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MASTERARBEIT

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„Role of the α5 nAChR subunit during pain processing mechanisms in spinal and supraspinal regions"

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Danksagung…

…an meine Familie, die immer für mich da ist und mich bedingungslos unterstützt.

…an Petra, die mich in dem Jahr meiner Masterarbeit im Zentrum für Hirnforschung immer mit vollem Einsatz betreut hat und mir beigebracht hat, wie man professionell Wissenschaft betreibt.

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Abstract

Nicotinic acetylcholine receptors (nAChR) are found in spinal cord dorsal horn and various brain areas associated with pain and analgetic responses to nicotine. These receptors are made up by 9α and 3β subunits that can assemble into a multitude of different homo- or hetero-pentameric receptors, with the biochemical and electrophysiological properties being greatly influenced by the subunit composition. The subtype(s) of nicotinic acetylcholine receptors, which are involved in maintaining neuropathic pain have not been unveiled up to date. One subunit suggested in playing an important role is α5 at the spinal and supraspinal level. The α5 nAChR subunit is a unique subunit because it cannot form functional nAChRs when expressed alone or with β nicotinic subunits, but its incorporation into the pentameric receptor has a major influence on the receptor function.

The aim of this study was to further resolve the role and function of the α5 nAChR subunit at the spinal and supraspinal level in neuropathic pain states.

Wild-type and α5 (-/-) mice were analyzed in an experimental model of neuropathic pain (partial sciatic nerve ligation; PSNL), but behavioral tests revealed no difference in nociception at any time after nerve ligation. In both mouse lines (wild-type and α5 (-/-)), nicotine, a potent analgetic was then injected subcutaneously 4 days post injury to examine the effect of α5 nAChR subunit deletion on nociception. Our data indicate a difference in the sensitivity of both mouse lines 4 days after PSNL induction to subcutaneously injected nicotine, when compared to vehicle-treated mice. When comparing wild-type and α5 (-/-) mice, however no different behavior to nicotine injection 4 days post PSNL could bee seen. In contrast, I could show a significant difference in the antinociceptive activity of nicotine between wild-type SHAM mice and α5 (-/-) SHAM mice at 15 and 20 minutes post injection.

The level of a5-containing receptors was analyzed in several different spinal and supraspinal regions using Immunoprecipitation (IP) of 3H-eptibatidine labeled nAChRs. No difference of the α5 nAChR subunit expression in spinal and specific supraspinal regions was observed between naïve wild-type mice and wild-type mice at particular days post PSNL.

Taken together, results of the study suggest that α5 nAChRs does not play a major role in pain-processing mechanisms in spinal and supraspinal regions in neuropathic pain states. However, based on our results and taken into account the data from previous studies, the α5 nAChR subunit seems to be involved in nicotine-mediated behaviors and further, its presence seems to be important to the perception of nociception in case of systemically applied nicotine.

Zusammenfassung

Nikotinische Azetylcholinrezeptoren (nAChRs) findet man in hoher Dichte in vielen Hirnregionen, die eine wesentliche Rolle in der Verarbeitung von Schmerzreizen spielen. Diese Rezeptoren sind Pentamere, wobei im Nervensystem 5 Untereinheiten aus einer Auswahl von 9α und 3β Untereinheiten assemblieren. Die Untereinheiten-Zusammensetzung bestimmt die pharmakologischen und biophysikalischen Eigenschaften des gebildeten Ionenkanals. Die bei der Erhaltung des neuropathischen Schmerzes beteiligten Untereinheiten des nAChRs, sind bis heute nicht bekannt. Eine Untereinheit, die in diesem Zusammenhang immer wieder genannt wird, ist α5. Die α5 nAChR-Untereinheit ist etwas besonderes, da sie alleine oder mit β nAChR-Untereinheiten exprimiert selbst keine funktionsfähigen nAChRs bilden kann. In einen pentameren Rezeptor integriert, verändert die α5 nAChR-Untereinheit allerdings die Eigenschaften des Rezeptors wesentlich.

Ziel dieser Arbeit war die Aufgabe und Funktion der α5 nAChR-Untereinheit in einem experimentellen neuropathischen Schmerzmodell sowohl auf spinaler als auch auf supraspinaler Ebene zu untersuchen.

Wildtyp und α5 (-/-) Mäuse wurden nach einer experimentell hinzugefügten Nervenverletzung (partial sciatic nerve ligaton, PSNL) in verschiedenen Schmerztests untersucht. Die Verhaltenstests zeigten jedoch zu keinem Zeitpunkt einen Unterschied in der Schmerzwahrnehmung beider Mauslinien.

In einem zweiten Experiment wurde Nikotin, ein bekannt starkes Analgetikum injiziert, um zu sehen, ob die experimentellen neuropathischen Schmerzen sowohl in wildtyp als auch in α5 (-/-) Mäusen gelindert werden können. Meine Ergebnisse deuten darauf hin, dass alle Mäuse 4 Tage nach der Nervenverletzung sensitiver auf subkutanes Nikotin reagieren als die SHAM operierten Kontrolltiere. Dies steht im Einklang mit schon existierenden Daten. Vergleicht man wild-typ Mäuse mit α5 (-/-) Mäusen, so konnte bei den PSNL-operierten Tieren nach Nikotininjektion kein Unterschied im Schmerzverhalten festgestellt werden. Bei den SHAM operierten Tieren konnte allerdings 15-20 min nach Nikotininjektion ein signifikant unterschiedliches Verhalten zwischen wild-typ und α5 (-/-) Mäusen gemessen werden.

Zum Abschluss wurden die Mengen der α5 nAChR-Untereinheit, sowie die Gesamtzahl an nAChR in Gehirn und Rückenmark mittels Immunpräzipitationstechnik untersucht. Es konnte allerdings in keiner der untersuchten Regionen ein signifikanter Unterschied zwischen operierten und nicht-operierten Tieren gefunden werden.

Zusammenfassend weisen unsere Ergebnisse darauf hin, dass α5 hältige nAChRs keine wesentliche Rolle in der Verarbeitung des neuropathischen Schmerzes spielen. Dennoch scheint die α5 nAChR Untereinheit in von Nikotin-induzierte Verhalten involviert zu sein, und des Weiteren scheint ihre Präsenz im Falle von subkutan injiziertem Nikotin wichtig für die Perzeption von Schmerz zu sein.

1 Introduction

1.1 The nicotinic acetylcholine receptor

The nAChR was discovered in 1914 by Henry H. Dale and Otto Loewi. In general, two main classes of pentameric acetylcholine receptors, which trigger various processes when bound by the endogenous neurotransmitter acetylcholine (ACh), exist in neuronal and nonneuronal cells. The first class is a G-protein coupled metabotropic receptor, the muscarinic acetylcholine receptor (mAChR), which gets activated by muscarine, a toxin found in *Amanita muscaria* mushrooms and blocked by a the toxin atropine from *Atropa belladonna* the deadly nightshade family, a very poisonous plant with small black shiny fruits, which grows in Europe, North Africa and West Asia. The second class is the nAChR, a ligand-gated ion channel, which, among other ligands, is opened by the tobacco alkaloid nicotine, (Figure 1). Although, muscarinic AChRs become activated relatively slowly (milliseconds to seconds), the activation of nAChRs takes micro to submicroseconds (Albuquerque *et al.* 2009). nAChRs can further be divided into the muscle receptors, positioned at the skeletal neuromuscular junction where they convey neuromuscular transmission, and the neuronal receptors present in the central and peripheral nervous systems (Hogg *et al.* 2003). Due to the fact, that ionotropic nAChRs conduct monovalent Na^+ and K^+ ions and further divalent Ca^{2+} ions, their action at the neuronal synapse is excitatory. Further, their capacity to change intracellular Ca^{2+} through the activation of diverse intracellular downstream pathways features a key role in neuron signaling. Although each group of nAChRs shares a common basic structure and different subunit combinations, they produce highly distinctive functional and pharmacological subtypes. nAChRs are therefore involved in a vast range of physiological functions in the peripheral and central nervous system and variations in the function and/or number correlate with a number of pathophysiological conditions (Gotti *et al.* 2009). Among others, one of the most important roles of nAChRs in the CNS has been suggested to be the regulation of transmitter release (Wonnacott 1997).

