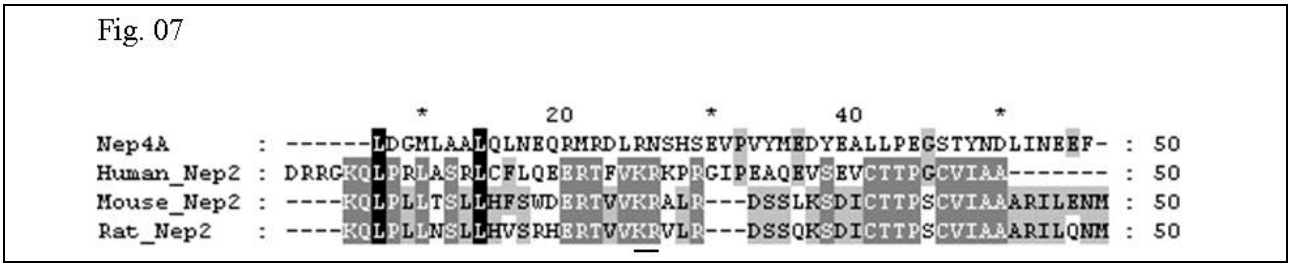

Fig. 7. Sequence alignment of amino acids following the transmembrane domains of *Drosophila* NEP4A compared to human, mouse and rat NEP2. *Drosophila* NEP4A does not harbor a characteristic Lys-Arg motif (black bar) which

is believed to be the site of proteolytic cleavage in mammalian NEP2. The respective transmembrane domains were predicted with TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0); the alignment was done with MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html).

Discussion

Neprilysin 4 exhibits enzyme characteristics similar to mammalian neprilysin 2.

A comparison of the enzymatic properties of neprilysin 4 from *Drosophila* with those of other neprilysins from different species reveals a clear analogy to mammalian neprilysin 2. Intriguingly, murine NEP2 was recently reported to be involved in sperm formation and embryonic development (Carpentier et al., 2004), two physiological functions that are likely to be shared by *Drosophila* NEP4, as the described expression pattern (Fig. 4 and 5) suggests such a functionality. Especially the expression in embryonic dorsal muscle founder and pericardial cells renders an involvement in developmental processes rather likely. However, further investigation is necessary to ascertain this issue properly. A remarkable similarity between *Drosophila* NEP4 and human NEP2 is the limited susceptibility to phosphoramidon and thiorphan, inhibitors that were shown to be effective against many neprilysins. Thiorphan in particular has frequently been used to inhibit neprilysin-like activity and is reported to be neprilysin specific at nanomolar concentrations (Turner et al., 2001). The observation that in contrast to human NEP, human NEP2 (Whyteside and Turner, 2008) but also *Drosophila* NEP4 (Fig. 6D) are relatively resistant against these two inhibitors, could be an indication for similarities in the structure of their active sites. Albeit distinct differences in substrate specificity and inhibitor sensitivity (Whyteside and Turner, 2008), a comparison of the amino acid residues that line the hydrophobic pockets of human NEP (Oefner et al., 2000; Oefner et al., 2004) and human NEP2 (Whyteside and Turner, 2008) showed identical ligand binding S1´ and S2´ subsites (table 1). NEP4 from *Drosophila* and human NEP2 on the other hand revealed considerably less homologies (S1´ subsite: five conserved residues, S2´ subsite: no conserved residue, table 1). Nevertheless, the two enzymes exhibit in addition to comparable inhibitor susceptibilities quite similar substrate specificities. In line with *Drosophila* NEP4 (this work), the main substrates cleaved by human NEP2 are substance P and angiotensin I (Whyteside and Turner, 2008). The fact that human neprilysin and neprilysin 2 despite completely conserved subsite residues exhibit major differences in their specificities clearly indicates that other residues than those mentioned above are responsible for regulating access to the catalytic center. Modelling of the active site of rat NEP2 recently proposed two additional critical residues (Ser-133 and Leu-739 (Voisin et al., 2004)) that are present in rat NEP2 whereas human NEP harbors glycines at the respective positions. Sequence alignments (http://bioinfo.genotoul.fr/multalin/multalin.html) indicate that *Drosophila* NEP4 shares one glycine (Gly-1005) with human NEP at the respective position, while the second glycine is replaced by Glu-416 in *Drosophila* NEP4 and Ser-133 in the case of rat NEP2. As glutamate as well as serine are, contrary to glycine, well known to be involved in hydrogen bond formation with the protein backbone and thereby stabilize the protein structure, this position could be of particular importance to substrate access. The identification of specific inhibitors, that act equally potent on both proteins, human NEP2 and *Drosophila* NEP4, would be a strong support for the hypothesis of structural relations between these two peptidases. Unfortunately, in neither case such an inhibitor is currently known.

With respect to other neprilysins from *Drosophila*, only neprilysin 2 (NEP2) has been characterized so far (Bland et al., 2006; Thomas et al., 2005). Contrary to *Drosophila* NEP2, *Drosophila* NEP4 does not cleave the peptide tachykinin from *Locusta migratoria*, demonstrating considerably different substrate specificities and thereby physiological functions within the *Drosophila* neprilysin family. Especially interesting in this context is the fact that *Drosophila* NEP2 and NEP4 are partially expressed in the same tissues. As mentioned, *Drosophila* NEP2 is expressed in the testis (Thomas et al., 2005), which is also true for *Drosophila* NEP4 (Fig. 4M). The obvious difference in substrate specificity together with

human NEP		human NEP2		*Drosophila* NEP4	
$S_1´$	$S_2´$	$S_1´$	$S_2´$	$S_1´$	$S_2´$
Phe-106	Arg-102	Phe-139	Arg-135	Thr-418	Pro-414
Ile-558	Phe-106	Ile-588	Phe-139	Ile-847	Thr-418
Phe563	Asp-107	Phe593	Asp-140	Phe-852	Lys-419
Met-579	Arg-110	Met-609	Arg-143	Val-868	Ser-422
Val-580		Val-610		Val-869	
Val-692		Val-722		Val-921	
Trp-693		Trp-723		Trp-922	

Table 1. Comparison of amino acid residues that line the hydrophobic pockets of human NEP, human NEP2 and *Drosophila* NEP4, respectively. While the critical amino acids in the ligand binding S1´ and S2´ subsites are identical between the human proteins, *Drosophila* NEP4 exhibits variations especially in the composition of its S2´ subsite. Correspondent amino acids were identified by sequence alignments (MultAlin, http://bioinfo.genotoul.fr/multalin/multalin.html).

their expression in the same tissue indicates a physiological requirement for highly specialized peptidases with individual substrate specificities. Although further investigation is necessary to ascertain this issue in more detail, the data presented in this study allow a direct comparison between two neprilysins from *Drosophila melanogaster* for the first time.

Noteworthy, former work with *Drosophila* head membranes already introduced an enzyme with neprilysin-like activities but low susceptibility to phosphoramidon (Isaac et al., 2002). Although the identity of this enzyme was not elucidated, it represented first evidence for the existence of neprilysin-like peptidases in *Drosophila* that are relatively resistant to common neprilysin inhibitors.

Isoform specific solubility is quite unusual among neprilysins

Neprilysins are generally considered to be membrane bound proteins (Turner et al., 2001). However, in individual cases, the existence of soluble neprilysins has been reported (Ikeda et al., 1999; Thomas et al., 2005), which apparently become soluble by proteolytic cleavage of a membrane bound precursor protein.

Our data demonstrate that neprilysin 4 from *Drosophila melanogaster* exists as both, a membrane bound and a soluble protein. In contrast to other neprilysins however, the solubility apparently depends on alternative splicing and corresponding protein biosynthesis instead of posttranslational proteolytic cleavage. In addition to the verification of transcript specific mRNAs (Fig. 2A), the fact that the NEP4A sequence does not contain a prohormone-convertase recognition site (Lys-Arg,) close to the transmembrane domain (Fig. 7) further contradicts the possibility of proteolytic release of isoform B from membrane bound isoform A. This recognition site is considered to be essential for proteolytic processing of neprilysin 2 in mammals (Ikeda et al., 1999). Albeit this difference, the mere existence of both, membrane bound and soluble NEP4 is quite unusual among neprilysins and bears potentially strong physiological relevance. Based on the current data, we expect a scavenger function for the soluble isoform that regulates peptide homeostasis in the hemolymph and in the nervous system independently of the more stationary membrane bound isoform. However, further studies on this issue are hindered by the fact that both isoforms share an almost identical structure (Fig. 1), which makes it difficult to distinguish between them. Efforts to utilize a *nep4A* specific RNA-probe for *in situ* detection of the corresponding mRNA in embryos were successful, however, while these data represent good evidence for the presence of transcript A in the embryonic nervous system, somatic muscle founders and the EPCs, the additional expression of

transcript B can be measured only indirectly by the stronger *in situ* signal generated by a probe against both transcripts (Fig. 4E). Nevertheless, together with a Western blot that shows expression of isoform B in embryonic tissues (Fig. 2C) we come to the conclusion that both splice variants are present in the central nervous system during embryogenesis indicating distinct physiological relevances, which are presumably unique to the respective isoforms. This interpretation is corroborated by the expression of both isoforms in the adult nervous system as shown by Western blot (Fig. 3B).

With respect to physiological functions, a potential *in vivo* relevance can be attributed especially to protein presence in the central nervous system. NEP4 expression in this tissue begins during embryogenesis and is maintained until adult stages, demonstrating a permanent role for neprilysin-like endopeptidases. As shown previously, members of the neprilysin family are responsible for terminating the actions of neuropeptides like enkephalins (Malfroy et al., 1978; Schwartz et al., 1980) or tachykinins (Barnes et al., 1993; Matsas et al., 1983) on neuronal surfaces and especially in the peri-synaptic region. A similar activity can be assumed also for *Drosophila* NEP4 as its potential to degrade neuropeptides (Fig. 6), together with a highly specific expression in glia cells (Fig. 5R,S) strongly indicate such a physiological relevance. This hypothesis is further corroborated by the general functions of the glia cell subtypes NEP4 is expressed in: while cell body associated glia are structurally similar to mammalian astrocytes (Freeman and Doherty, 2006), lateral glia cells are similar to oligodendrocytes (Stork et al., 2008), with both mammalian cell types reported to be responsible for ion and neurotransmitter homeostasis (Mentlein and Dahms, 1994; Vilijn et al., 1989; Stacey et al., 2007). As no system responsible for the reuptake of neuropeptides at the nerve terminal is known in *Drosophila*, the biological activity of these transmitters is presumably controlled by extracellular degradation. In this context, glia cells and corresponding peptidases might play a decisive role in modulating secreted peptides present in the extracellular spaces of the CNS. While the majority of this processing is presumably accomplished by membrane bound peptidases like NEP4A, the identification of a soluble NEP4 isoform expressed at least in the adult nervous system (Fig. 3B) is a strong indication for the additional requirement of soluble endopeptidases. Due to an efficient insulation of the nervous system that allows a fine tuned homeostasis of ions, peptides and other small molecules (Stork et al., 2008) soluble NEP4 secreted from glia cells is likely to remain within the nervous system instead of diffusing into the hemolymph.

Independent of the isoform distribution, the mere expression of NEP4 in testes (Fig. 4M) suggests an involvement in reproductive physiology. A potential role in reproduction has already been stated for *Drosophila* NEP2 which is also expressed in testes (Thomas et al., 2005) and shown for mammalian peptidases, where male mice lacking germinal angiotensin-converting enzyme reveal strongly impaired fertility, while female knockouts behave like wildtype (Krege et al., 1995). This sex-specific relevance can also be anticipated with respect to *Drosophila* NEP4 as protein expression was found to occur only in male but not female reproductive organs (Fig. 4M, 3B). The result that in testes the membrane bound isoform of NEP4 is expressed (Fig. 3B) together with the apparently soluble neprilysin 2 (Thomas et al., 2005) is another indication for distinct but concerted functions of different neprilysins in certain tissues.

With respect to the expression in even-skipped positive pericardial cells (EPCs), a possible physiological relevance for NEP4 could be the processing or degradation of signals sent from the pericardium to the dorsal vessel. Recently it was shown, that the heart function in *Drosophila* is likely to be modulated non-autonomously by secreted molecules from neighbouring cells, eventually the EPCs (Buechling et al., 2009). The central position of the pericardial cells together with the capability of NEP4 to hydrolyze the tissue hormone angiotensin I (Fig. 6A) furthermore suggests that NEP4 expressed in EPCs might be responsible for the homeostasis of different signaling peptides circulating the hemolymph and passing the heart of *Drosophila* with the respective pericardial cells functioning as a checkpoint for peptide clearance. Such a physiological function would be the first ever attributed to this cell type. Indeed, in addition to the apparent occurrence of *nep4A* mRNA in EPCs, at high magnifications a plasma membrane bound immunosignal is visible in these cells, strongly indicating expression of isoform A. In addition to this signal, tiny round shaped structures are also stained, presumably vesicles of the secretory pathway containing NEP4A (not shown). Due to the fact that the applied antisera do not discriminate between NEP4A and NEP4B, we obviously cannot exclude the possibility that the stained vesicles also contain isoform B which could eventually be exocytosed and serve its purpose as a soluble enzyme in the

hemolymph. In any case, based on the current data we propose that NEP4 expression in the pericardial cells is presumably required for the homeostasis of signaling peptides circulating the hemolymph of *Drosophila*.

A noteworthy observation is the transient expression of at least NEP4A in a particular set of dorsal somatic muscle founders. Muscle founders are crucial for the formation of the somatic body wall musculature and a pivotal step during myogenesis is myoblast fusion, a process that is initiated by the muscle founder cells. Expression of NEP4A in founder cells rather than in multinucleated myofibers indicates an early role for the protein in these cells. One possibility is, that all NEP4A expressing cells in the dorsal mesoderm, muscle founders and EPCs, are involved in peptide clearance. On the other hand, it could be that the enzyme harbors a specific but yet unknown function for myogenesis. However, future investigations on a specific neprilysin 4 mutant will be crucial for the identification of physiological processes NEP4 is involved in.

