Screening for touch receptor genes

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Dedicated to my family and well wishers…

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ABSTRACT

Somatosensory neurons of Dorsal Root Ganglia (DRG) can be divided in to three types, low threshold mechanoreceptors, nociceptors, responding to harmful mechanical, thermal and chemical stimulations and proprioceptors detecting muscle tension and joint position. Conversion of mechanical force into electrical force is attained by putative mechanotransduction complexes, which could be formed by extracellular matrix (ECM) proteins, ion channels and other associated membrane proteins, and intracellular cytoskeletal proteins. In *C.elegans* it is known that proteins from all the three different categories like TRP-4, OSM-9/OCR-2, MEC-4/MEC-10, MEC-2/MEC-6, UNC-24, MEC-1, MEC-5, MEC-9, MEC-7, MEC-12 affect mechanosensation (Reviewed O'Hagan and Chalfie, 2006). In mammals many ion channels like ASIC1, ASIC2, ASIC3, TRPA1, TRPM8, TRPV1, TRPV2, TRPV3, TRPV4, TREK-1, CaV3.2 (Reviewed by Lumkin and Caterina, 2007) and membrane associated protein like SLP3 (Wetzel, et al., 2007) were shown to affect mechanotransduction. DRG neurons require neurotrophins (NT) for their development, differentiation and (or) survival. DRG neurons are diverse and can be classified broadly as Aß-, Aδ-, or C-fibers according to myelination and conduction velocities. Different types of fibers are reported to be affected by the lack of different NTs. NGF affects Aδ-fiber (Lewin, et al., 1992), C-fiber heat nociceptors (Lewin, and Mendell, 1994), NT3 is necessary for the survival of proprioceptive neurons (Ernfors, et al., 1994), slowly adapting mechanoreceptors (SA) and D-hairs (DH) (Airaksinen, et al., 1996), BDNF for SA functions (Koltzenburg, et al., 1997), and NT4 affects survival of DH (Stucky, et al., 1998, 2002). As different NTs affect neurons with different functional modalities, we tried to isolate genes altered in their expression and from DRGs of NT mutants. NT3+/-//NT4-/- animals (NT34 mutants) were used. We assumed that if the gene expressed in a subpopulation of cells, they might be involved in their function.

In the first part of my Ph.D work, a high through put *in situ* hybridization technique was developed for adult wholemount DRGs using an enriched library for the transcripts influenced by BDNF in DRG neurons. We could isolate many genes that had a subpopulation specific expression pattern. We found out that many of these genes had an altered expression in BDNF+/- animals in comparison to their wild type littermate DRGs. In the DRGs of adult BDNF +/- animals, genes like Zwint, Tubb5, Spock1, Prkar1a, Hsp90ab1, and Aurkaip 1 were found to be down regulated and Paqr5 and Fstl1 were upregulated. Some other genes like Vbp1, Tmem50a, Sncg, mKIAA0528, Hspa8, ATPsynthase 6, Anxa6, and A230083H22 did not show any change.

In the second part of my work, NT34 mutants were characterized by electrophysiological, anatomical, molecular and behavioral experiments. Electrophysiological characterization of different sensory afferents was done using an *in vitro* skin nerve preparation for their sensitivity to different stimulus strengths. Aß sensory afferent were also tested for their sensitivity for the movement of the probe. There seemed to be no change in the functional properties of NT34 mutant neurons in comparison to the control neurons. Electron microscopy results show a profound loss of both myelinated (62.57%) as well as unmyelinated (68%) neurons. Many genes Tuba1, Thymosin beta, Hsp90ab1, etc., in the subtracted library were found to be expressed by subpopulation of DRG neurons. Experiments using affymetrix gene chip yielded 83 down regulated genes and 50 upregulated genes in the NT34 mutant DRGs compared to control DRGs. Among these, 20 genes from the down regulated and 38 genes from the up regulated genes showed a subpopulation specific expression pattern. Two genes, Secreted phosphoprotein 1 (SPP1) and Synaptogamin11 (Syt11) that were down regulated in the gene chip experiment were observed to be increased in the subtracted pool of transcripts enriched for the down regulated transcripts in NT34 mutants. qPCR results showed that two genes ramp2 and non annotated transcript (114131_at) to be up regulated in the NT34 mutants. Behavioral experiments to test tactile discrimination of surfaces did indicate deficits.

A null mutation of Secreted phosphoprotein (SPP1) was characterized with respect to mechanotransduction as the third part of my thesis. SPP1 was observed to be expressed mostly in medium sized cells. Electrophysiological characterization by using *in vitro*.skin nerve preparation showed a putative increase in the sensitivity of AM fibers to increasing stimulus strength (increasing the magnitude of indentation). But there was no other change observed in the mechanical withdrawal or thermal latencies. Initial results suggest that these animals do not seem to recognize fine textures (250 µm) unlike the controls in the grid based tactile acuity tests, which has to be verified.