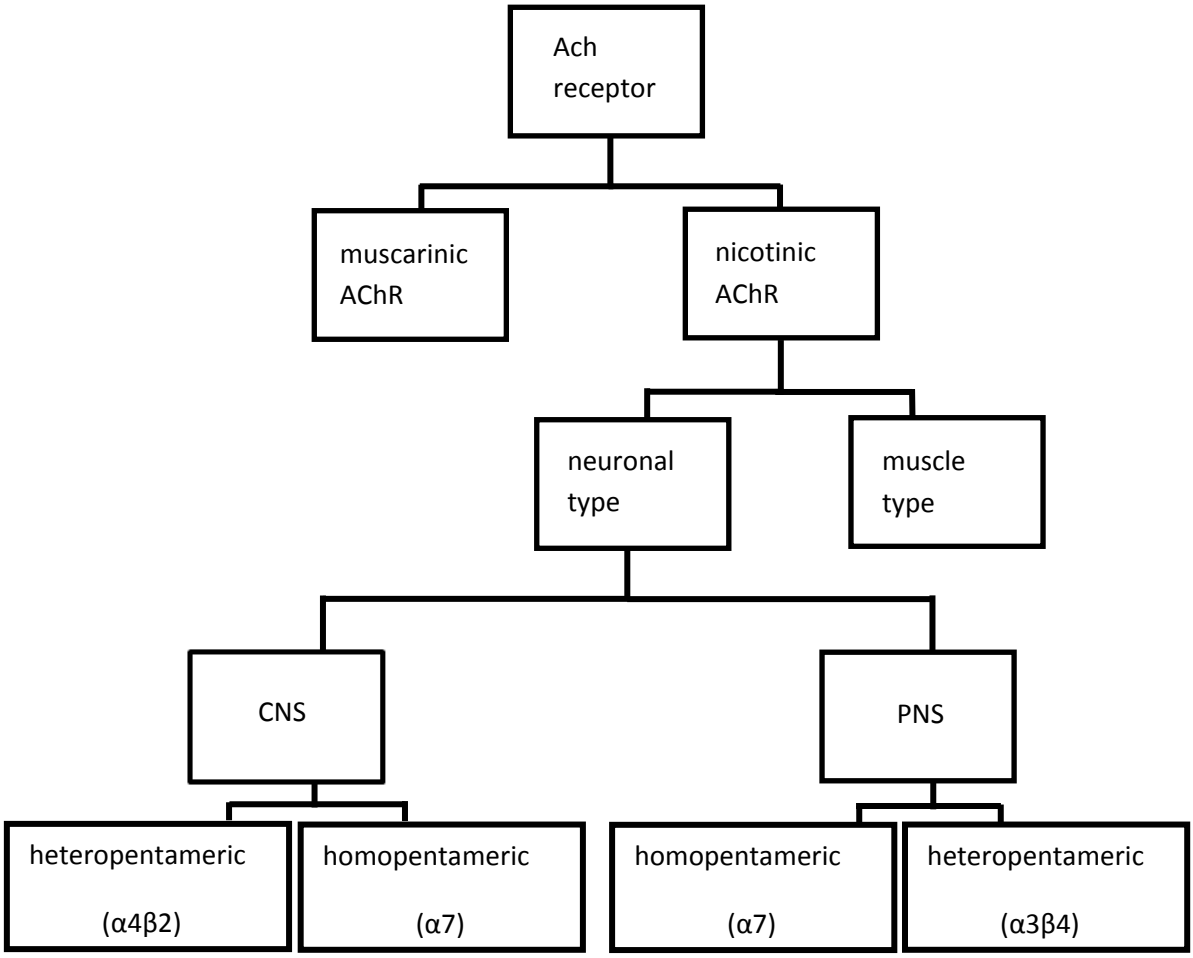

Figure 1: Family of the acetylcholine-receptor: Acetylcholine-receptors are divided in the mAChRs, also defined as metabotropic acetylcholine receptors and particularly responsive to muscarine, and the nAChRs or ionotropic acetylcholine receptors specificially responsive to nicotine. The nAChRs can further be divided in muscular and neuronal type of receptors. The neuronal type of nAChR can build homo-pentameres and hetero-pentameres in regions of the CNS as well as the PNS.

1.2 Structural composition and diversity of the hetero-pentameric neuronal nicotinic acetylcholine receptor

Neuronal nAChRs are a heterogeneous family of subtypes composed out of five distinct membrane-spanning subunits (α and β) surrounding a central aqueous pore (Gotti *et al.* 2009). Each of these five subunits features a conserved hydrophilic extracellular NH_2-terminus, three conserved hydrophobic transmembrane domains (M1-M3), a vast intracellular loop and a fourth hydrophobic transmembrane domain with a relatively short and diverse extracellular COOH-terminal sequence (Albuquerque *et al.* 2009)

Activation of nAChRs in response to the binding of two agonists, e.g. the endogenous neurotransmitter Acetylcholine (ACh) and an exogenous ligand such as nicotine, causes the temporary reorganization of each of these five nAChR subunits for a few milliseconds leading to cationic trafficking via the central pore. After the opening of a pentameric nAChR, the receptor closes to a non-conducting state.

Neuronal nAChRs additionally belong to the Cys-loop superfamily, which include $GABA_A$, $GABA_C$, glycine and serotonin 5HT3-receptors. At present, twelve neuronal nAChR subunits have been identified, nine diverse α subunits (α2- α10) and three different β subunits (β2-β4) which are expressed in the nervous system and in various non-neuronal tissues (Gotti *et al.* 2006). These subunits can assemble into homopentamers by incorporating five times the same α subunit, (α7- homopentamers represent the most common form in the mammalian nervous system) or heteropentamers by integrating different α (α2-α6) and β-subunits (β2-β4) (Figure 2).

Two adjacent cysteine residues of an α subunit build the primary face of the binding pocket for nicotinic agonists, further essential for ACh binding. The β subunits β2 and β4, which lack these cysteine residues, form the complementary face of the binding site, which is hence positioned between α and β subunits (Fowler *et al.* 2008). Therefore, even though all hetero-pentameric nAChRs can assemble into a magnitude of various subtypes, every hetero-pentameric nAChR has to be comprised of α- and β-subunits.

The stoichiometry of pentameric nAChRs is restrained to either $(\alpha)_3(\beta)_2$ or $(\alpha)_2(\beta)_3$, based on the fact that the two ligand binding sites of hetero-pentameric nAChRs are positioned at the junction between an α- and a β-subunit. Due to the fact that, some subunits avoid co-integration into a native functional hetero-pentameric nAChR, whereas others show a specific affinity for particular subunits according to a previous investigation, there are less hetero-pentameric nAChR subtypes expressed in vivo than theoretically possible (Gotti *et al.* 2006).

The α3β4-receptors have been demonstrated to be highest frequent subtype in the PNS (´ganglionic type´), whereas theα4β2 -receptors represent the brain type, due to the fact that they have been shown to dominate nearly all regions of the brain.

The subunits β3 and α5 by contrast are not able to take part in building a binding site with a partner subunit and hence can only be incorporated into receptors, which exhibit at the minimum two more subunits (e.g. α4β2β3, α4β2α5). Based on these properties, the two subunits are known as ´supplementary subunits´ (Gerzanich *et al.* 1998; Ramirez-Latorre *et al.* 1996; Fucile *et al.* 1997). Therefore, the role of the α5 subunit is limited to a modulatory when assembled into functional nAChRs, controlling the activation and desensitization kinetics and other features of nAChRs (Girod *et al.* 1999; Nelson and Lindstrom 1999). In the superior cervical ganglion (SCG) of wild-type C57BL/6J mice, the α5 subunit integrates only into receptors containing the subunits α3 and β4 (David *et al.* 2010). However, in various parts of the CNS such as the thalamus, the cerebral cortex, the hippocampus or the striatum, α5 only assembles into α4β2 receptors (Mao *et al.* 2008). In the mouse habenula, all α5 subunits congregate into α3α5β4β2-receptors (Grady *et al.* 2009; Scholze *et al.* 2012).

Recent studies have begun to distinguish the nAChR subunits expressed by individual neurons in the brain. Hence, the subunits α2, α3, α4, α5, α6, α7, β2, and β3 have been demonstrated to be expressed in brain regions that are known to play a role in the

reinforcing effects of addictive drugs and in the cognitive and emotional deficits linked to diverse neuropsychiatric disorders (Fowler *et al.* 2008).