Footnotes

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The abbreviations used are: BSA, bovine serum albumin; CNS, central nervous system; eGFP, enhanced green fluorescent protein; EPC, even-skipped positive pericardial cell; EST, expressed sequence tag; Eve, even-skipped; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranosid; Kr, Krüppel; NEP, neprilysin; Pa, phosphoramidon; PBS, phosphate buffered saline; PBT, PBS containing 0,1% Tween 20; PDF, pigment dispersion factor; Repo, reversed polarity; RT, reverse transcription; s. d., standard deviation; Tp, thiorphan

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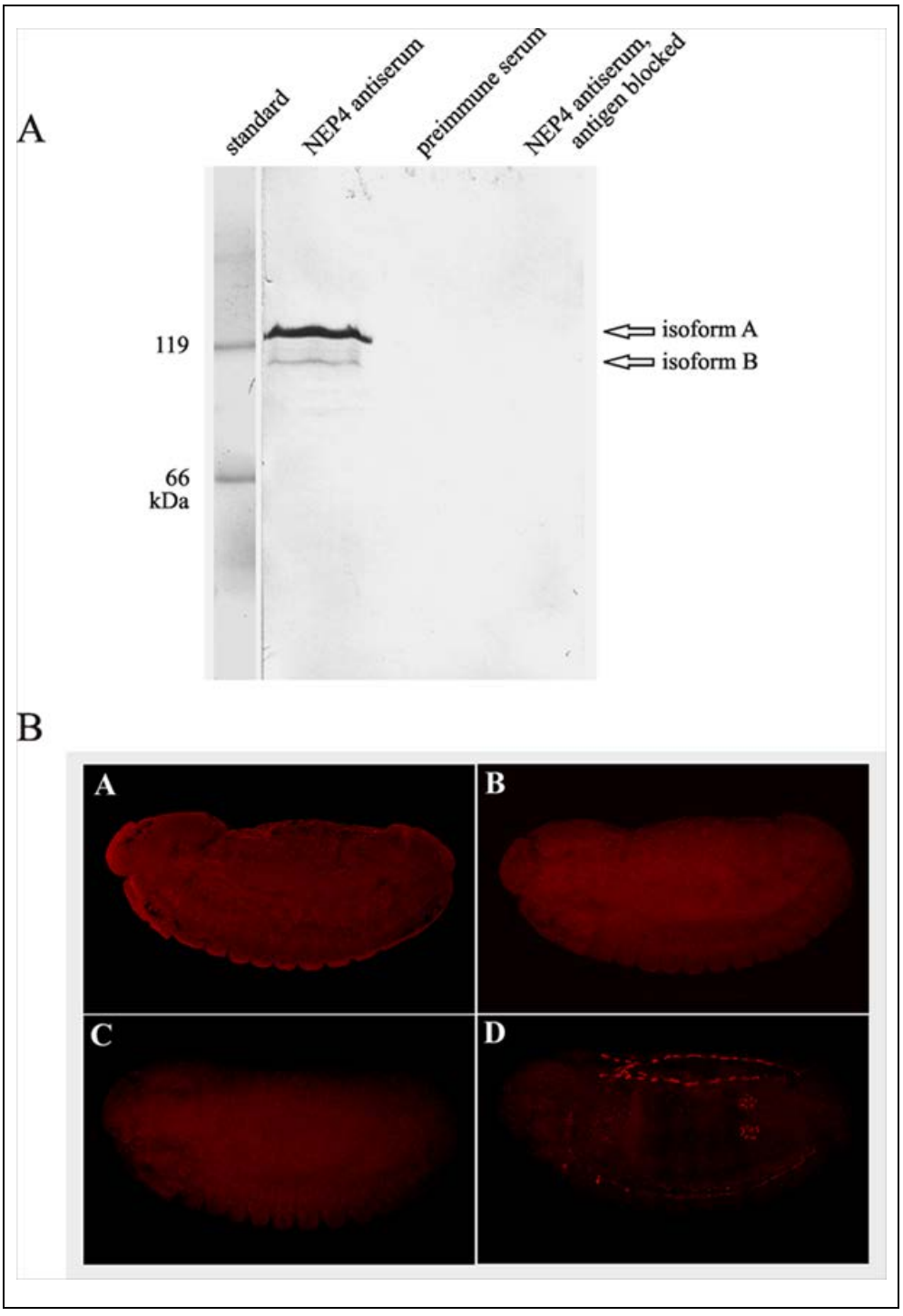
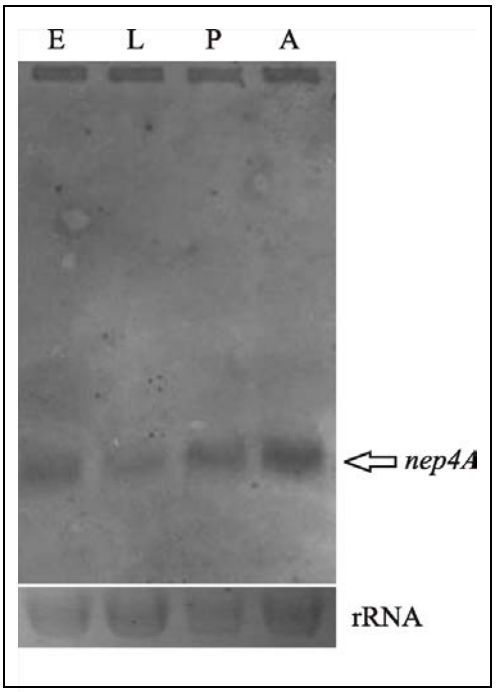


Supplemental Fig. 1. Demonstration of antibody specificity. *A*, Western blot of adult *Drosophila* protein preparations probed with NEP4 antiserum or control sera. While the application of preimmune serum or NEP4 antiserum preincubated with the antigen used for immunization do not yield any immunosignal, both isoforms are detected by the untreated antiserum (arrows). *B*, immunostainings of late staged *Drosophila* embryos. A distinct staining is visible only in embryos treated with the NEP4 antiserum (*D*). Control stainings with preimmune serum (*A*), secondary antibody only (*B*) or NEP4 antiserum preincubated with the antigen used for immunization (*C*) do not show any signal.



Supplemental Fig. 2. Northern blot probed with antisense riboprobes raised against a coding region which is unique to *nep4A*. The bottom panel shows part of the radiant red stained gel with ribosomal RNA (rRNA) visible to demonstrate the loading of comparable RNA amounts (15µg / lane). *nep4A* transcripts are detectable in embryonic (E), 3rd instar larval (L), pupal (P) and adult (A) RNA preparations.

3.3. A novel role for the non catalytic intracellular domain of Neprilysins in muscle physiology

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Running title: Neprilysin 4 is crucial for muscle integrity

Key words:
Drosophila melanogaster, metalloendopeptidase, muscle degeneration, muscle integrity, peptide metabolism

Abstract

Neprilysins are membrane bound M13-endopeptidases responsible for the inactivation and/or activation of peptide signaling events on cell surfaces. By hydrolyzing their respective substrates, mammalian neprilysins are involved in the metabolism of numerous bioactive peptides especially in the nervous, immune, cardiovascular and inflammatory systems. Based on such multifaceted requirements in significant physiological processes, proteins belonging to the family of neprilysins are considered as therapeutic agents as well as targets in different disease patterns, including Alzheimer's disease.
Here we report on the capacity of Neprilysin 4 (Nep4) from *Drosophila melanogaster* to induce a severe muscle degeneration phenotype upon overexpression. While an excess of full length Nep4 in somatic muscles resulted in strong tissue degeneration combined with severely impaired motility of larvae and lethality in late larval development, downregulation of expression mimicked mainly the latter two effects. Endogenous expression in the respective tissue was confirmed by reporter gene expression combined with immunostainings and semi-quantitative PCR. By expressing several mutated and truncated forms of Nep4 in transgenic animals, we identified the intracellular domain to be responsible for the observed phenotype while catalytic activity of the enzyme was apparently insignificant. Yeast two-hybrid screening identified a yet uncharacterized carbohydrate kinase as a first interaction partner of the intracellular domain of Nep4A. Our data represent the first report of an intracellular neprilysin domain being involved in muscle integrity.

Introduction

Neprilysins are type 2 transmembrane proteins that consist of a short cytoplasmic domain, a membrane spanning region and a large extracellular part which contains a highly conserved zinc-binding motif (HExxH) that classifies members of the neprilysin family as M13 zinc metallopeptidases (MEROPS database, http://merops.sanger.ac.uk). Other members of this family are e.g. endothelin converting enzymes (ECE-1, ECE-2), PEX or KELL (reviewed by (Turner et al., 2001)). In general, neprilysins act as ectoenzymes which cleave a multitude of physiologically relevant peptides to activate or inactivate them, and thereby contribute to the maintenance of the extracellular peptide homeostasis. Disruption of this balance may result in various diseases, e.g. hypertension (Molinaro et al., 2002), analgesia (Whitworth, 2003), cancer (Turner et al., 2001) or Alzheimer's disease (Iwata et al., 2000). Therefore, neprilysins are object of different clinical investigations regarding the mentioned pathologies and, in recent years, especially the potential of human neprilysin to cleave the neurotoxic amyloid ß-peptide (Iwata et al., 2000) has attracted great attention. Up to now however, research on neprilysins has focused almost completely on the hydrolytic activities exerted by the enzymes' extracellular part. Hence, the vast majority of data published on the respective peptidases has dealt with these activities and the associated physiological relevance. In contrast, little attention has been given to the intracellular domain. Nevertheless, there is growing evidence that the biological and regulatory effects exerted by neprilysins do not entirely depend on the catalytic processing of peptide substrates. Recent data indicate that interaction of the enzymes´ intracellular domain with cytoplasmic proteins or with the cytoplasmic domain of other membrane resident proteins is apparently also highly relevant for the physiological processes and diseases neprilysins are involved in.

Concerning human Neprilysin (Nep), it was shown to regulate cell migration via mechanisms independent of its catalytic functions. Apparently, the intracellular domain of Nep associates with tyrosine-phosphorylated Lyn kinase which then binds the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) resulting in an Nep-Lyn-PI3-K protein complex. This complex blocks focal adhesion kinase-PI3-K interaction in a competitive manner which eventually inhibits migration of prostate cancer cells (Sumitomo et al., 2000). Furthermore, the intracellular domain of Nep was reported to interact with the tumor suppressor PTEN (phosphatase and tensin homolog), thereby recruiting it to the plasma membrane which in turn increases protein stability and phosphatase activity of PTEN. This interaction ultimately results in a constitutive downregulation of Akt/PKB kinase activity and a suppression of prostate cancer cell growth (Sumitomo et al., 2004).

In *Drosophila*, the existence of four neprilysins was experimentally proven with a fifth being annotated (http://flybase.org) but not verified yet. However, so far no data regarding the respective intracellular termini are available and distinct information on expression patterns or enzymatic activities have been reported only for Neprilysin 2 (Nep2, (Thomas et al., 2005; Bland et al., 2007)), Neprilysin 3 (Nep3, (Meyer et al., 2011)) and Neprilysin 4 (Nep4, (Meyer et al., 2009; Meyer et al., 2011)). We have previously shown that Nep4 is an active peptidase with expression mainly in embryonic heart and muscle cells but also in glia cells of the central nervous system and in male germ cells of embryonic and later stages (Meyer et al., 2009). In the present work we demonstrate that the physiological relevance of Neprilysin 4 is not limited to its function as an active peptidase but that the enzyme's intracellular N-terminus has a certain potential to induce muscle degeneration in *Drosophila* larvae independently of the protein's enzymatic activity. Overexpression of wild type but also of catalytically inactive Nep4A causes a severe muscle degeneration phenotype concomitant with impaired movement and lethality in late larval development. By contrast, expression of truncated Nep4 lacking the complete intracellular domain does not induce any of the phenotypes.

Results

Neprilysin 4 is expressed in larval somatic musculature

While embryonic expression of Nep4 has been analyzed in detail (Meyer et al., 2009; Meyer et al., 2011) expression of the enzyme in post-embryonic stages was characterized only at a preliminary level. Particularly mesodermal expression in stages other than embryonic has remained elusive up to now. In order to ascertain whether mesodermal expression of Nep4 persists also in later stages of development, we raised and analyzed a reporter line that expresses nuclear eGFP under the control of the native mesodermal *nep4* enhancer, thereby mimicking the expression pattern of Neprilysin 4 not only in embryonic but also in later stages of development (Meyer et al., 2011). Utilizing this line, we found reporter gene expression in somatic muscles of *Drosophila* larvae (Fig. 1A), pupae (not shown) and adults (Fig. 1B arrow). Subsequent to identifying somatic muscles as a tissue presumably expressing Neprilysin 4, the validity of expression was confirmed by semi-quantitative PCR using total RNA preparations isolated from 3rd instar larval body wall muscles as templates. To exclude the possibility that the analyzed PCR amplicons had originated from contaminating mRNAs co-isolated from tissues other than muscles, e.g. from neuronal cells in which Nep4 is also expressed (Meyer et al., 2009), in addition to wild type we isolated mRNA also from transgenic animals that express *nep4*-specific hairpin RNA in muscle tissue (Mef-Gal4 driver), thereby reducing the amount of mature *nep4* mRNA exclusively in this tissue. If the obtained amplicons were based on transcripts from contaminating tissues, muscle specific downregulation of *nep4* would have had no effect. The fact that compared to body wall muscles from wild type 3rd instar larvae, considerably less *nep4* coding sequence is amplified from respective *nep4* knock-down muscle preparations is additional strong evidence for the expression of *nep4* in larval somatic muscles. Moreover, as anticipated, overexpression of *neprilysin 4* using the same driver line causes a strong increase in *nep4* transcript abundance (Fig. 1D).

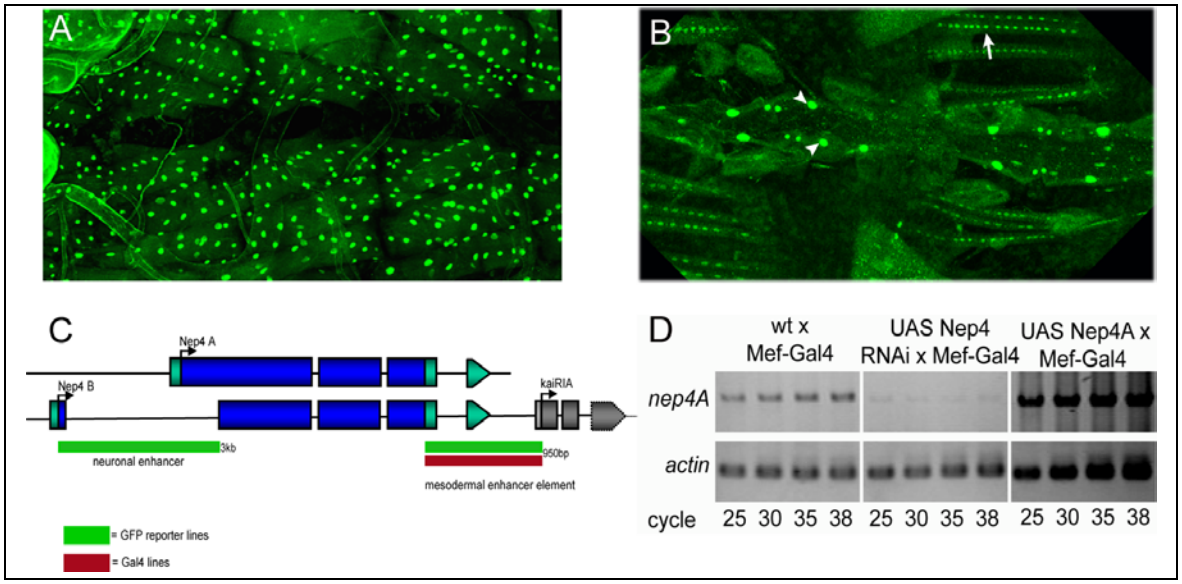


Fig. 1: **Mesodermal expression of Nep4** *A*: The mesodermal *nep4* enhancer element drives eGFP reporter gene expression in body wall muscles of third instar larvae. eGFP was visualized utilizing a GFP antibody. *B*: Adult flies of the respective line display eGFP expression in body wall muscles (arrow) and in certain heart cells (arrowheads) (A, B: magnification 100x). *C*: Schematic overview of *nep4* neuronal (2941 bp) and mesodermal enhancer elements (950 bp), respectively. For the latter element, in addition to GFP reporter lines, Gal4 driver lines were established. *D*: Nep4 transcript is detected in body wall muscles of third instar larvae by semi-quantitative PCR. Samples were taken at PCR cycles 25, 30, 35 and 38, respectively. Muscle specific knock-down of *nep4* results in a considerable reduction in of *nep4* transcript abundance which is significantly increased under overexpression conditions using the same driver line. As a control, *actin* coding sequence was amplified analogously to *nep4*.