The fourth part of my work was by using a T7 phage display library of the skin, identification of putative binding partners in the extracellular matrix for mechanosensitive ion channel ASIC3. ASIC3 was expressed in *X. leavis* oocytes and phages were adsorbed and amplified repeatedly for enriching the phages that could bind to the ion channel. After four rounds, some phages that displayed ECM protein parts were isolated that were adsorbed only by the oocytes expressing the ion channels. Some of these putative binding proteins of our interest are keratin 10, SPARC, an Ankyrin repeat containing protein (Ankrd44), and CLIC5.

ZUSAMMENFASSUNG

Somatosensorische Neurone des Dorsalwurzelganglions (DRG) lassen sich in drei Klassen einteilen: solche, die drucksensitiv sind, Nozizeptoren, die auf schädliche mechanische, thermische und chemische Stimuli reagieren und Propriorezeptoren , die Muskelspannungen und Gelenkpositionen erkennen. Umwandlung von mechanischer in elektrischer Kraft wird von dem Mechanotransduktionskomplex bewerkstelligt, welcher aus Proteinen der Extrazellulären Matrix (ECM), Ionenkanälen und anderen membranassoziierten Proteinen, sowie Proteinen des intrazellulären Zytoskeletts bestehen könnte. Bei *C. elegans* sind Proteine aller drei Kategorien beschrieben worden, die die mechanische Empfindlichkeit beeinflussen, wie TRP-4, OSM-9/OCR-2, MEC-4/MEC-10, MEC-2/MEC-6, UNC-24, MEC-1, MEC-5, MEC-9, MEC-7 und MEC-12 (Übersicht in O'Hagan und Chalfie, 2006). Bei Säugetieren wurde gezeigt, dass viele Ionenkanäle, wie ASIC1, ASIC2, ASIC3, TRPA1, TRPM8, TRPV1, TRPV2, TRPV3, TRPV4, TREK-1, CaV3.2 (Übersicht bei Lumkin und Caterina, 2007) und membranassoziierte Proteine, wie SLP3 (Wetzel, et al., 2007) die Mechanotransduktion beeinflussen. DRG-Neurone benötigen Neurotrophine (NTs) für ihre Entwicklung, Differenzierung und / oder ihr Überleben. Es gibt verschiedene DRG-Neurone, die grob in Aβ-, Aδ- und C-Fasern unterteilt werden können, je nach dem Grad der Myeliniserung und der Leitungsgeschwindigkeit. Es wurde berichtet, dass unterschiedliche Fasern vom Fehlen unterschiedlicher NTs betroffen sind. NGF betrifft Aδ-Fasern (Lewin, et al., 1992) und C-Faser-Hitze-Nozizeptoren (Lewin, and Mendell, 1994), NT-3 ist für das Überleben von propiorezeptiven Neuronen (Ernfors, et al., 1994), langsam adaptierenden Mechanorezeptoren (SA) und D-Hairs (DH) (Airaksinen, et al., 1996) notwendig, BDNF für die Funktion von SAs (Koltzenburg, et al., 1997) und NT4 beeinflusst das Überleben von DHs (Stucky, et al., 1998, 2002). Da verschiedene NTs Neurone mit unterschiedlichen Funktionsweisen beeinflussen, versuchten wir Gene zu isolieren die in ihrer Expression in DRGs von NT-Mutanten verändert sind. Dazu wurden NT3+/-/NT4-/- -Tiere (NT34-Mutanten) verwendet. Wir nahmen an, dass ein Gen, das in einer Subpopulation von Zellen exprimiert ist, an der Funktion dieser Subpopulation beteiligt ist.

Im ersten Teil meiner Doktorarbeit wurde eine *high throughput-in situ*-Hybridisierungstechnik für ganze adulte DRGs entwickelt, die mit einer cDNA-Bank durchgeführt wurde, die mit Transkripten angereichert war, die in DRGs durch BDNF beeinflusst werden. Wir konnten viel Gene mit einem Subpopulationsspezfischen Expressionsmuster isolieren. Wir fanden heraus, dass viele dieser Gene eine veränderte Expression in BDNF +/- -Tieren im Vergleich zu Wildtyp -Tieren des gleichen Wurfs hatten.