Further, the role of the supplementary subunits has been examined in the α4β2* (where the * denotes the possible inclusion of additional nAChR subunits) subtypes in which the presence of different accessory subunits (α5, β3, α4, β2) alters their pharmacological and biophysical characteristics, their sensitivity to allosteric modulators and their sensitivity to up-regulation (Kuryatov *et al.* 2008; Moroni *et al.* 2006; Tapia *et al.* 2007; Moroni *et al.* 2008).The $(\alpha4\beta2)_2\alpha5$ subtype has been shown to have the highest Ca^{2+} permeability (Kuryatov *et al.* 2008).

Furthermore, in the α3β2*and α3β4* subtypes the inclusion of the α5 subunit increases desensitisation and Ca^{2+} permeability, and further alters agonist-stimulated responses (Tapia *et al.* 2007). Interestingly, data is emerging that proposes a role for the α5 subunit in nicotine's behavioral effects. The α4β2α5 nAChR subtype has been shown to be implicated in nicotine-stimulated dopamine release in the striatum (Salminen *et al.* 2004), and to be expressed in brain areas involved in nicotine-induced behaviors (Jackson *et al.* 2010). In addition, the α4β2α5 nAChR subtypes have been demonstrated to mediate an important portion of the α-conotoxin MII-resistant dopamine release in striatal synaptosomes (Grady *et al.* 2010). Further, a decreased sensitivity to nicotine-mediated seizures and hypolocomotion has been seen in α5 (-/-) mice (Salas *et al.* 2003; Kedmi *et al.* 2004). Finally, α5 (-/-) mice display diminished somatic signs of nicotine withdrawal compared with wild-type littermates (Jackson *et al.* 2008; Salas *et al.* 2009) indicating moreover a potential role for α5* nAChRs in nicotine dependence.

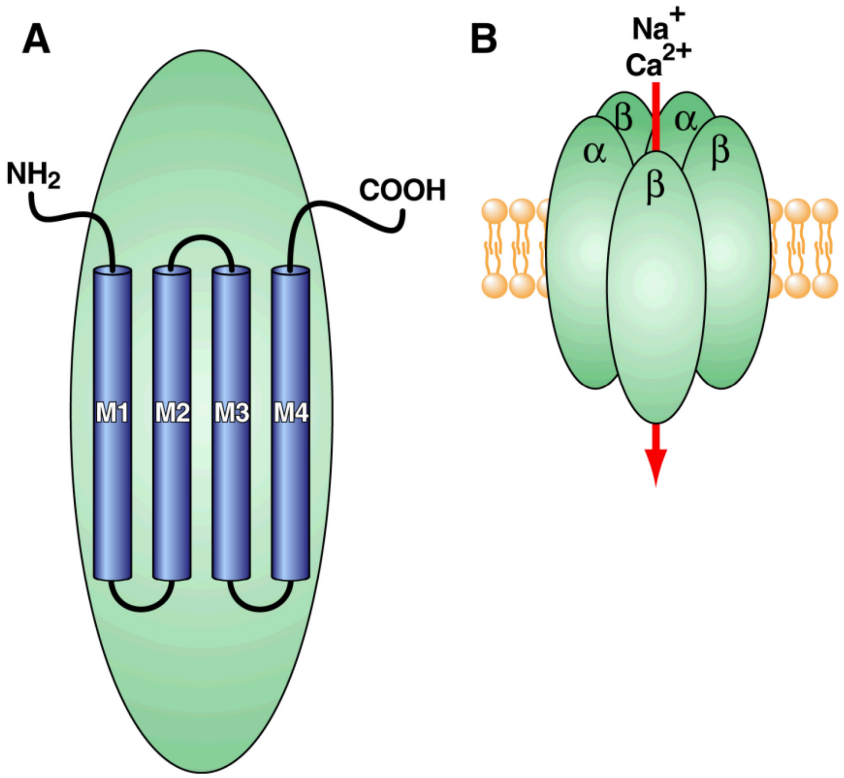

Figure 2: Structure of the hetero-pentameric nicotinic acetylcholine receptor: A demonstrates the four trans-membrane domains (TM1-TM4) of one of normally five nAChR subunits, spanning four times through the plasma-membrane which results in one extracellular and two intracellular loops. Each nAChR subunit further exhibits an extracellular N-and C-terminus.**B** shows a pentameric nAChR composed of five distinct membrane-spanning subunits. Depicted is further the possibility of cationic trafficking in case of receptor activation (Scheme retrieved from (Fowler *et al.* 2008).

1.3 Current tools to identify individual hetero-pentameric neuronal nicotinic acetylcholine receptor subtypes

1.3.1 Ligands – Agonists and their affinity to various hetero-pentameric nAChR

In addition to acetylcholine, many diverse plant and animal toxins bind the hetero-pentameric nAChR in the PNS and CNS. Therefore, a vast array of various synthetic ligands has emerged, due to the prospect of nAChRs as possible therapeutic targets. Besides nicotine, epibatidine a chloropyridine natural product isolated from the venom of the "poison arrow" frog (Epipedobates tricolor) is among the most potent nAChR ligands known to date. It binds the hetero-pentameric nAChR with very high affinity and therefore depicts a useful agonist for immunoprecipitation studies (Badio and Daly 1994). The affinity of nACh receptors to nicotinic agonists is crucial and can be exploited to differ between various subtypes of the hetero-pentameric nAChR. All agonists vary in their affinity (K_d = dissociation constant) for the binding sites of hetero-pentameric nAChR. Hence, previous investigations show that all nicotinic agonists bind the α4β2 receptor (´brain type´) with higher affinity than the α3β4 receptor (ganglionic type´) (Xiao *et al.* 1998; Perry *et al.* 2002). However, the α-

subunit is only minimally influencing the K_d of nicotinic agonists for hetero-pentameric nAChR. Hence, diverse α-associations with the same β-subunit cannot be easily identified. Moreover, up to date there are no identified α5 subunit selective ligands (Vincler and Eisenach 2004). Even though specificity of different synthetic ligands for selective hetero-pentameric nAChR subtypes has been previously proposed, this synthetic ligand specificity has been shown to be contradictory and deficient (Gao *et al.* 2010). A vast array of nAChR ligands may be more selective for certain nAChR subtypes, but they nevertheless display binding affinities for other hetero-pentameric nAChR-subtypes (Fowler *et al.* 2008).

1.3.2 Hetero-pentameric nAChR subunit specific antibodies

Due to the lack of specific ligands, subunit-specific antibodies are crucial for assessing the subunit composition of hetero-pentameric nAChR subtypes. However, the specificity of commercially available antibodies for nicotinic subtypes has been questioned in the literature and by previous studies. For example antibodies raised against the α3-, α4-, α7-, β2-, and β4-nicotinic acetylcholine receptor subunits on brain tissues of the respective knock-out mice displayed the same immunoreactivity in wild-type and in knockout (KO) mice (Moser *et al.* 2007; Herber *et al.* 2004). Hence, our group has previously generated polyclonal IgG antibodies specifically recognizing only defined subunits of the hetero-pentameric nAChR (Scholze *et al.* 2011; David *et al.* 2010). All of these antibodies target an epitope on the intracellular loop between the trans-membrane domains 3 and 4. The trans-membrane domains represent the most diverse protein sequence between α- and β-subunits. With the exception of anti-α3, all antibodies were tested on native receptors of WT mice (positive controls) and further on neuronal materials of appropriate KO animals (negative controls). Those controls have shown to be essential in order to eliminate false-positive results (Gotti *et al.* 2006; Moser *et al.* 2007; David *et al.* 2010; Scholze *et al.* 2011).

1.3.3 Knockout mice for the hetero-pentameric nAChR

In order to identify the function of selective subtypes, several constitutive KO mice for one or more of the hetero-pentameric nAChR have also been generated. Therefore, the consequences of the deletion of a specific subunit can be analyzed, as it has been done in previous studies (Jackson *et al.* 2010). Additionally, via the use of KO mice, analysis of specific receptor subtypes in certain tissues can be performed and properties of specific subtypes can be assessed in vivo.