To assess the subcellular localization of Nep4, we used a previously described Nep4 specific antibody (Meyer et al., 2009) to stain larval body walls. However, application of this antibody did not result in a pattern distinguishable from background staining, presumably due to a rather weak expression of the protein in this tissue that is still detectable by RT-PCR but not by our polyclonal antibody. To overcome this problem, we raised transgenic animals that express Nep4A fused to a C-terminal HA-tag (Nep4A-HA) under the control of the native *nep4* enhancer. This approach held the advantage that we could detect this protein by applying a monoclonal anti-HA antibody that is expected to have a higher affinity towards its epitope than our Nep4 antibody. Significantly, by using the endogenous enhancer to drive expression, the possibility of overexpression derived mislocalization could be minimized. Furthermore, expression of Nep4A-HA under the control of this enhancer did not induce any apparent phenotype. As shown in Fig. 2A´ staining of larval tissue with the anti-HA antibody resulted in a weak, yet distinct immunosignal that occurred in a repetitive and regular pattern. In addition, strong accumulation around the nuclei was apparent, possibly continuous with the nuclear membrane. In control experiments without primary antibodies the tissue remained completely unstained (not shown). To identify the subcellular compartments Nep4A-HA is expressed in, we performed double labelling experiments with an antibody being specific to the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). As depicted in Fig. 2B´´, HA-tagged Nep4A colocalizes extensively with SERCA at the sarcoplasmic reticulum (SR), especially in membranes continuous with the nuclear membrane.
In addition to somatic muscles, eGFP reporter gene expression was apparent in certain cardioblasts of third instar larvae (not shown) and persisted also in adult flies (Fig. 1B, arrowheads). However, due to a highly challenging preparation, it was not possible to obtain sufficient tissue in order to confirm expression of *neprilysin 4* in these distinct heart cells by semi-quantitative PCR. Therefore, in subsequent experiments we focused on the somatic musculature and the physiological function Nep4 exerts in this tissue.

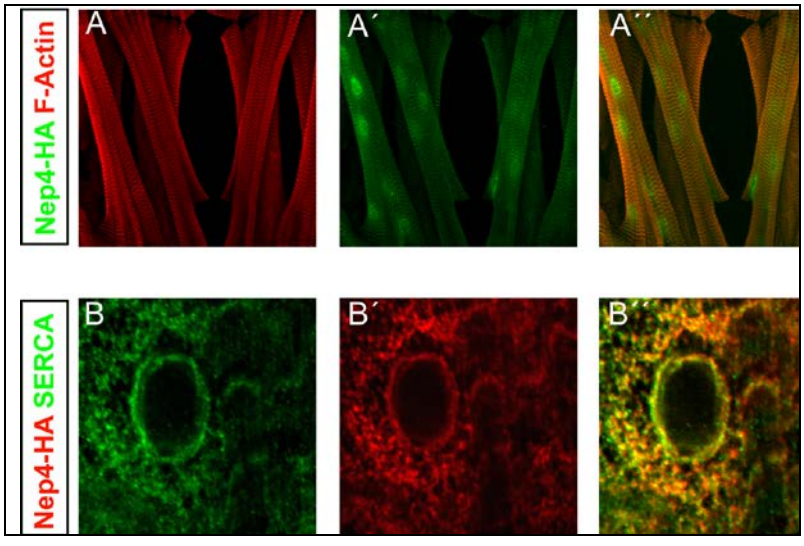


Fig. 2: **Subcellular localization of Nep4 in somatic muscles**
Expression of HA-tagged Nep4A (Nep4A-HA) under the control of the native *nep4* enhancer. *A*: Nep4-HA is detected in somatic muscles of 3rd instar larvae. Strong accumulation around the nuclei, possibly continuous with the nuclear membrane, is apparent (magnification 250x). Larvae were stained for Nep4-HA with an anti-HA antibody (A´) and for F-Actin using phalloidin-TRITC (A). A´´: merge *B*: Detail screen of the musculature of respective larvae stained for Nep4-HA (B´) and SERCA (B) to label the sarcoplasmic reticulum (SR). B´´: merge (magnification 630x). Nep4A-HA colocalizes extensively with SERCA at the SR.

Altering Neprilysin 4 expression levels in somatic muscles severely impairs larval movement
To understand the physiological functions of Nep4 in larval somatic muscles, we used the UAS-Gal4 system to either increase or reduce expression of the enzyme in a tissue specific manner. With respect to overexpression, we generated nine independent UAS-lines harboring full-length Nep4A as a transgene and selected three of them with each one displaying distinct

efficiencies in ectopic Nep4A expression as assessed by Northern and Western analyses: while lines one and two show strong expression of Nep4, line three displays a rather moderate increase in mRNA and protein abundance (Fig. 3A, B). These different expression levels are highly beneficial in identifying dose dependent effects that may remain undiscovered if only one transgenic line or individual lines with similar expression levels were used. Noteworthy, in addition to full length Nep4A a second band of lower molecular mass is labelled by our antibody indicating partial proteolytic digestion of the mature protein (Fig. 3B, asterisk). To realize a muscle specific knock-down of the enzyme, we utilized a *nep4* specific RNAi line and analyzed the effects of *nep4* downregulation. In order to generally classify RNAi mediated knock-down capability of the respective line, expression of hairpin RNA was initially driven by the strong ActinC5-Gal4 driver with high knock-down efficiency being demonstrated by Northern blot (Fig. 3C). In subsequent experiments however, the muscle specific Mef-Gal4 driver was applied.

As depicted in Fig. 4A, both, increasing as well as reducing Nep4 expression levels causes severely impaired larval movement; however, apparently strong overexpression of the enzyme is necessary to manifest this phenotype since only larvae of UAS Nep4A lines #1 and #2 show movement defects (about 55% reduced crawling speed) while larvae of line #3, that evidently expresses less ectopic Nep4A than the other two lines (Fig. 3A, B), show a movement speed comparable to wild type. Since in UAS Nep4A line #1 the strong ectopic expression of Nep4A causes second instar larval lethality, all measurements were done with larvae of this respective stage. RNAi mediated downregulation of *nep4* expression also causes a reduction in movement speed. Larvae of this genotype move approximately 38% slower than wild type controls (Fig 4A). To ascertain if the observed reduction in crawling speed is caused by weaker muscle contractions, by fewer contractions, or by the combined effect of both factors, we also counted the number of contractions the individual larvae perform during a straight run of 10 seconds. As shown in Fig. 4B, with respect to overexpression the results of this assay are similar to the previous approach with the lines #1 and #2 that show very strong Nep4A expression, displaying the fewest contractions. However, while crawling speed in these two lines is reduced by approximately 55% compared to wild type, the number of contractions is reduced by only about 28% which indicates that the overall reduction in crawling speed is caused by a combination of fewer and of weaker contractions. On the other hand, *nep4* knock-down larvae display an even stronger effect than the overexpression lines. Compared to wild type, animals with reduced *nep4* expression levels exhibit about 32% fewer contractions during a straight run of 10 seconds (Fig. 4B). This observation together with the fact that the overall movement speed of *nep4* knock-down larvae is reduced by only 38% indicates that decreased *nep4* levels predominantly reduce the number of muscle contractions and exert only limited influence on contraction power.

Noteworthy, the depicted effects of Nep4 overexpression are only valid for the membrane bound isoform A. Transgenic animals overexpressing soluble Nep4B in amounts comparable to Nep4A lines #1 and #2 (as confirmed by Western blot, not shown) behave completely like wild type. The same isoform specificity was observed regarding viability: while transgenic flies overexpressing Nep4B develop into adulthood without any apparent phenotype, animals expressing an excess of Nep4A (lines #1 and #2) die at 3rd instar larval stage at the latest. Noteworthy individuals of the weakest Nep4A overexpression line (line #3) developed into adulthood without any apparent delay; however, animals of this transgenic line were unable to fly.

Muscle specific downregulation of *nep4* on the other hand also causes lethality with the respective animals dying not later than pupal stage.

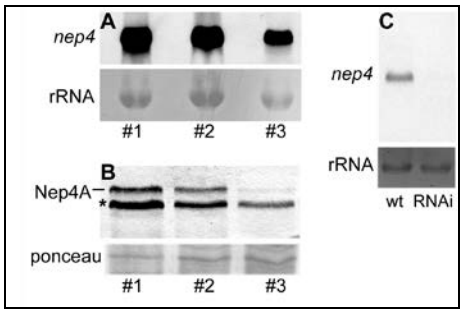


Fig. 3: **Overexpression and knock-down of *nep4* is highly efficient** *A, B*: Individual transgenic UAS Nep4A lines display significant variations in overexpression efficiency if driven by Mef-Gal4. UAS Nep4A line #1 exhibits the strongest, line #2 a medium and line #3 the weakest overexpression level as demonstrated by Northern (A) and Western blot (B). The detection of a second band of less molecular weight in the respective protein preparations (B, asterisk) indicates partial proteolytic digestion of the mature protein. The bottom panel of the respective images shows either part of the radiant red stained gel (A) or part of the Ponceau stained nitrocellulose membrane (B) to demonstrate loading of comparable RNA (15 µg / lane, A) and protein amounts (10 µg / lane, B). *C*: RNAi-mediated knock-down of *nep4* is highly efficient. Knock-down was driven with Actin-Gal4/TM6B, tb. While RNA extracted from F1 tubby shaped pupae was considered as wild type, none tubby pupae corresponded to knock-down animals. *nep4*-specific antisense RNA probes clearly detect *nep4* transcripts in wt, while expression of *nep4* in knock-down pupae is reduced below detection limits. The bottom panel shows part of the radiant red stained gel to demonstrate loading of comparable RNA amounts (15 µg / lane).

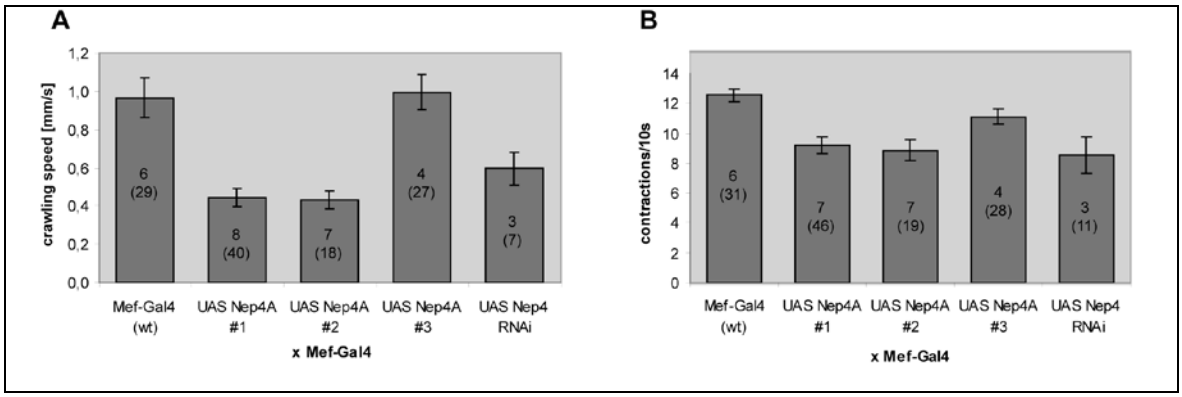


Fig. 4: **Altering Nep4 expression levels severely impairs larval motility** *A*: Crawling speed of wild type (wt), Nep4A overexpressing (UAS Nep4A #1/2/3) and *nep4* knock-down 2nd instar larvae (UAS Nep4 RNAi). Larval speed was monitored individually by calculating the distance covered within a continuous run of 10 sec. Mef-Gal4 was used as a muscle specific driver line for all crossings and also as wt control. For each genotype, the number of individuals tested (upper number) and the number of total runs performed (lower number in brackets) are depicted in the respective bar. For each single individual, at least 2 runs were recorded. Bars represent mean values ± s.e.m. *B*: Number of contractions 2nd instar larvae perform in the course of 10 seconds. Number of individuals and number of runs counted for the respective genotypes are depicted as described in A. Bars represent means ± s.e.m.