In den DRGs von adulten BDNF +/- -Tieren waren Gene, wie Zwint, Tubb5, Spock1, Prkar1a, Hsp90ab1, und Aurkaip 1 herunterreguliert und Paqr5 und Fstl1 waren heraufreguliert. Einige andere Gene, wie Vbp1, Tmem50a, Sncg, mKIAA0528, Hspa8, ATPsynthase 6, Anxa6, und A230083H22 zeigten keine Veränderung.
Im zweiten Teil meiner Arbeit wurden NT34-Mutanten mit elektrophysiologischen, anatomischen, molekularen und Verhaltensexperimenten Charakterisiert. Die elektrophysiologische Charakterisierung der verschiedenen sensorischen Afferenzen erfolgte mit einer *in vitro*-Hautnerv-Präparation mit der die Sensitivität für verschieden starke Stimuli bestimmt wurde. Aß sensorische Afferenzen wurden außerdem auf ihre Sensitivität hinsichtlich der Bewegung der Sonde untersucht. Es schien keine Veränderung der funktionellen Eigenschaften von Neuronen von NT34-Mutanten gegenüber denen von Kontroll-Neuronen zu geben. Die Ergebnisse der Elektronenmikroskopie zeigten einen schweren Verlust von myelinisierten (62,75 %), sowie unmyelinisierten (68%) Neuronen. Viele Gene der subtrahierten cDNA-Bank, wie Tuba1, Thymosin beta, Hsp90ab1, etc., zeigten eine Expression in einer Subpopulation von DRG-Neuronen. Experimente mit Affymetrix Gen-Chips ergaben 83 herunterregulierte Gene und 50 heraufregulierte Gene in den DRGs von NT34-Mutanten im Vergleich zu denen von Kontroll-DRGs. Von diesen zeigten 20 von den herunterregulierten und 38 von den heraufregulierten Genen ein subpopulationsspezifisches Expressionsmuster. Zwei Gene, die im Chip-Experiment herunterreguliert waren, Secreted phosphoprotein 1 (SPP1) und Synaptogamin11 (Syt11), waren verstärkt in der subtrahierten cDNA-Bank vorhanden, die mit Transkripten angereichert waren, die in NT34-Mutanten herunterreguliert waren. qPCR-Ergebnisse zeigten, dass zwei Gene, ramp2 und ein nicht benanntes Tanskript (114131_at) in NT34-Mutanten heraufreguliert waren. Verhaltensexperimente zur Oberflächendiskriminierung lieferten keine zuverlässigen Ergebnisse, da diese Tiere weniger aktiv waren.
Eine Secreted phosphoprotein (SPP1) Doppelmutante wurde im dritten Teil meiner Arbeit hinsichtlich der Mechanotransduktion charakterisiert. SPP1 zeigt vorwiegend eine Expression in mittelgroßen Zellen. Die elektrophysiologische Charakterisierung mittels der *in vitro*-Hautnerv-Präparation zeigte eine mutmaßliche Verstärkung der Sensitivität von AM-Fasern bei ansteigender Stimulusstärke (Vergrößerung des Ausmaßes des Eindrückens). Eine Veränderung der Vermeidungsreaktion von mechanischen Stimuli oder der Latenz bei der Vermeidung von Hitze-Stimuli konnte nicht beobachtet werden. Vorläufige Ergebnisse lassen vermuten, dass die Tiere feine Oberflächentexturen (250 μM), anders als die Kontroll-Tiere, nicht wahrnehmen können. Dies muss noch bestätigt werden.

Der vierte Teil meiner Arbeit bestand darin, mit Hilfe einer *T7 phage display library* der Haut, potentielle Bindungspartner des mechanosensitiven Ionenkanals ASIC3 in der extrazellulären Matrix zu identifizieren. ASIC3 wurde in Oozyten von *X. leavis* exprimiert und Phagen wurden adsorbiert und wiederholt die Phagen vermehrt, die an den Ionenkanl binden können. Nach vier Runden wurden Phagen isoliert, die Teile von ECM-Proteinen exprimierten und nur an die Oozyten adsorbierten, die den Ionenkanl exprimierten. Einige der dieser potentiellen Bindungspartner sind Keratin 10, SPARC, ein *Ankyrin-repeat* enthaltendes Protein namens Ankrd44, und CLIC5.

Der vierte Teil meiner Dissertation beschäftigt sich mit der Identifikation von mutmaßlichen Bindungspartnern in der Extrazellulären Matrix für den mechanosensorischen Ionenkanal ASIC3 unter Verwendung einer T7 „phage display library“ der Haut. ASIC3 ist in *X. leavis* Oozyten exprimiert; die Phagen wurden zur Anreicherung adsorbiert und wiederholt verstärkt, um den Ionenkanal zu binden. Nach vier Runden wurden Phagen isoliert, die ECM-Protein-Anteile von Ionenkanälen aufwiesen, die nur durch die Oozyten exprimiert werden konnten. Einige dieser mutmaßlichen Bindungsproteine interessieren uns besonders: Keratin 10, SPARC, ein Ankyrin-beinhaltendes Protein (Ankrd44), CLIC5.