1.4 Anatomical distribution of the hetero-pentameric neuronal nicotinic acetylcholine receptor in the CNS

The diversity and regional distribution of nAChR subtypes in the brain and the entire gray matter of the spinal cord contribute to the different functions that these receptors play in the central nervous system (CNS). With the use of immunoprecipitation (IP) and/ or immunopurification assays in tissue from rat and/or WT and/or KO mice the main nicotinic receptor subtypes present in the cortex, cerebellum, hippocampus, interpeduncular nucleus medial habenula and pineal gland have been identified (Gotti *et al.* 2006). The nAChR subtypes present in the amygdala, hypothalamus, locus coeruleus, olfactory bulb, raphe nuclei, substantia nigra-ventral tegmental area and thalamus have been identified via single cell-PCR, *in situ* hybridization and binding studies of tissues from rat and/or WT and/or receptor subunit KO mice (Hogg *et al.* 2003; Champtiaux *et al.* 2003; Gotti *et al.* 2005; Zoli *et al.* 2002). Although nAChRs are scattered over the entire gray matter of the spinal cord, they show their highest density in the dorsal horn of the spinal cord (Khan *et al.* 2003). With the help of various selective agonists and antagonists, limited data about the existence of a few subtypes in the spinal cord has been provided (Khan *et al.* 2001; Young *et al.* 2008). A detailed overview of the frequency and combination of subunits and their distribution in the entire spinal cord of 18 day old WT mice was recently performed (Grössl *et al.* 2013). It was demonstrated that 85% of the receptors consist of the subunits α4 and β2, whereas α3 co-assembles with β4 to represent 10% of receptors. The subunits α2 and α5, on the other hand, occur at low numbers only 2, 5% and 2%, respectively. The α6 and α7 subunits do not exist at all in the spinal cord of 18 day old WT mice. Further, the α5 nAChR subunit in the spinal cord mostly associates with the receptor subunits α4 and β2 (Grössl *et al.* 2013). This is in analogy to several parts of the CNS such as the hippocampus, the striatum, the cerebral cortex and the thalamus, which also express receptors that contain α5 in combination with the α4 and β2 subunits (Mao *et al.* 2008). In contrast, in the superior cervical ganglion (SCG) of WT mice, α5 assembles only into α3β4 receptors (David *et al.* 2010). In the habenula, α5 co-assembles not only with β2 but also with β4 (Grady *et al.* 2009), Scholze et al 2012).

1.5 Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”

(http://www.iasp-pain.org/Content/NavigationMenu/GeneralResourceLinks/PainDefinitions/default.htm#Pain).

Pain is usually evoked by noxious stimuli, which activate specialized pain receptors (nociceptors) in the peripheral nerve. Pain is crucial for avoiding damaging situations, the maintenance of homeostasis and most importantly for survival. Specifically, nociception (neural process of encoding and processing noxious stimuli) and the awareness of pain can

be elicited by various stimuli such as inflammatory mediators and toxic molecules, and in addition by temperatures and pressures high enough to potentially injure tissue. Specific nociceptors for chemical, mechanical, or thermal stimuli exist.

Chemical nociceptors possess TRP channels that respond to a wide variety of spices, irritants like acrolein, a World War I chemical weapon and a component of cigarette smoke and/or to various endogenous ligands. Mechanical nociceptors are activated by mechanical deformation or to excess pressure. The cortex processes the complex reaction further to a stimulus. In addition, nociceptors frequently have polymodal characteristics. Therefore, it is possible that some of the transducers for chemical, thermal or mechanical stimuli are all the same for mechanical stimuli. For example, the presence of the TRPA1 receptor in nociceptors appears to detect both mechanical and chemical changes. Thermal nociceptors respond to noxious heat or cold at various temperatures. There are specific nociceptor transducers that are responsible for how and if the specific nerve endings respond to the thermal stimulus. TRPV1 was the first to be discovered and it has a threshold that coincides with the heat pain temperature of 42 °C. Any other temperature in the warm–hot range is conveyed by more than one TRP channel. Each of these channels expresses a particular C-terminal domain that corresponds to the warm–hot sensitivity. Cool stimuli are sensed by TRPM8 and TRPA1 channels. The C-terminal domain from the TRPM8 channels differs from the heat sensitive TRPs. Although this channel corresponds to cool stimuli, it is still unknown whether it also contributes in the detection of intense cold. An interesting finding related to cold stimuli is that tactile sensibility and motor function deteriorate while pain perception persists (Woolf and Ma 2007). The sensory inflow produced by nociceptors activates neurons in the spinal cord which project to the cortex by an interconnection in the thalamus, causing pain. Hence, the pain experience not only involves spinaly-mediated reflex withdrawals, but also an enhancement in arousal, as well as autonomic, emotional, and neurohumeral responses all connected to the brain (Woolf and Salter 2000).

1.5.1 Acute vs. chronic pain

There are many differences between acute and chronic pain, which suggests that they must be considered separate entities (Auvenshine 2000).

Acute pain is usually transitory, which generally diminishes rapidly when the noxious stimulus is removed or the underlying damage or pathology has healed. It serves a crucial, protective purpose. It alerts the body in case of danger and limits the use of injured or diseased body parts (Carr and Reuben 2005).

Chronic/maladaptive pain in contrast, may be considered a disease state. It lasts a long time and is due to painful conditions such as cancer, peripheral neuropathy or rheumatoid arthritis. It has minimal protective aspects and persists despite normalization after injury or disease (Table 1). Conventionally, the differentiation between acute and chronic pain

depends on the interval of time from onset; although definitions can vary, chronic pain is usually defined as pain that lasts longer than the injury (Carr and Sendil 2002).

Characteristic	Acute Pain	Chronic Pain
Duration	Hours to weeks	Months to years
Pathology	Present	Little or none
Causes	Surgery, trauma, medical procedures	Nerve injury, Drug-induced neuropathy, Medical procedures, Arthritis, Diabetic neuropathy, Post-herpetic neuralgia
Prognosis	Predictable	Unpredictable
Complicating issues	Uncommon	Depression, anxiety and financial issues
Nerve conduction	Rapid	Slow
Treatment	Primarily analgesics	Multimodal

Table 1: Characteristics of acute and chronic pain. Acute and chronic pains are different clinical entities. Acute pain is evoked by a particular disease or injury, serves a useful biologic purpose, is associated with sympathetic nervous system activation and is self-limited. In contrast, chronic pain may be considered a disease state. It is pain that outlasts the normal time of healing, if associated with a disease or injury. Chronic pain may arise from psychological states, serves no biologic purpose, and has no recognizable end-point.

1.5.2 Neuropathic pain

The term "neuropathic pain" is a form of chronic/maladaptive pain (Figure 3). It refers to pain that originates from pathology of the nervous system and is defined by the IASP as "Pain caused by a lesion or disease of the somatosensory nervous system" (Campbell and Meyer 2006)

(http://www.iasppain.org/Content/NavigationMenu/GeneralResourceLinks/PainDefinitions/default.htm#Pain).

Neuropathic pain reflects both central and peripheral sensitization mechanisms. The syndromes are clinically characterized by burning, shooting pains along with tingling, crawling, or electrical sensations. Further, neuropathic pain syndromes can be divided in two different types, the spontaneous types of pain (ongoing, paroxysms) and evoked types of pain (hyperalgesia, allodynia) (Baron 2009). In the presence of hyperalgesia, individuals usually display an increased sensitivity to nociceptive stimuli whereas by exhibiting allodynia, individuals often experience normally innocuous stimuli as painful (Payne 1986). Thermal hyperalgesia and tactile allodynia constitute two essential manifestations of neuropathic pain (Ossipov et al. 2000).

Innumerable diseases may be the cause of neuropathic pain, such as cancer, metabolic diseases, autoimmune diseases, infections, vascular diseases and trauma. The clinical manifestations differ with the type of disease, indicating diverse mechanisms (Campbell and Meyer 2006).