Alterations in Neprilysin 4 expression levels cause severe muscle degeneration

Based on the initial observation that both, an excess and a lack of Nep4A in muscle tissue severely impairs larval movement, we analyzed muscle integrity in the respective animals. To exclude the possibility that overexpression of Nep4A interferes with its correct subcellular localization, we initially assessed protein distribution under overexpression conditions by immunostainings. As shown in Fig. 5B´, the subcellular localization of the overexpressed peptidase is consistent with the pattern we observed in animals expressing Nep4A-HA under the control of the native enhancer (Fig. 2A´) with strong accumulation around the nuclei and continuous with the nuclear membrane. Using TRITC-conjugated phalloidin, we could show that overexpression of Nep4A (line #2) causes a strong muscle degeneration phenotype with the myofilaments as well as the T-tubular structure being severely impaired. Apparently, in the affected muscles the Actin-Myosin distribution is disorganized and the I-band pattern characterizing the sarcomeric structure is completely disrupted (Fig. 5B, B´, F). This strong muscle degeneration phenotype is also obvious at the ultrastructural level. As revealed by transmission electron microscopy, muscles of third instar larvae overexpressing Nep4A have completely lost their sarcomeric structure and display a severe disorganization of the Actin-

Myosin filaments (Fig. 6B). In addition, muscles of these animals exhibit a number of morphological changes that are characteristic for necrosis, e.g. nuclei with dispersed chromatin (Fig. 6B´) swollen mitochondria (Fig. 6B´´) and swollen SR (Fig. 6B´´´). By contrast, abnormalities being characteristic for apoptotic processes like condensed mitochondria, fragmented SR or nuclei forming apoptotic bodies were not observed that frequently. In order to assess the specificity of the phenotype, we also performed overexpression studies with Neprilysin 1 (Nep1) from *Drosophila*. Despite significant overexpression of Nep1, as quantified by Western blot, animals of the respective transgenic lines developed into adulthood without any obvious defects (not shown) which indicates that muscle degeneration is elicited highly specifically by Neprilysin 4.
Muscles from animals with reduced *nep4* expression on the other hand showed considerably less severe effects compared to tissue from Nep4A overexpressing larvae. In fact, phalloidin stainings of body wall muscles from *nep4* RNAi animals did not reveal any apparent phenotype (not shown). In these animals, Actin distribution as well as T-tubular structure (Fig. 5G) appears to be comparable to wild type. At the ultrastructural level however, some nuclei with dispersed chromatin as well as swollen mitochondria are apparent (Fig. 6C´, C´´), again indicating ongoing necrotic processes, yet to a far lesser extent. In contrast to overexpression conditions, SR appeared to be normal (Fig. 6C´´´). Features characteristic for apoptosis were not observed at all in the respective muscles. These data demonstrate that primarily increasing but also reducing Neprilysin 4 expression levels in larval somatic musculature result in muscle degeneration and indicate that this phenotype is predominantly based on necrotic rather than apoptotic processes. This indication is further confirmed by TUNEL-assays which showed that neither increasing nor reducing *nep4* expression levels results in significant formation of apoptotic nuclei (supp. Fig. 1).

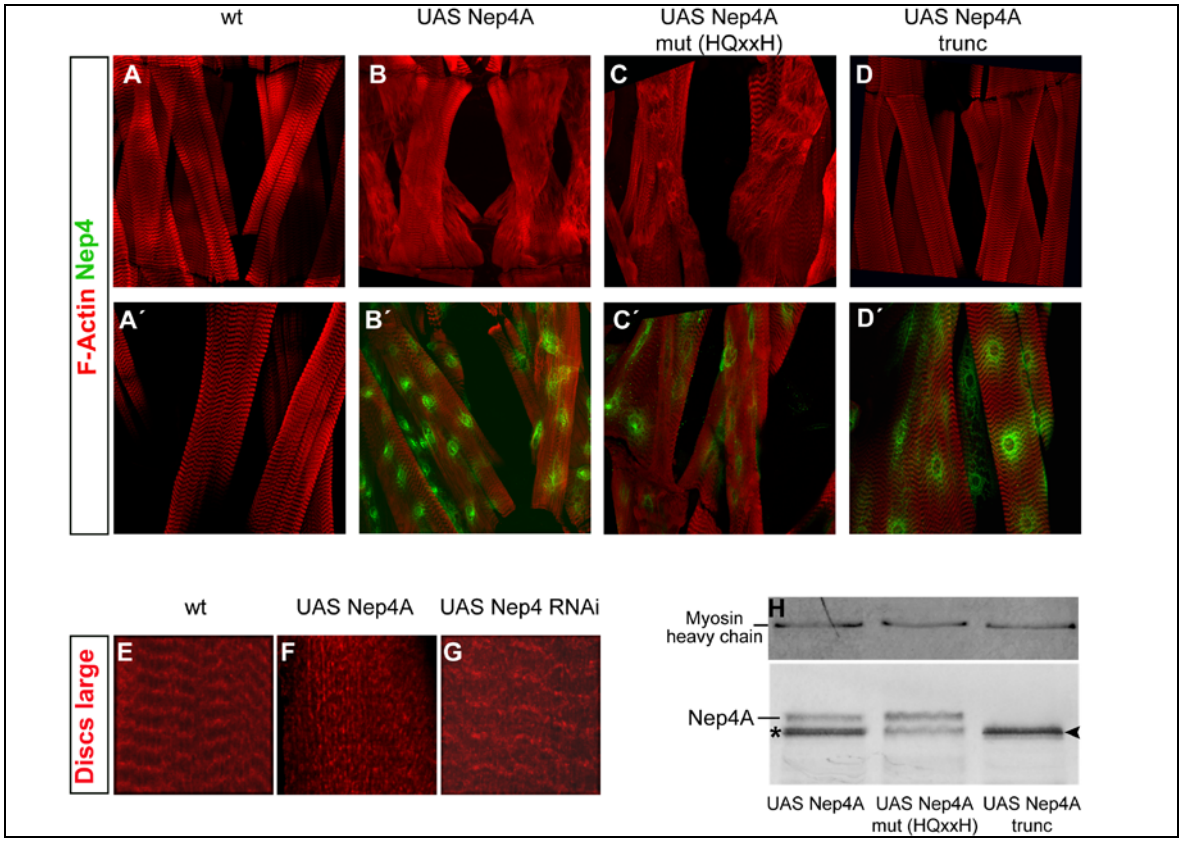


Fig. 5: **Altering Nep4 expression levels severely impairs muscle integrity** Body wall muscles of transgenic 3rd instar larvae were stained with phalloidin-TRITC to label filamentous Actin (red) and with anti-Nep4 antibodies (green) to visualize expression of the protein (A-D´). Anti-Discs large antibodies were applied to label T-tubular structure (E-G). In addition to driving expression of the respective constructs, the Mef-Gal4 driver line was also used as wt control. In the latter line F-Actin is distributed in a regular pattern while endogenous Nep4 can not be detected by the antibody, presumably due to a weak expression level (A, A´). Muscle specific overexpression of wild type Nep4A (UAS Nep4A, line #2) results in muscle degeneration and a loss of sarcomeric structure. Strong accumulation of ectopically expressed Nep4A around the nuclei, continuous with the nuclear membrane is apparent (B, B´). T-tubular structure also appears to be disordered (F) compared to wt (E). Ectopic expression of mutated, catalytically inactive full length Nep4A induces the same phenotype with severely disarranged muscles (C, C´). By contrast, muscles expressing truncated Nep4A that lacks its intracellular domain look completely like wild type (D, D´). Significantly, the subcellular localization of truncated Nep4A is apparently identical to the localization of overexpression constructs that induce the phenotype (B´, C´, D´). In contrast to overexpression of Nep4A (B, F), muscle specific knock-down of the protein does not induce neither Actin (not shown) nor T-tubular structure misarrangements (G). Images A-D collected at 200x, and A´-D´ at 250x magnification. Detail screen of E-G was generated from pictures of 250x magnification. *H*: The extent of construct specific overexpression by the individual transgenic lines was assessed by Western blot. The respective lines display comparable levels of ectopic protein expression. Concerning the lines expressing either full length Nep4A (UAS Nep4A) or a catalytically inactive full length construct (UAS Nep4A mut), in addition to Nep4A a second band is detected by the Nep4 antibody (asterisk), indicating partial proteolytic digestion of the mature protein. Regarding the truncated protein (UAS Nep4A trunc) strong expression with no indications of proteolytic degradation is obvious (arrowhead). Myosin heavy chain was used as loading control (10 µg / lane).

Muscle degeneration is independent of enzymatic activity but caused by the intracellular domain of Neprilysin 4A

To understand the physiology of the observed muscle degeneration we analyzed whether or not enzymatic activity of Nep4 is responsible for the apparent phenotype. In the course of these experiments we show that overexpression of an inactivated Nep4A that holds a glutamine instead of an essential glutamate (E873) in the zinc binding domain of the enzyme causes an identical degeneration phenotype as overexpression of the wild type peptidase (Fig 5C, C´). To obtain further evidence that muscle degeneration is not associated with enzymatic activity we also expressed mutated versions of Nep4A that held phenylalanins instead of the two zinc-coordinating histidins in the conserved HExxH motif (supp. Fig. 2). Since overexpression of these constructs did, like the Glu / Gln exchange mutant, elicit the same muscle degeneration phenotype that was obvious in larvae overexpressing wild type Nep4A (not shown), we conclude that enzymatic activity is not the major factor responsible for muscle degeneration. The imperative necessity of the mutated amino acids for enzymatic activity of M13 metallopeptidases has been shown extensively (Devault et al., 1988; Kubo et al., 1992; Klimpel et al., 1994; Fushimi et al., 1999; Rioli et al., 2003).

To eventually identify the protein domain causing the observed phenotype, we generated transgenic lines that express truncated versions of Nep4A. Unfortunately, a protein construct lacking the complete extracellular domain could not be generated, presumably due to limited stability of the resulting protein. On the other hand, transgenic lines expressing a protein version lacking the intracellular part but still harbouring the transmembrane and the extracellular domain (amino acids 46-1040) were established with an expression rate being at least equal to that of the full length Nep4A overexpression line #2 (Fig. 5H). In contrast to the full length constructs that are apparently prone to proteolysis, as indicated by the detection of a second protein of less molecular weight (Fig. 5H, asterisk), truncated Nep4A is not susceptible to proteolytic degradation (Fig. 5H, arrowhead), which indicates that the cleavage site of the full length construct is located in the intracellular domain. Significantly, muscle specific overexpression of this N-terminally truncated protein did, in contrast to all other Nep4A constructs, not elicit any degeneration phenotype. We analyzed transgenic animals from 10 independent lines, all of them displaying a musculature that looked completely like

wild type (Fig. 5D). Furthermore, lifespan of these animals was also identical to wild type. Since the subcellular localization of truncated Nep4A was consistent with the pattern observed for the overexpressed wild type as well as mutated constructs (Fig. 5B´, C´, D´), we can exclude the possibility that the construct specific phenotypes are due to mislocalization effects.

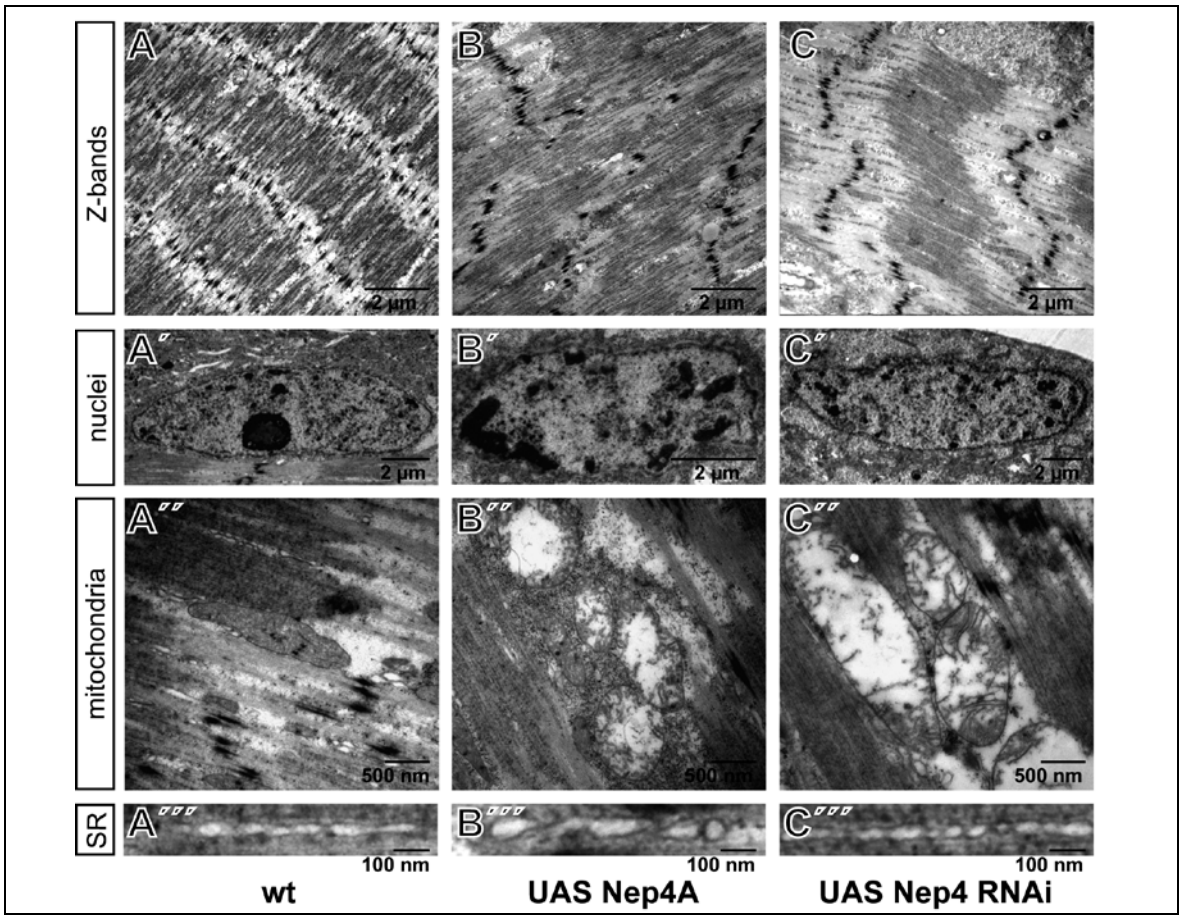


Fig. 6: **Altering Nep4 expression levels induces necrotic muscle degeneration as revealed by ultrastructural analysis** Transmission electron microscope sections of transgenic 3rd instar larval body wall muscles. Animals of the following genotypes were crossed to Mef-Gal4: Mef-Gal4 (wt); UAS Nep4A, line #2; UAS Nep4 RNAi. Muscles overexpressing Nep4A exhibit staggered Z-bands (B) as well as nuclei with dispersed chromatin (B´), swollen and severely fragmented mitochondria (B´´) and swollen sarcoplasmic reticuli (SR, B´´´). Muscle specific knock-down of *nep4* has apparently significantly less detrimental effect on sarcomeric architecture with the Z-bands looking comparable to wild type (C). Nevertheless, nuclei with dispersed chromatin (C´) and swollen mitochondria with ragged cristae (C´´) were observed. Sarcoplasmic reticuli appearance on the other hand was comparable to wild type (C´´´).