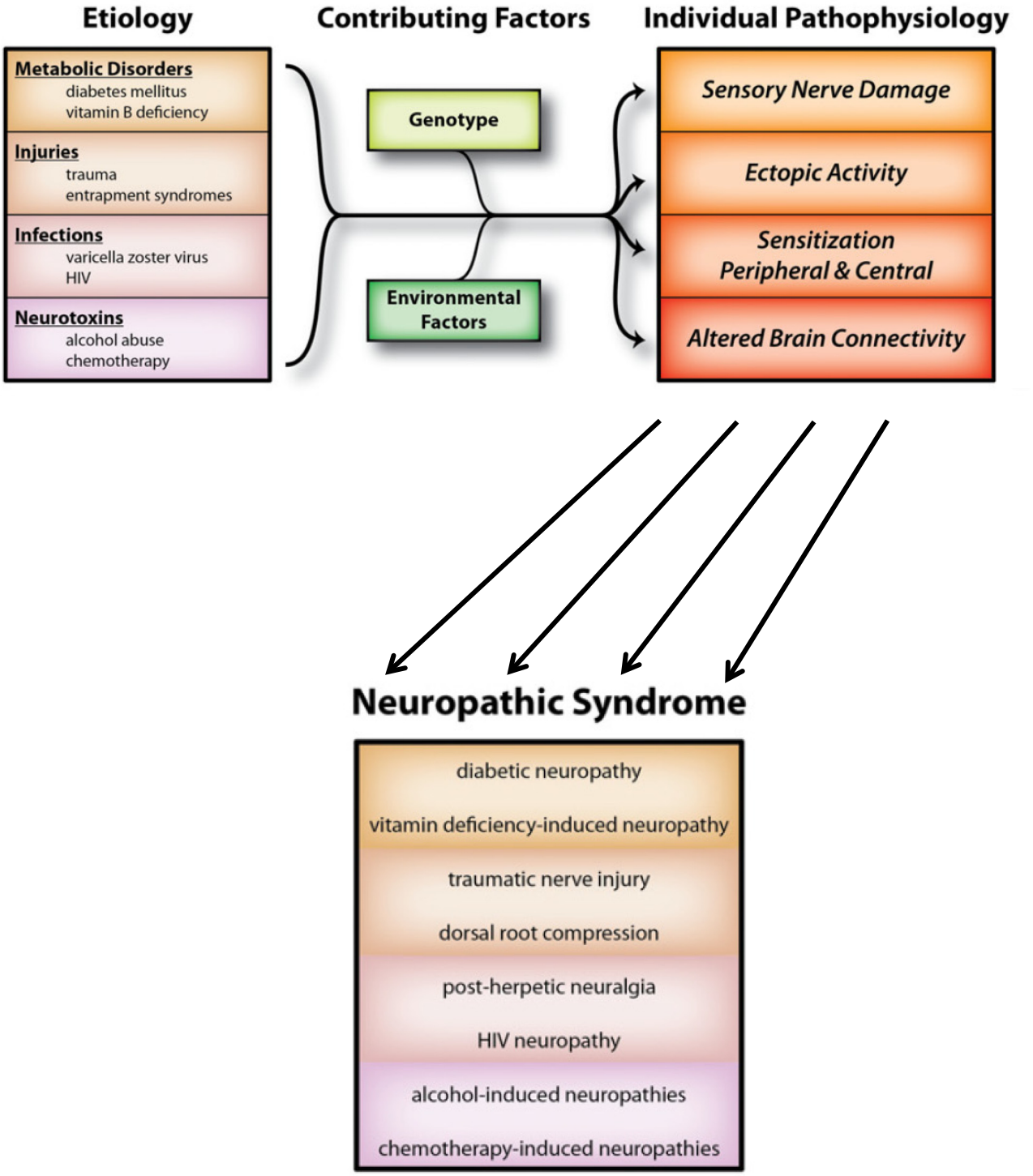

Figure 3: From Etiology to the Neuropathic Syndrome. The neuropathic syndrome is the result of an initiating disease where the genotype and environmental factors all contribute to the pathophysiological outcome. As a consequence various individual neuropathic pain phenotypes exist. (Illustration retrieved and altered from: (von Hehn *et al.* 2012)).

1.6 The spinal cord and neuropathic pain

The spinal cord is part of the CNS and depicts the first relay site in the transmission of nociceptive information, as demonstrated in Figure 4 (Dickenson *et al.* 2005).

Generally, nociception begins in the peripheral terminals of nociceptors with the activation of nociceptive transducer receptor/ion channel complexes, which produce depolarizing currents in response to noxious stimuli. Transducer proteins that respond to extrinsic or intrinsic irritant chemical stimuli are selectively expressed in sensory neurons. If threshold is reached, following transduction, action potentials are generated. Via conduction of the action potentials to the central nervous system (CNS), transmitter is released induced by central nociceptor terminals in the spinal cord. This fast excitatory synaptic transmission in pain is mediated by kainate ligand-gated ion channels and glutamate acting on AMPA. Secondly, this fast excitatory synaptic transmission is facilitated via the segmental and descending activation of inhibitory neurons, most of which co-release gamma-aminobutyric acid (GABA) and glycine (Woolf and Salter 2000). The nociceptors substance P and calcitonin-gene-related peptide are usually expressed by nociceptor C- and A- fibres, and are strongly involved in sensory transmission between nociceptive transducer receptor/ion channel complexes, and the CNS, and the generation of central sensitisation. Upon peripheral nerve injury, the expression of these neuropeptides is diminished. However, large myelinated Aβ fibres begin to express these neuropeptides - a so called phenotypic switch occurs (Woolf and Mannion 1999).

Nerve injury results in a delayed loss of sensory neurons, an effect more often seen in C- than A-fiber neurons (Coggeshall *et al.* 1997). Additionally, the A-fibers start to reorganize, sprouting from their deep dorsal horn laminar location up into the spinal cord where the C-fibers normally terminate and make functional synaptic contacts (Woolf *et al.* 1992; Koerber *et al.* 1999; Kohama *et al.* 2000). This modulated connectivity might be the cause for the irresolvable nature of many neuropathic pains (Woolf and Salter 2000). Further, nerve injury induces changes in transcription in dorsal horn neurons, which may be facilitated via activation of the MAPK/pCREB cascade. These transcriptional changes include changes in receptors (NK1, TrkB, GABA-R), and transmitters (dynorphin, enkephalin, GABA), and the induction of COX2 in dorsal horn neurons (Noguchi and Ruda 1992; McCarson and Krause 1994; Hay and de 1997).

So, to summarize two different mechanisms seem to occur upon nerve injury: 1) an increased expression of receptors in dorsal horn neurons for transmitters elevated in primary sensory neurons, in order to prepare the system for an enhanced excitation (Woolf and Costigan 1999) 2) a decline in the segmental and descending activation of inhibitory neurons. This reduced inhibition or disinhibition might be induced by a loss of inhibitory interneurons or via a decrease in receptors or transmitters (Castro-Lopes *et al.* 1993; Fukuoka *et al.* 1998).

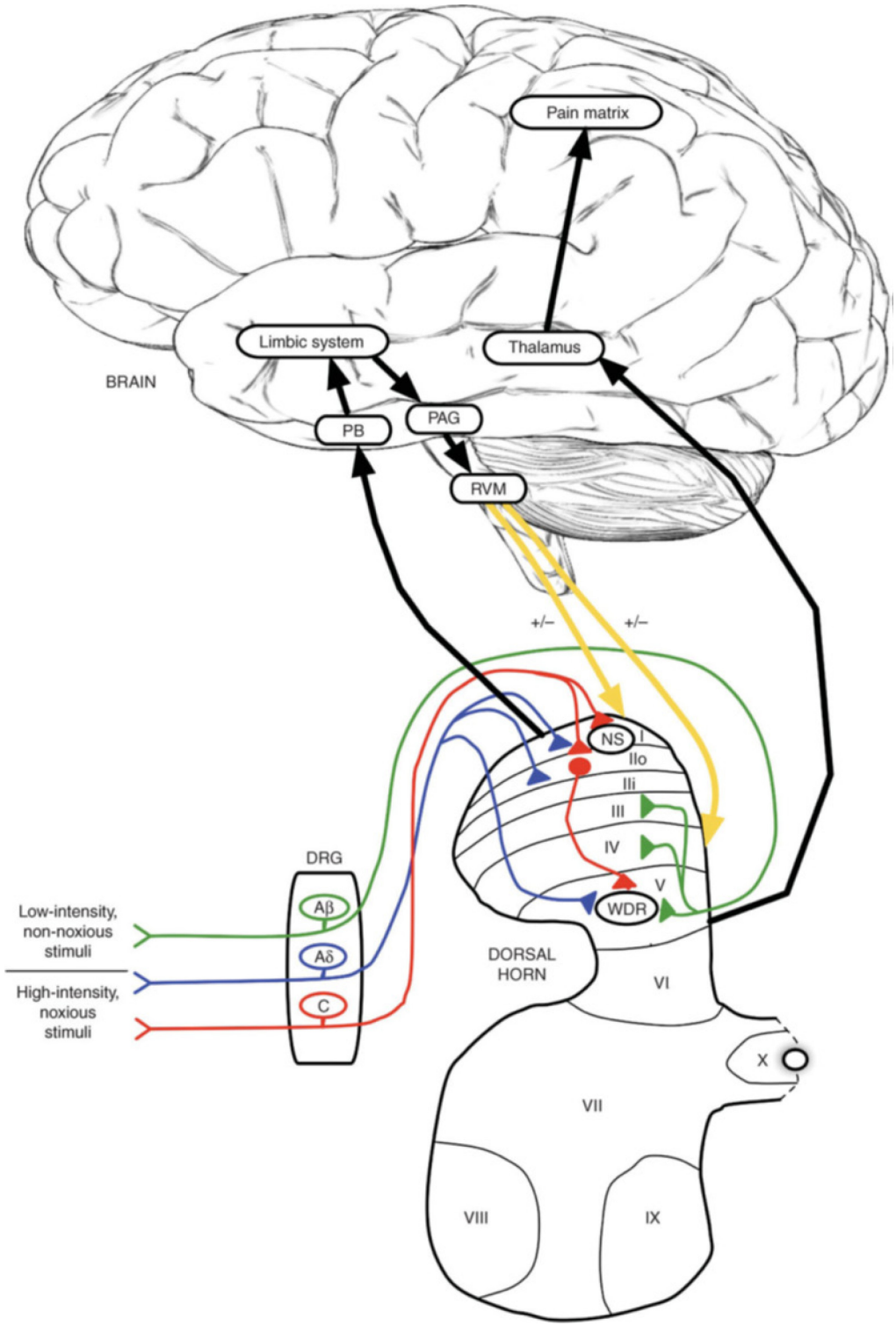

Figure 4: Pain pathways from the periphery via the spinal cord to the brain. Primary afferent fibres (Aβ-, Aδ-, and C-fibres) conduct stimuli from the periphery, through the dorsal root ganglion (DRG) and into the dorsal horn of the spinal cord. Nociceptive specific (NS) cells are mainly located in the superficial dorsal horn (laminae I–II); most wide dynamic ranges (WDRs) though are found deeper (lamina V). Projection neurones from lamina I affect the limbic system via the parabrachial area (PB) and periaqueductal grey (PAG). Spinal processing is regulated and activated from descending pathways (here: yellow arrows) from brainstem nuclei, e.g. the rostral ventromedial medulla (RVM). Lamina V neurons mainly project to the thalamus (spinothalamic tract), from which diverse cortical regions composing the 'pain matrix' (primary and secondary somatosensory, insular, anterior cingulate, and prefrontal cortices) are activated. (Figure obtained from: (D'Mello and Dickenson 2008)).

1.7 Supraspinal regions and neuropathic pain

A change in the chemistry, structure of neurons (neural plasticity) and function certainly underlies the generation of the modulated sensitivity characteristics of neuropathic pain. These changes have been thoroughly studied in sensory and spinal cord neurons but homogeneous alterations also take place in supraspinal regions (Woolf and Mannion 1999). Previous functional imaging studies have shown a number of individual brain structures that enhance their activity due to noxious stimulation (Derbyshire *et al.* 1997).

For example, a previous study revealed that persistent neuropathic pain is associated with significant changes in the anatomy of a number of brain regions traditionally associated with nociceptive processing including the VP region of the thalamus, PFC, insular cortex, amygdala, and premotor cortex (Gustin *et al.* 2010). The amount of change in all these regions is significantly correlated with pain severity, further strengthening an association with pain perception.

Additionally, a different study investigating the effect of chronic neuropathic pain via the use of the spared nerve injury (SNI) model, an animal model of neuropathic pain which has been described previously (Decosterd and Woolf 2000), on the hippocampus, could show that neuropathy results in multiple hippocampal abnormalities. They could demonstrate a potential underlying mechanism for hippocampal-mediated behavioral abnormalities in animals through the reduction of hippocampal neurogenesis and modulated short-term synaptic plasticity (Mutso *et al.* 2012).

Under chronic pain conditions, the homeostasis of the habenula seems to be disrupted. For example the reward system of the habenula has been demonstrated to become dysfunctional; therefore outputs from the pain modulator regions, which normally inhibit pain, may now facilitate pain. Moreover, few studies have evaluated analgesic responses of the habenula in neuropathic pain models. It is likely that drugs that target the habenula may provide a useful model for neuropathic pain because of its integrative role in pain and analgesia (Shelton *et al.* 2012). However, the role of the habenula in neuropathic pain in the human condition has not been determined so far. Given the insights of this structure on conditions that are seen with chronic pain including addiction (Fowler *et al.* 2011) and depression (Sartorius *et al.* 2007), abnormal functioning in the habenula may have a crucial role during neuropathic pain.

Another supraspinal region, the striatum has been demonstrated to produce analgesic actions during pain, via the activation of its dopamine D_2 receptors and transmitted through the indirect pathways of the basal ganglia and the medullary dorsal reticular nucleus (RVM) to the sensorial nuclei of the trigeminal nerve (Barcelo *et al.* 2012).

To summarize, various discrete supraspinal regions have been demonstrated to associate with long lasting significant changes and reorganization in the anatomy during neuropathic pain. However, until present there is still very little data about the function of nicotinic

receptors, particularly alpha5 containing receptors, in specific processes and changes that occur in several supraspinal regions during neuropathic pain.

1.8 How do nAChRs influence pain-mediated pathways in the spinal cord and supraspinal regions?

nAChRs are located in the whole grey matter of the spinal cord, but they have their highest density in the dorsal horn of the spinal cord (Khan *et al.* 2003). Previous studies showed via the use of subunit specific KO mice, that not only one specific, but diverse nAChR subtypes seem to play a role in the anti-nociceptive activity during pain-processing mechanisms in the spinal cord (Marubio *et al.* 1999; Matsumoto *et al.* 2007; Gao *et al.* 2010).

The anti-nociceptive activity in the spinal cord during pain-processing mechanisms is thought to be conferred by spinal α4β2* receptors, which exist in large quantities (Khan *et al.* 2001; Rashid *et al.* 2006). This is in line with the finding that mechanical and thermal nociceptive thresholds are considerably reduced in β2 KO mice. Nevertheless, by using the tail flick test, a previous report showed that subcutaneously applied nicotine still mediates analgesia (however at higher doses) in α4 and β2 KO mice (Marubio *et al.* 1999). Furthermore, ispronicline a selective α4β2 agonist does not confer analgesia in the complete Freunds' adjuvant and formalin models of pain, whereas the non-selective nAChR agonists' varenicline and ABT-594 demonstrated to be effective analgesiscs in these tests (Gao *et al.* 2010).

Depending on dose, nicotinic agonists exert either pro-nociceptive or anti-nociceptive effects when applied intrathecally into the spinal cord or via microdialysis into the dorsal horn of the spinal cord (Khan *et al.* 1998; Khan *et al.* 2001). Recent clinical studies have examined the anti-nociceptive effect of nicotine. They could demonstrate efficacy but also inefficiency with higher dose, displaying side effects (Gao *et al.* 2010). Nicotine exerts its antinociceptive action at the site of the spinal cord (Aceto *et al.* 1986) and at supraspinal regions. Via the use of spinal-specific pain tests of animals, which received subcutaneous nicotine injections, this analgesic effect of intrathecally administered nicotine agonists could be supported. This antinociceptive action could be detected as a result of multiple diverse nicotinic agonists, moreover indicating a participation of nAChRs in the spinal cord during pain. Since subcutaneous applied nicotinic agonists like ABT-594 or epibatidine exhibit anti-nociceptive activity with potencies largely exceeding those of morphine, these pain-processing mechanisms in the spinal cord involving nAChRs are of clinical interest (Bannon *et al.* 1998).