The intracellular domain of Neprilysin 4A interacts with a yet uncharacterized carbohydrate kinase

To comprehend the physiology causing the observed muscle degeneration phenotype, we conducted a yeast two hybrid (Y2H) screen using the coding sequence of the intracellular domain of Neprilysin 4 as bait and a normalized *Drosophila* cDNA library as prey. As a result we identified the putative carbohydrate kinase CG3534 as a first interaction partner of the intracellular domain of Nep4. To support the Y2H data and to confirm a physical interaction between these two proteins, we did pull down assays using the immobilized Nep4 intracellular domain fused to an 8x histidin-tag as bait and full length CG3534 fused to an

HA-tag as prey. As shown in Fig. 7A, subsequent to immobilization on an affinity column, the intracellular domain of Nep4A is able to pull CG3534 out of a protein preparation extracted from SF21 insect cells expressing HA-tagged CG3534. If the same extract is applied to a column loaded with a preparation lacking the respective Nep4 domain, only negligible background binding of CG3534 is apparent. These data confirm the initial Y2H results and demonstrate a physical interaction between the two proteins. Noteworthy, expression of CG3534 mRNA in 3rd instar larval body wall muscles was confirmed by RT-PCR (Fig. 7B) using the same RNA preparations that were already applied to confirm expression of *nep4* in this tissue.

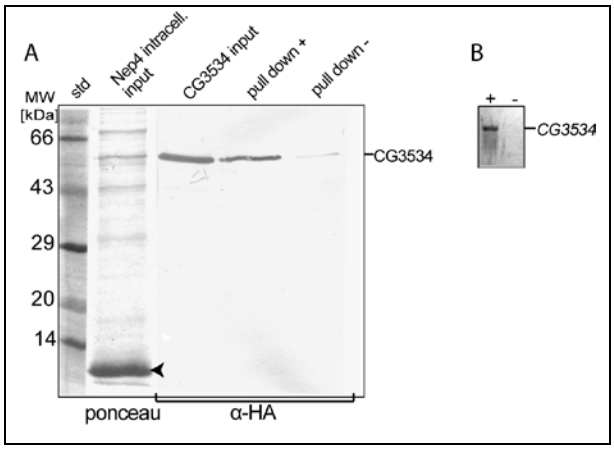


Fig. 7: **The intracellular domain of Nep4 interacts with CG3534** A: Pull down of CG3534-HA by the immobilized Nep4A intracellular domain was confirmed by Western blot using an anti-HA antibody (pull down +). As a control a Ni-NTA column loaded with the same protein preparation, yet lacking the intracellular domain, was incubated with CG3534-HA (pull down -). CG3534-HA efficiently binds to the intracellular domain of Nep4A but not to a control protein preparation lacking this domain. Prior to incubation, expression of CG3534-HA was confirmed (CG3534 input). The amount of immobilized intracellular domain was assessed by Ponceau staining of the first fraction eluted from the Ni-NTA column (Nep4 intracell. input, arrowhead).B: *CG3534* is expressed in 3rd instar larval body wall musculature as confirmed by RT-PCR (+). In a control reaction lacking the respective cDNA no amplification product is visible (-).

Discussion

Muscle degeneration is induced highly specifically by Neprilysin 4, isoform A

Due to their ability to degrade the amyloid-ß peptide, which is one of the major factors causing Alzheimer's disease, mainly the catalytic activities of neprilysins have been of principal interest while non-catalytic protein domains were considered as being less physiologically relevant. In the present study, we report on the potential of Neprilysin 4 from *Drosophila melanogaster* to induce a severe muscle degeneration phenotype upon overexpression. Significantly, this breakdown of sarcomeric structure is not caused by modified enzymatic activities of Nep4 but is rather based on the proteins´ intracellular N-terminus. As mentioned previously, up to now only few studies are available that focus on the interaction between the intracellular domain of neprilysins and cytoplasmic proteins. Notwithstanding, the data reported so far relate the respective domain of human Neprilysin to essential physiological processes such as cell migration (Sumitomo et al., 2000) or the suppression of prostate cancer cell growth (Sumitomo et al., 2004), emphasizing the physiological relevance of this domain. The observation that in somatic muscles of *Drosophila* an excess of the intracellular Nep4 domain induces a severe breakdown of sarcomeric architecture unravels a novel physiological process these domains are involved in. Significantly, muscle specific overexpression of the soluble Nep4 isoform B or of a truncated Nep4A version that lacks the intracellular domain but still holds its transmembrane region does not interfere with lifespan or muscle integrity of the animals, which clearly implies that the intracellular part of Nep4 is responsible for the observed phenotype.

In order to assess the specificity of this phenotype, in addition to Nep4 we also did overexpression studies with Nep1 from *Drosophila*. Apart from Nep4 isoform B every *Drosophila* Neprilysin is predicted to be membrane bound and to hold an intracellular domain

with sizes ranging between 20 and 112 amino acids, respectively (TMHMM v2.0, http://www.cbs.dtu.dk/ services/TMHMM-2.0/). Noteworthy, comprising 112 amino acids, the intracellular part of *Drosophila* Nep1 represents the largest of these domains. As expected, in contrast to Nep4, muscle specific overexpression of Nep1 did not elicit any muscle degeneration phenotype. Despite significant overexpression, transgenic animals developed into adulthood without any obvious defects (not shown). Furthermore, data by (Bland et al., 2009) demonstrated that *actin* driven overexpression of Nep2 does also not evoke any phenotypes comparable to Nep4 overexpression. The respective animals reached adult stage without displaying any obvious muscle degeneration. Based on their data, the authors concluded that observed locomotory and geotactic behavioural defects were due to changes in peptide homeostasis and therefore related to enzymatic activities of the peptidase rather than to its intracellular terminus. Finally, overexpression of human Neprilysin in plasma membranes of murine skeletal muscles again did not result in any muscle degeneration phenotype (Liu et al., 2009). Based on these results we conclude that the phenotypes observed after overexpression of Nep4A are caused highly specifically by the intracellular domain of this distinct enzyme. While clearly further research is necessary to analyze this issue in more detail, we consider this finding as a first indication favouring the assumption that the respective domains of neprilysins are acting in individual physiological pathways with little redundancy present. This is in contrast to the substrate specificity of neprilysins which is apparently rather broad. Thus, such specific functions for the intracellular domain of neprilysins might help to explain why in many organisms several closely related and therefore catalytically redundant neprilysins are expressed simultaneously (Turner et al., 2001; Meyer et al., 2011).

The intracellular domain of Nep4 interacts with the putative glycerol kinase CG3534

By combining Y2H data with pull down assays we could identify a first interaction partner of the intracellular Nep4 domain, named CG3534. Unfortunately, this interacting protein is currently rather uncharacterized with little to none experimental data being available. However, based on sequence similarities it is highly probable that this protein represents a carbohydrate kinase, presumably a glycerol kinase (Motif Scan, http://hits.isb-sib.ch/cgi-bin/motif_scan) which renders an involvement in energy metabolism rather likely. Glycerol kinases are enzymes that catalyze the formation of glycerol 3-phosphate from ATP and glycerol, which represents the rate-limiting step in glycerol utilization (McCabe, 1975). Significantly, in insects glycerol 3-phosphate has been shown to be one major metabolite for mitochondrial ATP-production in flight muscles (Hansford and Johnson, 1975). Furthermore, in *Drosophila* it has been demonstrated that flight muscle function requires a highly specific localization pattern of enzymes involved in ATP-synthesis, such as glycerol 3-phosphate dehydrogenase or glyceraldehyde 3-phosphate dehydrogenase, with a mislocalization of these enzymes causing flightlessness. This inability to fly occurred even though the full complement of yet mislocalized enzymes was present in the muscles, which demonstrates that the mere presence of those enzymes is not sufficient for muscle function; correct localization in the sarcomere is crucial (Wojtas et al., 1997). However, up to now, the physiological reason for this need for distinct localization is not completely understood. One favoured explanation suggests that the respective enzymes are components of a functional complex which has to be complete to support energy production for flight with the functional significance being an optimization of ATP production efficiency (Wojtas et al., 1997).

Intriguingly, under conditions of mild Nep4A overexpression (UAS Nep4A line #3, Fig. 3A, B) transgenic flies were also unable to fly which indicates that the physiological function of the Nep4 intracellular domain might also be linked to energy metabolism in muscles. Binding of CG3534 to the respective domain of Nep4A could cause a mislocalization of the putative

kinase which in turn results in a similar phenotype as mislocalization of other enzymes involved in ATP-production evokes. This result is clearly expected if CG3534 was indeed part of such a functional enzyme complex as described above. Thus, in line with data from other enzymes involved in ATP-synthesis, we propose that activity of CG3534 requires correct localization of the protein and that interaction with the intracellular domain of Nep4A results in a mislocalization that severely impairs its activity and thereby flight muscle functionality. In addition to flight muscles, glycerol kinase activity was also detected in somatic muscles of vertebrates as well as of invertebrates such as insects, where it is involved in fat metabolism and thereby again supports sustained muscular activity (Newsholme and Taylor, 1969; Guo and Jensen, 1999; Montell et al., 2002).
Based on these data it appears conceivable that the intracellular domain of Nep4 is involved in regulating activity of CG3534 in both, body wall but also flight musculature. In body wall muscles, apparently strong expression of the intracellular Nep4 domain is necessary to induce the observed degeneration phenotype. This high abundance may be necessary to recruit and thereby presumably inactivate sufficient CG3534 to significantly interfere with muscle physiology. Since in somatic muscles Nep4 is located mainly in membranes of the sarcoplasmic reticulum (Fig. 2B´´), recruitment of CG3534 to this particular compartment is expected. Milder Nep4A overexpression on the other hand impairs mainly flight muscle activity while the somatic musculature remains functional. In this context, the facts that insect flight muscles are on one hand among the most energy-demanding tissues known in nature and on the other hand highly labile (Marden, 2000) may be of particular relevance since they indicate that this type of muscle is considerably more prone to metabolic or structural perturbations than other muscles are. Therefore, minor modifications in CG3534 activity may already significantly impair flight muscle physiology while somatic muscles are still functional. Thus, the observed inability to fly is considered to be the consequence of a mislocalization of only part of the CG3534 pool which in turn accounts for a minor reduction in catalytic activity that is yet sufficiently disturbed to induce flightlessness. In future studies the availability of a CG3534 specific antibody and the analysis of CG3534 mutant lines will be highly beneficial in corroborating these implications.

Nep4 expression has to be tightly regulated

The observation that compared to overexpression, RNAi mediated knock-down of *nep4* causes a similar, yet significantly weaker phenotype appears in the first instance counterintuitive. If both, increasing and reducing expression of Nep4, would interfere with energy metabolism, discriminative phenotypes would be expected unless an excess of ATP had the same impact on muscle physiology as a limitation. However, this conflict is only valid if both expression level alterations were in fact interfering with the same physiological process. In lieu thereof, upon closer inspection significant differences become apparent that clearly discriminate the overexpression phenotype from RNAi-mediated effects, thereby corroborating the assumption that increasing and reducing Nep4 expression levels do not target the same physiological process. In addition to the distinct extents of muscle impairment, especially the differences observed in motility assays indicate discrete physiological events overexpression and downregulation of Nep4 interfere with: while reduced protein levels influence primarily the contraction frequency, an excess of Nep4 reduces both, frequency and power of contraction. This leads to the assumption that under overexpression conditions the muscles are not able to contract exhaustively any longer, possibly due to a lack of accessible energy sources. The physiology causing the RNAi mediated phenotype on the other hand is currently less clear and requires further research. Especially the fact that we currently cannot discriminate between phenotypes caused by reduced expression of the intracellular domain and those evoked by reduced catalytic

activities exerted by the extracellular part of the protein, renders an analysis of this issue more complicated. Therefore, in order to understand the physiological processes Nep4 overexpression and downregulation interfere with in more detail, the identification of additional proteins interacting with the intracellular part of Neprilysin 4 will be a main objective in future studies.

Ectopic expression of neprilysins as a therapeutic strategy

Extracellular deposits of insoluble amyloid ß-peptide are considered to be one of the central indications for Alzheimer's disease. To counteract accumulation of this peptide and therefore exacerbation of the disease, application of peptidases capable of cleaving and inactivating it are of great interest. While e.g. overexpression of human Neprilysin in skeletal muscles of trangenic mice was reported to be without adverse effects (Liu et al., 2009), expression of the same Neprilysin in the *Drosophila* nervous system caused a dramatic loss of neurons, axon degeneration and a significantly shortened life span of affected flies (Iijima-Ando et al., 2008). Nevertheless, in both transgenic model systems accumulation of amyloid ß-peptide could be effectively reduced. In this context, Iijima-Ando and colleagues (Iijima-Ando et al., 2008) argued that all overexpression derived detrimental effects are due to ectopic enzymatic activities since expression of a catalytically inactive Neprilysin did not induce any side effects. However, in addition to their function as enzymes balancing peptide homeostasis, neprilysins are apparently also regulating distinct physiological processes by means independent of their catalytic activities. As mentioned previously, the intracellular domain of human Neprilysin is able to recruit the phosphatase PTEN to the plasma membrane, thereby activating the protein and inhibiting oncogenic pathways (Sumitomo et al., 2004). The same is true for interactions between the intracellular domain and ERM (ezrin/radixin/moesin) proteins, with the former competing with CD44 for binding ERMs, thereby regulating CD44 dependent cell migration and invasion (Iwase et al., 2004). Regarding these diverse functions it appears conceivable that in addition to reducing amyloid ß-peptide levels, ectopic expression of Neprilysin could be beneficial in the treatment of other diseases as well. Unfortunately, based on our data it is rather probable that binding of ectopically expressed neprilysins to cytosolic or membrane bound factors will also have considerable adverse effects. The result that even mild overexpression of Nep4 induces severe detrimental effects on muscle physiology which in turn cause flightlessness emphasizes this issue in particular. Thus, in addition to identifying further substrates of neprilysins, appreciation of the physiological processes being affected by the enzymes´ intracellular termini is essential prior to applying this family of proteins in therapeutic approaches.

Materials and Methods

Fly strains

The following *Drosophila* lines were used in this work: w1118 was used as wild type. For knock-down experiments Nep4 RNAi line 100189 (Vienna *Drosophila* RNAi Center, VDRC, http://stockcenter.vdrc.at) was used and crossed to either Mef-Gal4 (H. Nguyen, Erlangen, Germany), or ActinC5-Gal4 (BL3954, Bloomington *Drosophila* Stock Center, BSC). Ectopic expression was achieved using the former drive line. UAS Nep1 (BL22465, BSC) was used for ectopic expression of the respective peptidase.