As mentioned above, nAChRs are not only widely distributed in the spinal cord but also throughout the brain. Like in the spinal cord, the α4β2* receptors constitute the most studied and common receptors in the brain (Flores *et al.* 1992). Cerebral blood flow changes in awake rats in the dorsal raphe, the thalamus, and the periaqueductal gray (PAG), the cingulate, and the motor, somatosensory, auditory and prefrontal cortexes, in response to

application of nicotinic receptor agonists (Skoubis *et al.* 2006). In addition, previous investigations have indicated a role of brain nAChRs in nociception and anti-nociception. Anti-allodynic effects of systemic and intracerebroventricularly administered nicotinic agonists during neuropathic pain are depicted to be timely and dose-dependently coordinated to the nicotine receptor occupancy rates in the thalamus. These effects can be blocked by i.v. applied antagonists (Ueda *et al.* 2010; Ueda *et al.* 2011). Further, acetylcholine-induced 86Rb+ efflux from synaptosomes in hindbrain and thalamus is considerably changed in α5 KO mice, whereas acetylcholine-stimulated 86Rb+ efflux from synaptosomes in spinal cord is not altered (Jackson *et al.* 2010).

1.9 The α5 nAChR subunit in spinal- and supraspinal regions during pain-processing mechanisms

Although most nAChR subunits can join to form ligand-activated channels via the use of diverse combinations of α and β subunits, the α5 nAChR subunit cannot yield functional receptors when expressed alone or as the sole α subunit expressed with either β2 or β4. Therefore, the function of the α5 nAChR subunit has often been confined to a regulative role via modulation of various characteristics of the nAChR (Jackson *et al.* 2010; Vincler and Eisenach 2005). However, according to a recent investigation by Jackson et al. 2010, the α5 nAChR subunit plays a role in pharmacological and behavioral effects of nicotine in mice. In this study, they show that subcutaneously administered nicotine in wild-type mice mediates analgesia in both the tail-flick test and the hot plate test. However, these effects are lost while using the tail-flick test, or significantly lowered via the use of the hot-plate test in α5 KO mice, indicating a crucial role of the α5 nAChR subunit both in the spinal cord and in supraspinal regions during pain-processing mechanisms (Figure 5) taken from (Jackson *et al.* 2010). Furthermore, a different study was able to show an upregulation of the α5 nAChR subunit in the dorsal horn of the rat spinal cord following spinal nerve ligation, an animal model of neuropathic pain, by the use of immunostaining (Figure 6) taken from (Vincler and Eisenach 2004). And moreover, antisense knockdown of α5 not only lowers α5 immunoreactivity but also hyperalgesia and mechanical allodynia (Vincler and Eisenach 2005). These findings were extended by another study (Yang *et al.* 2004), which could also demonstrate an upregulation of the α5 nAChR subunits in the dorsal horn 14 days post peripheral rhizotomy in rats.

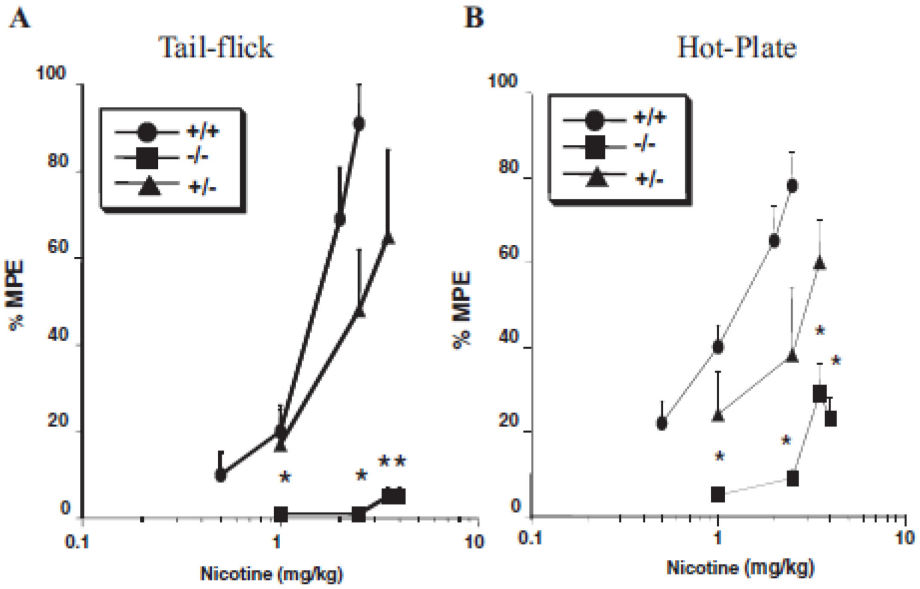

Figure 5: Antinociceptive activity of nicotine in α5 KO mice. Antinociception was analysed in wild-type, α5 (-/+) and α5 (-/-) mice using the tail-flick and the hot-plate tests**.** In response to an acute, subcutaneous nicotine injection a significant antinociceptive response in both wild-type and α5 (-/+) mice was measured. However a gene-dosage effect was observed, because α5 (-/+) and α5 (-/-) mice were progressively less sensitive to both behavioral tests in comparison to the wild-type mice, indicating that nicotine has a less acute antinociceptive effect in α5 (-/-) mice. Data shows an experiment from a recent study (Jackson *et al.* 2010).

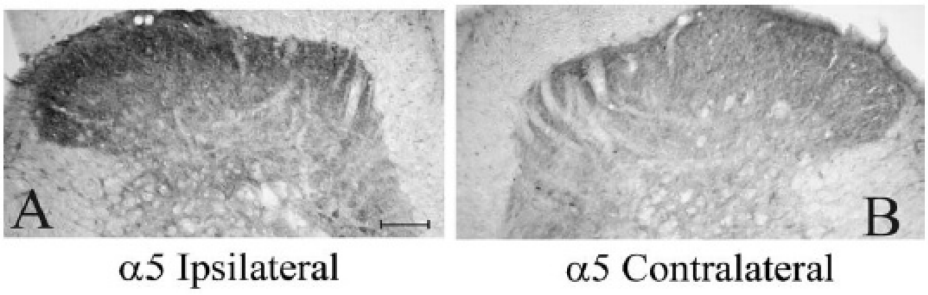

Figure 6: α5 Immunoreactivity in the rat dorsal horn post SNL. After spinal nerve ligation, α5 immunoreactivity is enhanced ipsilateral (A) to the nerve-injury in the outer laminae. This increase was not seen contralaterally (B) to the ligation (Scheme retrieved from: (Vincler and Eisenach 2004).

Although the role and participation of spinal and supraspinal nAChRs during pain-processing mechanisms is of interest, due to its potential clinical benefit, relatively few studies have examined changes in inflammatory or neuropathic pain. The characterization of the diverse subtypes of hetero-pentameric nAChR and in particular the α5 nAChR subunit at the level of the spinal cord and supraspinal regions in pain-mediated pathways has been surprisingly neglected so far. Only four studies exist to date (Jackson *et al.* 2010; Vincler and Eisenach 2004; Vincler and Eisenach 2005; Yang *et al.* 2004). In one of these studies (Jackson *et al.* 2010), the role of the α5 nAChR subunit was investigated by the assessment, among other aspects, of its sensitivity to selected pharmacological effects of nicotine including, antinociception. In this study it was proposed that systemically applied nAChR agonists do

not induce analgesia, 5 minutes post induction, in naïve α5 (-/-) mice in contrast to naïve WT mice. The other study stated (Vincler and Eisenach 2004) that the introduction of spinal nerve ligation (SNL) increases the expression of the α5 nAChR subunit on the ispilateral side to the ligation in the dorsal horn of the spinal cord. In contrast, the expression of the contralateral side was not increased when measured by immunoreactivity. The third study (Vincler and Eisenach 2005) suggested that the knock down of the α5 subunit in SNL rats moderately enhances mechanical allodynia, suggesting that the presence of the α5 nAChR subunit contributes to this condition. The study furthermore stated that antisense treatment in SNL rats decreases pCREB immunoreactive nuclei in the outer laminae, proposing that the α5 subunit functions to increase ACh-evoked excitatory amino acid release. The last study mentioned (Yang *et al.* 2004), could demonstrate an upregulation of the α5 nAChR subunits in the dorsal horn 14 days post peripheral rhizotomy in rats. Hence, these studies suggest a prominent role of the α5 nAChR subunit in spinal and supraspinal regions during pain processing mechanisms.