UAS Nep4 constructs were established by cloning the corresponding coding sequences of isoforms A or B, respectively, into the pUAST vector (Brand and Perrimon, 1993). A C-terminal hemagglutinin (HA)-tag was inserted into the *nep4A* sequence by appropriate primer design using the following reverse primer: tactcactcgagctaagcgtaatctgga acatcgtatgggtaccaaacgctgcacttttttctg. Mutated Nep4A constructs were created by PCR utilizing the Phusion site-directed mutagenesis kit (Finnzymes, Finland) and wild type *nep4A* sequence as a template. Mutagenesis primers used were: gtcattggcttcgaactgacccac (forward, fw, mutation 1: FExxH), gtcattggccaccaactgacccacggt (fw, mutation 2: HQxxH) and gtcattggccacgaactgaccttcggtttcgat (fw, mutation 3: HExxF). In any case the reverse (rv) primer cacaccgatgccgccgaagtt was used. To generate a truncated Nep4A construct (Nep4A trunc), primers tactcagaattcatggtgcagtggtgtcccggtctg (fw) and tactcactcgagctacca aacgctgcactt (rv) were used.

nep4 specific GFP reporter and Gal4 driver lines were generated by cloning an intronic region (950 bp) of the *nep4* gene into the pHStinger vector (Barolo et al., 2000) or the pPTGal vector (Sharma et al., 2002), respectively (see fig.1C). Primers used were: tactcagaattctcggagggaaccaaatcaata (fw) and tactcaggtacccgccttcaattattgaagttt (rv).

The constructs were subjected to P-element based transformation using commercial services (Best Gene, CA, USA). For each construct several individual lines were tested for ectopic expression (see fig. 3A, B), driver capacity or reporter activity.

Larval motility and contraction analysis

Nep4 overexpression and knock-down was driven by Mef-Gal4, with the driver line itself being also applied as a control. *nep4* knock-down, gain of function or wild type F1 larvae were kept at 29°C until time of investigation. For movement and contraction assays, 2nd instar larvae were transferred into a glass petri dish with millimeter paper placed underneath. All movements were recorded with a standard video camera (Canon UC X10Hi). Larval movement speed was determined by calculating the distance covered in a continuous run of 10 sec. The same run was used to count larval body contractions.

Immunohistochemistry and western blot

Larval antibody stainings were performed as previously described (Monier et al., 2005). In brief, larvae were dissected in cold PBS, fixed in 4% formaldehyde for 30 min and washed extensively in PBT (PBS + 0.1% TWEEN20). Subsequently, tissues were incubated in PBS containing 1% Triton X-100 (30 min) and afterwards blocked with 1% BSA in PBT (30 min) before application of antibodies. Embryonic antibody stainings were done according to standard protocols. Antibodies and dyes used were: phalloidin-TRITC (1:50, Sigma-Aldrich, Munich, Germany), DAPI (1:200, Roth, Karlsuhe, Germany), anti-Discs large (1:500, Developmental Studies Hybridoma Bank, DSHB, Iowa, USA), anti-Nep4 (1:500, (Meyer et al., 2009)), anti-SERCA (1:500, M. Ramaswami) and anti-HA (1:500, Sigma-Aldrich). Secondary antibodies used were either conjugated to Cy2 (1:200) or Cy3 (1:100; Jackson ImmunoResearch, Newmarket, Suffolk, UK) and diluted in PBS. TUNEL assay was performed with *in situ* cell death detection kit TMR red (Roche, Basel, Switzerland). Briefly,

larvae were dissected in cold PBS, fixed in 4% formaldehyde for 20 min, permeabilized in 0.1% Triton X-100 for the same period, incubated with Proteinase K (20 µg/ml, 30 min), post-fixed for 20 min (4% formaldehyde) and incubated with TUNEL labeling mix at 37°C for 1h in the dark. For positive controls, subsequent to permeabilization wild type larval body walls were treated with DNAse I (20U, 15 min). For Western blot analysis equal amounts of larvae were homogenized (glass-teflon homogenizer) in PBS containing protease inhibitor mix M (Serva, Heidelberg, Germany). Subsequently, Laemmli buffer was added and samples were boiled at 99°C for 3 min. Protein samples (10µg/lane) were separated by SDS-PAGE (12%) and transferred to nitrocellulose membranes. Immunodetection was done using anti-Nep4 (1:2000), anti-Myosin heavy chain (1:300, (Kiehart and Feghali, 1986)) and anti-HA antibodies (1:1000, Sigma-Aldrich), respectively.

Northern blot, semi-quantitative PCR and RT-PCR

Northern blots were conducted as described (Meyer et al., 2009) with 15µg of total RNA being loaded per lane and a hybridization temperature of 65°C (20h).

To obtain total RNA for semi-quantitative PCR, the following lines were used: 1. Mef-Gal4 x wild type; 2. Mef-Gal4 x Nep4 RNAi (line 100189, VDRC); 3. Mef-Gal4 x UAS Nep4A #2. Subsequent to isolation of total RNA from F1 larval body wall musculature, cDNA was synthesized using the AMV First Strand cDNA Synthesis Kit for RT-PCR (Roche). In the course of isolating body wall musculature, all other tissues were removed as accurately as possible.

PCR was done with the following primers. *actin* (control): gagcaccccgtgctgctgacc (fw), ctcggggcagcggaaacgctc (rv); *nep4*: atgagtcgccacagccaactg (fw), ctaccaaacgctgcacttttttctg (rv). By the end of cycles 25, 30, 35 and 38, respectively, the reaction was temporarily halted and 2 µl aliquots were taken from each tube.

To confirm *CG3534* expression in 3rd instar larval body wall musculature by RT-PCR, the same muscle tissue derived wild type cDNA template was applied that was already used for confirming expression of *nep4* and *actin* in the respective tissue. Primers used were the same as for cloning CG3534 into the pFastBacDual vector. Identity of all amplificates was confirmed by sequencing.

Histological sections and transmission electron microscopy

For histological cross sections, *Drosophila* larvae were opened from the ventral side in ice cold PBS containing 2% glutaraldehyde and only the gut was removed. Subsequently, animals were firstly postfixed in 1% OsO_4 + 2% glutaraldehyde in 0.05 M cacodylatbuffer (4°C, 2h) and then in 1% OsO_4 in the same buffer (4°C, 1h). Washing steps in cacodylatbuffer were done after each fixation. Before embedding the larvae in Epon 812 resin (Serva) tissues were dehydrated by a series of ethanol washes. Ultra-thin sectioning (70 nm) was done using an Ultracut UCT ultramicrotome (Leica, Wetzlar, Germany) with diamond knives. Sections were mounted on grids and contrasted with uranyl acetate (30 min) and lead citrate (20 min) using a Nanofilm Stainer (Nanofilm, Goettingen, Germany). Sections were analyzed with a transmission electron microscope (model 902, Zeiss, Jena, Germany).

Laser scanning microscopy

Confocal images were captured with either a LSM 5 Pascal confocal microscope (Zeiss) or a LSM 510 Meta (Zeiss). Z-stacks are displayed as maximum projections if not stated otherwise.

Yeast-2-Hybrid

Yeast-2-hybrid screening was conducted using the Matchmaker Gold Yeast two hybrid system (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. As bait, the intracellular domain of Nep4A was cloned into the pGBKT7 vector using the

following primers: tactcagaattcatgagtcgccacagccaactg (fw); tactcaggatccctatttgcagcaggtcagaccggg (rv). Subsequent to mating with a Mate & Plate™ library (normalized universal *Drosophila*, Clontech) positive clones were isolated and sequenced.

Heterologous expression and purification
The intracellular domain of Nep4A fused to an 8x histidin-tag was cloned into the pET-29b vector (Novagen, Darmstadt, Germany) using the primers tactcacatatgagtcgccacagc caactgaag (fw) and tactcaggatccttaatgatgatgatgatgatgatgtttgcagcaggtcagacc (rv). Expression in *E. coli* Rosetta (DE3) cells (Novagen) was done essentially as described (Meyer et al., 2009). In parallel, as a negative control untransformed *E. coli* Rosetta cells were grown under equal conditions including the addition of IPTG. Subsequent to a final OD determination, cells were harvested by centrifugation, resuspended in 10 ml/g (wet weight) binding buffer (500 mM NaCl, 20 mM Tris, 5 mM imidazole, pH 7.9) and frozen at -20°C. For further processing, the respective cells were thawn on ice and lyzed by sonication (Branson sonifier 250, Branson Ultrasonics, Danbury, CT, USA). Following centrifugation (15 min, 10.000 x g), the supernatant was subjected to affinity chromatography using Ni-NTA affinity resin (Qiagen, Hilden, Germany). Proteins bound to the column were not eluted immediately but used as bait for subsequent pull down assays.
For baculovirus based expression of CG3534 in SF21 insect cells, the complete coding sequence was cloned into the pFastBacDual vector (Invitrogen, Carlsbad, CA, USA) downstream of the polyhedrin promoter using the primers tactcagtcgacatgg gtccgccaaagcagctg (fw) and tactcatctagactaagcgtaatctggaacatcgtatgggtatgtgttaggattgga caggac (rv). To allow detection of the expressed protein, an HA-tag was fused to its C-terminus by appropriate primer design. To track infection efficiency, an enhanced GFP (eGFP) reporter gene was inserted into the same vector downstream of the p10 promoter. Subsequent to reaching an infection rate of at least 80%, SF21 cells were harvested, resuspended in incubation buffer (100 mM NaCl, 20 mM Tris, pH 7.5) and homogenized with a glass-teflon homogenizator. To remove cell debris and other insoluble material, the homogenate was centrifuged (10.000 x g, 15 min, 4°C) and the supernatant was directly applied in pull down assays.

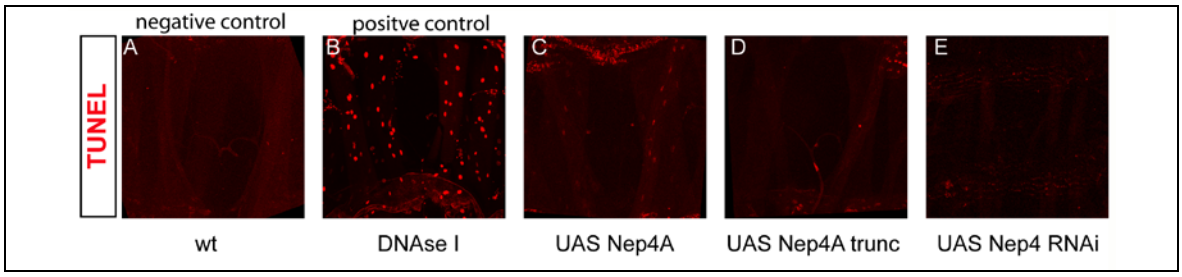
Pull down assay
Ni-NTA affinity resins, loaded with equal amounts of protein extracts obtained from either *E. coli* Rosetta cells expressing the intracellular domain of Nep4A or from untransformed control cells, were equilibrated with incubation buffer (10 bed volumes). Resins were then incubated with a fraction of soluble proteins isolated from SF21 cells expressing CG3534-HA. Incubation was done under gentle agitation at 4 °C, over night. Subsequently, the resins were washed with incubation buffer (20 bed volumes). Elution was done with binding buffer containing 400 mM imidazole and corresponding samples were further processed as stated above prior to being separated by SDS-PAGE. Interactions between the intracellular Nep4 domain and CG3534-HA were assayed by Western blot.

List of abbreviations

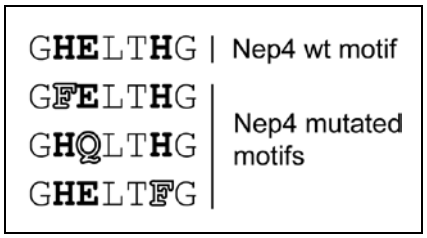
CNS	central nervous system
ECE	endothelin converting enzyme
eGFP	enhanced green fluorescent protein
fw	forward primer
HA	hemagglutinin
His	histidin
Nep	neprilysin

PI3-K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
rv	reverse primer
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SR	sarcoplasmic reticulum
Y2H	Yeast-2-Hybrid

Supplementary



Supplementary Fig. 1: **Muscle degeneration is not caused by apoptosis** TUNEL assays on body wall musculature of 3rd instar larvae of different genotypes (magnification: 20x). In any case, expression of transgenes was driven by Mef-Gal4. While DNAse I treatment of wild type larvae body walls results in strong staining of nuclei (B), neither in non treated wild type larvae (A) nor in larvae expressing truncated Nep4A (D) or *nep4* specific hairpin-RNA (E), the appearance of apoptotic nuclei is observed. In muscles of larvae overexpressing full-length Nep4A a faint staining of some nuclei is visible (C).



Supplementary Fig. 2: **Mutations introduced into the catalytic center of Nep4** Transgenic lines expressing mutated, catalytically inactive Nep4A constructs were generated. The wild type motif (HExxH, first row), was either mutated into **F**ExxH, H**Q**xxH or HExx**F**. For all contructs at least five independent transgenic lines were firstly tested for protein expression by Western blot and secondly for their ability to induce muscle degeneration. Muscle specific overexpression of all mutated Nep4A constructs resulted in significantly increased premature lethality, mainly in 2nd instar larval stage, and strong muscle degeneration in those animals that developed into 3rd instar larval stage, concomitant with late larval or pupal lethality.

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Author contributions

Heiko Meyer and Achim Paululat conceived the project and interpreted the data. Mareike Panz and Heiko Meyer designed and carried out most of the experiments, analyzed the data and wrote the manuscript. Jessica Vitos-Faleato established and carried out the motility assay and Arne Jendretzki and Jürgen Heinisch did the yeast-2-hybrid screen.

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4. Unveröffentlichte Ergebnisse und Resümee

4.1. Die Neprilysin 4 Isoformen und ihre subzelluläre Lokalisation

Die Metalloendopeptidase Neprilysin 4 aus *Drosophila melanogaster* wird aufgrund von alternativem Splicing in Form von zwei Transkriptvarianten exprimiert. Die 1040 AS umfassende Isoform A setzt sich aus einer 56 AS langen N-terminalen Domäne, einer Transmembrandomäne von 19 AS Länge und aus einer C-terminalen, extrazellulären Domäne zusammen (965 AS). Der Isoform B fehlt sowohl die intrazelluläre als auch die Transmembrandomäne, wodurch sie als lösliches Enzym exprimiert und vermutlich in den Extrazellularraum sekretiert wird. In Bezug auf die jeweilige Sequenz der extrazellulären Domäne und somit auch bezüglich des katalytischen Zentrums, unterscheiden sich beide Proteine nicht.

Während die Expression beider *nep4* Transkripte in allen Entwicklungsstadien (Embryo, Larve, Puppe, Adult) nachgewiesen werden konnte, ist das gewebespezifische Expressionsmuster der Isoformen noch weitgehend ungeklärt (Tab. 1). Im Embryo markiert ein Nep4–spezifischer Antikörper Gliazellen im ZNS, Keimzellen, sowie Herz- und Muskelvorläuferzellen. Da der eingesetzte Antikörper beide Isoformen erkennt, kann jedoch keine Aussage darüber getroffen werden, welche der beiden Isoformen in den entsprechenden Geweben vorkommt. Sowohl die Expression in den Gliazellen als auch in den männlichen Keimzellen bleibt bis in das adulte Stadium bestehen. Vermutlich gilt dies auch für die Nep4 Expression in der somatischen Körperwandmuskulatur und im Herzen, allerdings konnten die eingesetzten anti-Nep4 Antikörper das Protein nicht in Geweben dieses Stadiums nachweisen. Dessen ungeachtet konnte das Vorkommen beider *nep4* Transkripte in der Muskulatur des dritten Larvalstadiums mittels semi-quantitativer PCR belegt werden, was darauf hindeutet, dass Nep4 in den genannten Geweben exprimiert wird, die Affinität des eingesetzten Antikörpers aber nicht ausreicht, um die geringe Expression des Proteins zu detektieren. Diese Annahme wird dadurch gestärkt, dass transgene Reporterlinien, die eGFP unter der Kontrolle des mesodermalen *nep4* Enhancers exprimieren, deutliche Reportergen-Expression im Herzen und in der somatischen Muskulatur aller Entwicklungsstadien aufweisen. Während der Embryogenese detektiert der anti-Nep4 Antikörper das Protein im Bereich der Plasmamembran von Herz- und Muskelvorläuferzellen. Doppelfärbungen mit anti-Spektrin Antikörpern im Herzen zeigen eine Kolokalisation beider Proteine (unveröffentlicht, diese Arbeit), sodass zumindest die membrangebundene Nep4 Isoform A im Herzen vorliegen dürfte. Im Gegensatz hierzu, führt die Expression der Nep4 Isoform A, gesteuert durch den

mesodermalen *nep4* Enhancer, in der Muskulatur zu einer perinukleären Anreicherung des Proteins. Wie beschrieben (Panz et al. submitted) kolokaliert Nep4A in diesem Fall mit dem Sarkoplasmatischen Retikulum (SR) und mit der Kernmembran.

Gewebe (Nachweismethode)	**Nep4 Expression**	**Isoform A**	**Isoform B**
ZNS (ISH, IHC, Western Blot)	X	X	X
Körperwandmuskulatur von 3. Larven (semi-quantitative PCR)	X	X	x
embryonales Herz und Muskulatur (ISH, IHC)	X	?	?
Männliche Keimzellen (ISH, IHC, Western Blot)	X	X	-
frühe Embryogenese (IHC)	X	?	?

Tab. 1: Expression von Nep4 in verschiedenen Geweben in *Drosophila*. Das Nep4 Protein konnte mit Hilfe von *in situ* Hybridisierungen (ISH) und/oder immunhistochemischen Färbungen (IHC) im zentralen Nervensystem (ZNS) und in männlichen Keimzellen sämtlicher Stadien, in embryonalen Herz- und Muskelzellen sowie in der frühen Embryogenese nachgewiesen werden. Der Nachweis beider *nep4* Transkripte in der larvalen Muskulatur erfolgte ausschließlich über semi-quantitative PCR. (X = deutliche Expression nachgewiesen, x = schwache Expression nachgewiesen, - = keine Expression nachweisbar, ? = nicht untersucht)

Neben der Lokalisation von Nep4 in der Plasmamembran und dem SR ist zusätzlich die Expression der Metallopeptidase in Zellkern nennenswert (unpubliziert, diese Arbeit). Im Verlauf der frühen Embryogenese (1-3 Std. nach Eiablage) markiert der Nep4 Antikörper spezifisch das Chromatin in den sich mitotisch teilenden Zellkernen des synzytialen Blastoderms (Abb. 6). Das Nep4 Protein scheint hierbei insbesondere mit der stark kondensierten DNA der Inter- und Prometaphase (Abb. 6 D, E) sowie der Ana- und Telophase (Abb. 6 E, F) zu kolokalisieren. Membranfärbungen mit dem Strukturprotein Spektrin zeigen, dass Nep4 auch noch im zellulären Blastoderm mit der DNA kolokalisiert (Abb. 6 H).

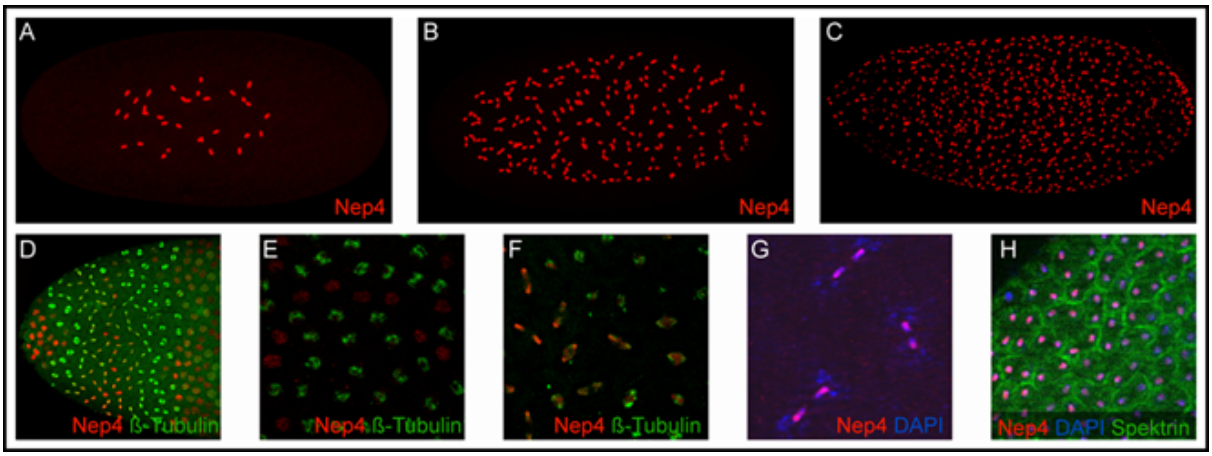

Abb. 6: Expressionsmuster von Nep4 im frühen Embryo A-C: In frühen Embryonen markiert Nep4 die sich teilenden Zellkerne im synzytialen Blastoderm. **A:** Fünfte mitotische Teilung (~32 Zellkerne); **B:** Achte Teilung (~240 Kerne); **C:** Zehnte Kernteilung (~860 Zellkerne); **D-H:** Wildtypische Embryonen wurden mit Nep4, ß-Tubulin (Spindelapparat), DAPI (DNA) und/oder Spektrin (Zellmembran) gefärbt. **D:** Anteriorer Teil eines Embryos der die typischen metazyklischen Mitosen durchläuft; **E-G:** Vergrößerte Bildausschnitte der unterschiedlichen Mitosestadien im Embryo. Nep4 kolokalisiert nicht mit dem Spindelapparat, sondern mit der DNA. **H:** Im frühen zellulären Blastoderm kolokalisiert Nep4 mit dem Zellkern.

Analog zur Expression von Nep4 in der frühen Embryogenese zeigt das Nep4 Protein auch in den männlichen Keimzellen (larvale / adulte Testis) eine nukleäre Lokalisation (Abb. 7). Im Embryo wird es dabei zunächst noch in beiden Geschlechtern exprimiert (Abb. 7 A, Pfeilspitzen), während es ab dem Larvalstadium nur noch in männlichen Keimanlagen zu finden ist (Abb. 7 B). Immunhistochemische Färbungen zeigen, dass das Protein hauptsächlich mit der DNA kolokalisiert oder in unmittelbarer Nähe des Zellkerns zu finden ist (Abb. 7 D-E´´). In reifen Spermatidenbündeln liegt Nep4 direkt an bzw. neben der stark elongierten, kondensierten Kern-DNA (Abb. 7 D, Pfeilspitze). Mittels Western Blot konnte gezeigt werden, dass es sich bei der im Testis exprimierten Isoform ausschließlich um das membrangebundene Nep4A handelt. Die frühe Nep4 Expression im Embryo konnte hingegen nur immunhistochemisch (unveröffentlicht, diese Arbeit) und nicht im Western Blot nachgewiesen werden (Meyer et al. 2009), so dass unklar ist, welche der Isoformen vorliegt. Negativkontrollen und alle bislang durchgeführten immunhistochemischen Färbungen belegen jedoch eine hohe Spezifität der eingesetzten polyklonalen Antikörper und bekräftigen somit die Validität der gezeigten Nep4 Expression im Verlauf der frühen Embryogenese.

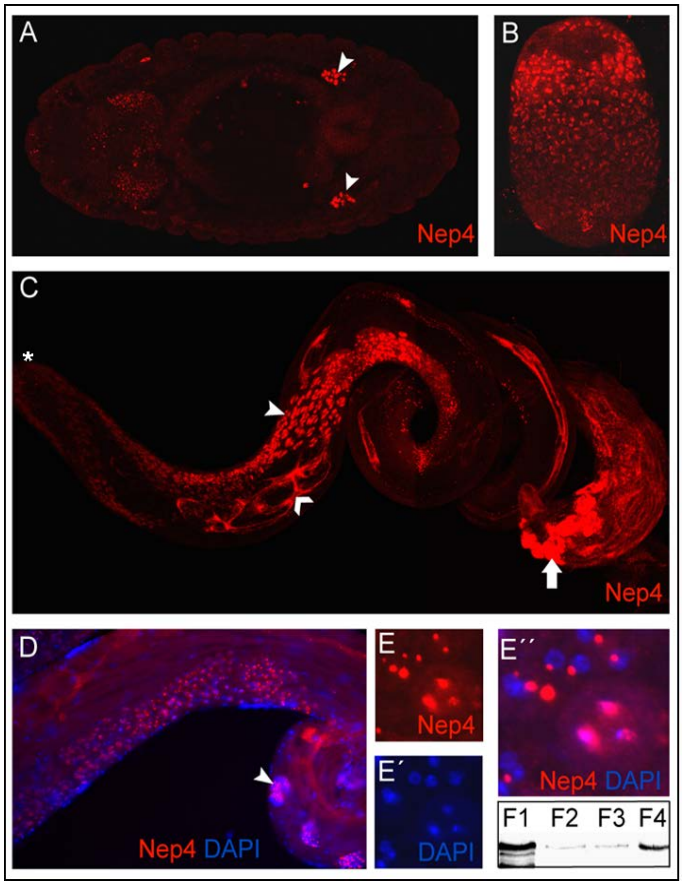

Abb. 7: Expression von Nep4 in den Geschlechtszellen. A-C: Nep4 Expression in den bilateralen Anlagen der Keimzellen im Embryo (A, Pfeilspitzen) und im Testis von Larven (B). Im adulten Männchen erstreckt sich die Expression von Nep4 angefangen von den mitotischen Keimzellen der Testisspitze (C, Sternchen), über die meiotischen Zellen in der Testismitte (C, Pfeilspitze: Keimzellen; offene Pfeilspitze: Zystenzellen) bis hin zu den post-meiotischen Zellen (C, Pfeil) am Ende des Testisschlauches. **D-E:** Während der gesamten Spermatogenese kolokalisiert das Nep4 Protein mit der DNA oder liegt in direkter Nachbarschaft zu dieser vor (D, E, E´, E´´; Pfeilspitze in D: Spermatozytenbündel). **F:** Nachweis von Nep4 in Testisgewebe mittels sub-zellulärer Fraktionierung. Fraktionen: F1, Zytosol; F2, Membran und Organellen; F3, Nuklei; F4, Zytoskelett.

Die physiologischen Funktionen, die Nep4 in den männlichen Geschlechtsorganen oder in der frühen Embryogenese von *Drosophila* ausübt, konnten innerhalb dieser Arbeit nicht abschließend geklärt werden. Dies ist in der Hauptsache dadurch begründet, dass die Funktion der Peptidase in Muskel- bzw. Herzgewebe im Mittelpunkt des Interesses stand. Nennenswert ist jedoch die Tatsache, dass die membrangebundene Isoform A aus Hodengewebe, basierend auf subzellulären Fraktionierungen, intrazellulär vorliegt und hauptsächlich in der zytoplasmatischen- und der Zytoskelett-Fraktion auftritt (Abb. 7 F1-F4).

Da es sich beim Zellzyklus und der Karyogenese um grundlegende Prozesse eukaryotischer Zellen handelt, ist der Nachweis von Nep4 in sich mitotisch und meiotisch teilenden Zellen von besonderem Interesse. Wie bereits beschrieben, weisen alle menschlichen Vertreter der Neprilysine, aber auch Nep2 aus *Drosophila*, eine Expression in den männlichen Geschlechtsorganen auf (Thomas et al. 2005; Ouimet et al. 2000; Ghaddar et al. 2000; Ikeda et al. 2002; Bonvouloir et al. 2001), wobei die subzelluläre Lokalisation dieser Neprilysine in den Testis bislang kaum untersucht wurde.

Im Gegensatz dazu konnten Neprilysine bereits in Zellkernen anderer Gewebe nachgewiesen werden: In humanen Muskelzellen kolokalisiert Neprilysin beispielsweise zum Teil mit dem Zellkern (Broccolini et al. 2006). In diesem Zusammenhang ist erwähnenswert, dass humanes Neprilysin zwar eine N-terminale Nukleäre-Lokalisationssequenz (NLS) aufweist, die eine Translokation in den Kern vermitteln könnte, Gomes et al. (2003) jedoch vermuten, dass diese Sequenz durch C-terminale Bereiche verdeckt wird und somit funktionslos ist. Im Fall von Neprilysin 4 aus *Drosophila* findet sich die entsprechende NLS zwar nicht wieder, es werden jedoch innerhalb der extrazellulären Domäne drei Bereiche mit möglichen NLS identifiziert (AS: 133-147, 206-223, 371-385; http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Ein weiteres Beispiel für kernlokalisierte M13-Metallopeptidasen stellt ECE-1 dar, das sowohl in transfizierten CHO-Zellen, als auch endogen in Endothelzellen, oder auch in Prostatakrebszellen im Zellkern bzw. im perinukleären Bereich nachgewiesen wurde (Jafri und Ergul 2003; Dawson et al. 2006). Die physiologischen Funktionen der jeweiligen Metalloproteasen im Zellkern konnten bislang von keiner der genannten Gruppen näher definiert werden. Dementsprechend ist zurzeit nicht bekannt, ob die enzymatische Aktivität oder eine hiervon unabhängige Eigenschaft der jeweiligen Proteine die nukleäre Lokalisation der entsprechenden Peptidasen erforderlich macht.

4.2. Die Funktion von Neprilysin 4 in der Muskulatur

Wie im Verlauf dieser Arbeit gezeigt wurde, führt die muskelspezifische Überexpression der Metallopeptidase Neprilysin 4 aus *Drosophila melanogaster* zu einer progressiven Degeneration der somatischen Körperwandmuskulatur. Dieser Prozess manifestiert sich phänotypisch in einer geringeren Beweglichkeit der Tiere, gekoppelt mit dem Verlust der myogenen Organisation und Letalität in der späten larvalen Entwicklung. Transgene Tiere, die eine katalytisch inaktive Form des Nep4 Proteins exprimieren, zeigen ebenfalls den genannten Phänotyp. Durch die Generierung eines verkürzten, katalytisch aktiven,

membranständigen Nep4 Konstruktes, dem lediglich die intrazelluläre Domäne fehlte, konnte diese als ursächlich für die Degeneration der Muskulatur identifiziert werden.

Um die physiologische Funktion der intrazellulären Domäne von Nep4 zu verstehen, wurden zunächst mit Hilfe eines Hefe-2-Hybrid Screens Proteine identifiziert, die mit dieser Domäne direkt wechselwirken. Aus den identifizierten Kandidaten wurden zunächst Proteine ausgewählt, die in der Muskulatur exprimiert werden oder die mit der Aufrechterhaltung der Muskelintegrität in Verbindung gebracht werden können. Zur Verifizierung der Interaktion wurden die entsprechenden Proteine heterolog in Insektenzellen (SF21) exprimiert und deren physikalische Interaktion mit der gereinigten intrazellulären Domäne von Nep4 mittels Pull-down Versuchen analysiert.
Unter Verwendung der genannten Methodik konnte zunächst die Wechselwirkung des Faktors CG3534 mit Nep4 bestätigt werden. Das identifizierte Protein wurde von den SF21-Zellen in löslicher Form exprimiert und fungiert vermutlich als Glyzerolkinase, die die Phosphorylierung von Glyzerol katalysiert (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Die Interaktion von CG3534 mit Nep4 ist insofern sehr interessant, als dass die Beteiligung von Glyzerolkinasen am Stoffwechsel in der Muskulatur von Vertebraten (Maeda et al. 2008, Montell et al. 2002; Watford et al. 2000), aber auch von Invertebraten (Candy et al. 1997; Newsholme und Taylor 1969), seit langer Zeit bekannt ist. Dabei verwendet das Enzym Glyzerol und ATP zur Synthese von Glyzerol-3-Phosphat, welches ein wichtiger Metabolit der mitochondrialen ATP Produktion in der Flugmuskulatur von *Drosophila* ist (Hansford und Johnson 1975). Da darüber hinaus bekannt ist, dass die Funktion dieser Muskulatur von der korrekten subzellulären Lokalisation der für den Energiemetabolismus relevanten Enzyme abhängig ist (Wojtas et al. 1997), liegt nahe, dass auch die spezifische Lokalisation der Glyzerolkinase essentiell ist. Die Interaktion von CG3534 mit der ektopisch exprimierten, intrazellulären Domäne von Nep4 könnte somit eine Fehllokalisation der Kinase und damit eine Inaktivierung des Enzyms oder zumindest eine verringerte Emzymleistung an der erforderlichen Stelle verursachen, was letztlich die Degeneration der Muskulatur bedingt. Dennoch kann nicht ausgeschlossen werden, dass abgesehen von CG3534 auch andere Faktoren mit der intrazellulären Domäne von Neprilysin 4 wechselwirken, die eine direktere Verbindung zu dem beobachteten Phänotyp aufweisen und entsprechend ursächlich für die Degeneration der Muskulatur sind.
Für die zukünftige Analyse von CG3534, sowie den weiteren Kandidatenproteinen aus dem Y2H, bieten sowohl Interaktionsstudien mittels Pull-down als auch Kolokalisationsstudien,

bzw. die Untersuchung von Knock-out oder Knock-down Mutanten, die Möglichkeit, die Funktionen der N-terminalen Domäne von Nep4A in *Drosophila* genauer zu bestimmen. Eine Interaktion dieser Domäne mit den *Drosophila* Homologen von PTEN und ERM, die mit der intrazellulären Domäne des menschlichen Neprilysins interagieren (Sumitomo et al. 2004; Iwase et al. 2004), wurde nicht gefunden.

In Übereinstimmung mit der Überexpression führt auch eine RNAi vermittelte Reduzierung der Expression von *nep4* zu Bewegungsstörungen, ultrastrukturellen Degenerationen der larvalen Muskulatur und zu frühzeitiger Letalität (pupales Stadium). Die Ursache für diesen Phänotyp konnte bislang nicht zweifelsfrei ermittelt werden. In diesem Zusammenhang ist von besonderem Interesse, ob das Fehlen der intrazellulären Domäne, der katalytischen Aktivität oder eine Kombination beider oder weiterer Faktoren den Knock-down Phänotyp verursachen (Panz et al. submitted).

4.3. Die Regulation der Neprilysin 4 Expression

Die Regulation der Genexpression ist die Grundlage einer Vielzahl von essentiellen biologischen Prozessen in Zellen bzw. Organismen. So ist die differentielle Genaktivität von zentraler Bedeutung um die Differenzierung von Zellen bzw. Geweben zu gewährleisten, stellt aber auch die wesentliche Antwort eines biologischen Systems auf sich ändernde Umweltreize dar. Während die Promotorregion die basale Expression eines Genes kontrolliert, wirken cis-aktivierende oder reprimierende Elemente als die eigentlichen Faktoren, die die Bindung des Transkriptionskomplexes an den Basalpromotor und damit die Expression des Gens grundlegend beeinflussen. Da der Genlocus und somit die Ausstattung eines Genes mit cis-Elementen jedoch gleichbleibend ist, wird die zeitliche und räumliche Regulation von Genen erst durch die Bindung spezifischer Transkriptionsfaktoren vermittelt.
Innerhalb dieser Arbeit konnten zwei regulatorische Bereiche des *nep4* Gens identifiziert werden: ein 2941 Bp umfassende Sequenz, die in transgenen Fliegen die Regulation des Nep4 Proteins im ZNS abbildet und eine Sequenz von 950 Bp Länge, die die mesodermale Nep4 Expression widerspiegelt (Abb. 8). Zusätzlich wurden auch größere Sequenzbereiche (insgesamt etwa 4 Kb) stromaufwärts des *nep4* Gens, sowie intronische *nep4* Sequenzen auf ihre Fähigkeit hin untersucht, die Expression eines Reportergens (eGFP) zu regulieren, jedoch

zeigten diese Konstrukte keine, bzw. nur von dem Nep4 Expressionsmuster abweichende Signale.

Da der Fokus dieser Arbeit auf der Expression von Neprilysin 4 im Herz bzw. in der Muskulatur von *Drosophila* lag, ist insbesondere die weitere Analyse des mesodermalen Enhancers von Interesse. Kolokalisationsstudien an mesodermalen GFP Reporterlinien ergaben, dass der Enhancer während der Embryonalentwicklung vollständig die Expression von Nep4 im Mesoderm steuert (Abb. 6 in Meyer et al. 2011). In den folgenden Entwicklungsstadien ist das Reporterprotein weiterhin in den Muskeln der Körperwand und in spezifischen Herzzellen nachweisbar (Panz et al. submitted, diese Arbeit). Die Tatsache, dass anti-Nep4 Antikörper das Protein in diesen Stadien nicht mehr detektieren können, ist vermutlich durch ein sehr niedriges Expressionslevel in den genannten mesodermalen Geweben bedingt. Diese Annahme wird insbesondere durch die Ergebnisse einer semi-quantitativen PCR, bzw. Muskel-spezifischer Knock-downs unterstützt, die zeigen, dass Nep4 auch in der Muskulatur postembryonaler Stadien exprimiert und durch den bekannten Enhancer reguliert wird (Abb. 1 in Panz et al. submitted, diese Arbeit).

Durch Sequenzvergleiche des mesodermalen *nep4* Enhancers mit homologen Regionen von fünf weiteren *Drosophila*-Arten (ECR Browser, http://ecrbrowser.dcode.org/) konnten evolutionär konservierte Bereiche innerhalb der 950 Bp langen Enhancersequenz identifiziert und zur Erzeugung weiterer Reporterlinien verwendet werden. Vorläufige Ergebnisse belegen beispielsweise, dass eine konservierte Sequenz von etwa 360 Bp Länge eine den 950 Bp entsprechende Regulationsaktivität aufweist. Sowohl die vollständige mesodermale Enhancersequenz als auch die neu identifizierte verkürzte Sequenz bieten die Möglichkeit, DNA-Protein Interaktionsstudien durchzuführen um somit *nep4*-spezifische Transkriptionsfaktoren zu identifizieren.

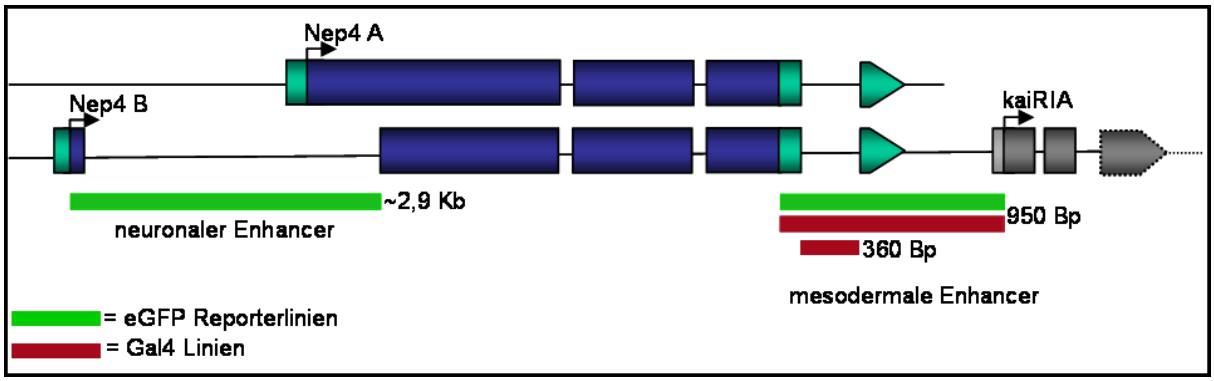

Abb. 8: Übersicht über die Lage der regulatorischen Elemente des *nep4* Gens. Das *nep4* Gen codiert für zwei Proteinisoformen. Die Transkripte der beiden Isoformen sind als blau plus türkis markierte Bereiche dargestellt, während nur der blau markierte Bereich translatiert wird. Die in grün bzw. rot dargestellten Sequenzabschnitte wurden zur Generierung transgener eGFP Reporter bzw. Gal4 Fliegenlinien verwendet.

Junion et al. (2007) konnten auf Basis von Mikroarray-Daten zeigen, dass die Expression von Nep4 vermutlich durch den Transkriptionsfaktor Ladybird reguliert wird. Das Homeobox Gen Ladybird gehört zu den Segmentpolaritätsgenen (Jagla 1994) und ist wie auch das Paarregelgen Even-skipped am Differenzierungsprozess von Herz und Muskelzellen beteiligt (Jagla et al. 1997; Jagla et al. 2002). Während Even-skipped aber teilweise mit den Nep4 positiven Muskelvorläuferzellen und den Perikardzellen kolokalisiert (diese Arbeit, Meyer et al. 2009), überlappt die Expression von Ladybird und Nep4 nicht, was die o.g. Ergebnisse des Mikroarrays stützt, wonach Ladybird die Expression des Nep4 Proteins im Herzen reprimiert (Junion et al. 2007). Erste Nep4 Antikörperfärbungen an Ladybird-Mutanten und Überexpressionstieren deuten des Weiteren darauf hin, dass Ladybird zusätzlich die Nep4 Expression in den Muskelvorläuferzellen inhibiert (unveröffentlicht, diese Arbeit).

Auf Basis von DNA- bzw. Protein-basierten Interaktionsdatenbanken (DroID, http://www.droidb.org/) wechselwirkt Nep4 möglicherweise ebenfalls mit den Faktoren Pox meso (Protein-Protein), sowie Chinmo, Knot, Scribbler und dem Zinkfingerprotein Zfh1 (Protein-DNA). Darüber hinaus werden Interaktionen mit einer Reihe von MikroRNAs vorhergesagt, wobei bislang keinerlei Informationen bezüglich der Effekte dieser Interaktionen vorliegen. Während die Untersuchung der Interaktion zwischen Nep4 und einigen der genannten Faktoren noch aussteht, deuten Fehlexpressionsexperimente von Zfh1 und Pox meso darauf hin, dass die Expression von Nep4 tatsächlich durch diese Faktoren reguliert wird (unveröffentlicht, diese Arbeit). Ob diese Regulation auf Transkriptions-, Translations- oder posttranslationaler-Ebene stattfindet, ist Gegenstand zukünftiger Untersuchungen. In diesem Zusammenhang erscheint sowohl die Verwendung der generierten Reporter- bzw. Gal4–Linien als auch die Analyse von Fehlexpressionsmutanten sinnvoll. Darüber hinaus ist die Durchführung von Interaktionsstudien auf Protein-Protein bzw. Protein-DNA Ebene notwendig.

5. Publikationsliste

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8. Lebenslauf

Persönliche Daten:

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Jan. 2007 - Febr. 2012:	Promotion, AG Entwicklungsbiologie, Universität Osnabrück, Thema: Funktionelle Charakterisierung der Metalloprotease Neprilysin 4 aus *D. melanogaster*
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9. Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Bei der Auswahl und Auswertung folgenden Materials haben mir nachstehend aufgeführte Personen in der beschriebenen Weise unentgeltlich geholfen:

Bei den Arbeiten an der Veröffentlichung: Meyer H, Panz M, Zmojdzian M, Jagla K, Paululat A (2009):

1. H. Meyer: Reinigung des Nep4-Antikörpers aus Serum; RT-PCR der *nep4* Transkripte (Abb. 2 A); Sequenzverleich (Abb. 7); Vergleich der katalytischen Taschen von humanem Nep, Nep2 und *Drosophila* Nep4 (Tabelle 1)
2. M. Zmojdzian, K. Jagla: *In situ* Hybridisierungen und Antikörperfärbungen der Abb. 5 A-L

Bei der Veröffentlichung: Meyer H, Von Ohlen T, Panz M, Paululat A (2010) führte ich folgende Arbeiten durch:

1. *In situ* Hybridisierungen Meltrin (Abb. 2)
2. Northern Blots Meltrin (Abb. 4)

Bei den Arbeiten an der Veröffentlichung: Panz M, Vitos Falleato J, Jendretzki A, Heinisch J, Paululat A, Meyer H. (2012) halfen mir folgende Personen bei den genannten Arbeiten:

1. J. Vitos Falleato: Larvale Kriech- und Kontraktionsassays
2. E. Hass-Cordes: Klonierung der intrazellulären Domäne von Nep4 in pET29b
3. K. Etzold: Nachfixierung und Weiterbearbeitung der Larven für das TEM, weitere TEM Arbeiten
4. A. Jendretzki und J. Heinisch: Durchführung des Y2H-Screens
5. H. Meyer: Vorversuche zur Reinigung der Histidin-markierten intrazellulären Nep4 Domäne

Weitere Personen waren an der inhaltlichen materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

(Ort, Datum) (Mareike Panz)