1.10 Aim of this study

The aim of my study was to specifically test the hypothesis that the α5-containing nAChR is involved in mouse models of neuropathic pain and the analgesic response to the parasympathomimetic alkaloid nicotine. In addition we wanted to assess the subunit composition of hetero-pentameric nAChR subtypes in non-treated mice and mice subjected to an animal pain model via the use of self-generated subunit-specific antibodies (David *et al.* 2010).

2 Materials & methods

2.1 Animals

For all behavioral and biochemical experiments, adult male wild-type C57BL/6J mice and mice with deletions of the nAChR subunit genes α5 (Wang *et al.* 2002) were used. The animals were bred in-house and were used at an age of 2-4 months. All experimental mice were littermates from heterozygous breeding pairs and were genotyped at weaning. Adult male mice (15-20 grams) were housed in Type IIL cages (~553cm^2) at a density of 4-6 per cage. Animals were kept in thermally stable environment at 20-24°C in a 12h light/dark cycle with food and water *ad libitum*. All the animals were age, weight and sex – matched.

2.2 Neuropathic pain model

For the neuropathic pain model, mice received a partial sciatic nerve ligation (PSNL) on the left hind paw, according to a standard method previously reported (Malmberg and Basbaum 1998), and based on the rat model developed by (Seltzer *et al.* 1990). Mice were briefly anaesthetized under gas anesthesia using a mixture of 2:1 [N_2O] and O_2 and additionally 2% isoflurane. Under sterile conditions the sciatic nerve was exposed at high thigh level, carefully freed from the surrounding, connective tissue and one tight ligature was applied via the insertion of a G-6 (8.0mm 3/8c) silk suture into the nerve. The wound was closed with 1-2 muscle sutures and 2-3 skin sutures. In the SHAM-operated mice, only the skin and the muscle were carefully exposed, while the sciatic nerve was left intact. The wound in the SHAM group was closed in the same way as in the PSNL-operated mice. Animals were housed individually after surgery. They were followed for 21 days during which there were no motor problems or other abnormalities observed.

2.3 Behavioral tests

Nociceptive responses to heat and cold stimuli were assessed. Baseline values were measured after the habituation to the experimenter and the testing equipment for 1-2 hours for three days prior to any experimental test.

2.3.1 The Hargreaves test

The Hargreaves test (Hargreaves *et al.* 1988) was used to test for cutaneous hyperalgesia in both hind paws. Mice were placed individually inside Plexiglas cylinders (7cm x 9cm x 7cm) on a glass floor (21°C) of a Hargreaves analgesiometer apparatus (Stoelting Co, Wood Dale, IL, U.S.A.) and were allowed to habituate for 20-30 minutes. Thermal stimulation of both hind paws, beginning with the contralateral paw and followed by the ipsilateral paw, was applied by a radiant heat source positioned beneath the glass floor. This procedure was repeated for 3 times with a spacing of 10 minutes. The paw withdrawal latency (PWL), i.e.

the time between the start of the thermal stimulation and the paw withdrawal behavior of the mice, was measured automatically by a digital timer. The light beam intensity was adjusted to measure baseline paw withdrawal latencies (PWLs) between 12 and 14 seconds. The cut-off was set to 20 seconds to avoid possible tissue damage.

2.3.2 The Acetone test

To assess the development of cold allodynia, the acetone test was used (Choi *et al.* 1994). Mice were again placed individually inside Plexiglas cylinders (7cm x 9cm x 7cm) standing on a wire mesh and again permitted to habituate for about 20 minutes. Using a pipette, 20µl of Acetone was then applied to the plantar surface of the hind paws, and nociceptive behavior was measured for 5 minutes. The contralateral paw was tested first, followed by the ipsilateral side. The total duration of nociceptive behaviors to the cooling effect of acetone was recorded via the use of a stopwatch. This process was repeated 3 times with a spacing of 10 minutes.

2.4 Nicotine injections

The analgesic impact of nicotine in wild-type and α5 (-/-) mice on nociceptive behavior was tested, in a 60 minute timecourse, at day 4 post PSNL induction. Male mice were injected subcutaneously with 2, 5 mg/kg nicotine at day 4 post PSNL or SHAM induction. Antinociception was measured using the Hargreaves test. Groups of 4 mice were used for each round of testing. Tests were performed every 5 minutes post subcutaneous nicotine injection. The antinociceptive response was calculated as percentage maximal possible effect (%MPE), where %MPE = [(test value - baseline)/ (cut-off time (20s) - control value)] x 100, where baseline represents the value prior to nicotine or other drugs.

2.5 Experimental design

Mice were tested in blocked groups of 8.

Experiment 1:

The effect of PSNL on nociceptive behavior at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes post nicotine injection was assessed 4 days after PSNL. A post-treatment protocol, using a single nicotine injection at 4 days post PSNL was tested in α5 (+/+) and α5 (-/-) males (Sham: n = 8; PSNL: n = 8).

2.6 Preparation of spinal and supraspinal regions

For all experiments in this study, mice were sacrificed by a deep CO_2 anesthesia followed by cervical dislocation and subsequent decapitation. The brain was rapidly exposed, freed from

the surrounding tissue, and transferred on a plate with Ca^{2+}-free Tyrode´s solution (4°C cold on ice): 150mM NaCl, 4mM KCl, 2.0 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4. The mouse body was then pinned dorsally on a polystyrene plate and dissected ventrally. Under a light microscope, the skin and organs were removed and the spine and the vertebral canal were exposed. The spinal cord was then carefully dissected and transferred on a plate with Ca^{2+}-free Tyrode´s solution (4°C cold) on ice. The lumbar (L3-L6) region of the spinal cord was isolated, the region in which nociceptive fibers from the affected hind paw terminate of the sciatic nerve, and subsequently divided into the contralateral sides, the control sides and the ipsilateral, the injured sides. Further, the thoracal region of the remaining spinal cord was also divided into ipsilateral and contralateral sides. Spinal cord regions lumbar/thoracal regions from the same genotype were collected and transferred into an Eppendorf tube with 1ml Ca^{2+}-free Tyrode´s solution (4°C cold). Then, the interpeduncular nuclei (IPN), habenulae, hippocampi, striatae as well as thalami of the brain, were dissected. 6-12 IPN, habenulae, hippocampi, striatae and thalami of the same genotype were collected and transferred into an eppendorf tube with 1 ml Ca_2+-free Tyrode´s solution (4°C cold). The supernatant was removed after a 1 minute centrifugation at 16 000g, and the tissue was then flash-frozen with liquid nitrogen and stored at - 80°C until immunoprecipitation.

2.7 Immunoprecipitation (IP) of [^{3}H]-epibatidine labeled hetero-pentameric nAChRs with subunit specific antibodies

Spinal and supraspinal nAChRs were solubilized in 2% Triton X-100 lysis buffer: 50 mM Tris–HCl pH = 7.5, 150 mM NaCl, 2% Triton X-100, supplied with one complete mini protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA) per 10 mL buffer. Subsequently the receptors were sonicated for 5 seconds with the ultrasonic homogenizer (Bandelin Sonoplus UW 2200), at 30% energy level, and were left at 4°C for 2 hours. Samples were then centrifuged at 16 000 g for 15 minutes at 4°C. The supernatant was collected and the pellet discarded. For later protein determination, an aliquot of 50 µl was collected and stored at – 18°C. 115 µl lysate was incubated with 20 µL 10 nM [^{3}H]-epibatidine and 7 µg antibody in 30 µL phosphate-buffered saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH = 7.4) on a shaker overnight at 4°C. Non-specific binding was defined by the addition of an excessive (in comparison to [^{3}H]-epibatidine) amount of nicotine (300 µM) to half of the samples.

Heat-killed, formalin-fixed *Staphylococcus aureus* cells (Standardized Pansorbin-cells; Calbiochem; San Diego, CA, USA) were centrifuged at 2300 g for 5 minutes at 4°C. Resulting Pansorbin-pellets were washed twice with IP-High (50mM Tris-HCl pH=8.3, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and once with IP-Low (50mM Tris-HCl pH=8.0, 150 mM NaCl, 1mM EDTA, 0.2% Triton X-100), and re-suspended with IP-Low. 20µl of the washed Pansorbin was added to the samples which were subsequently incubated on a shaker for 2 hours at 4°C. After the incubation with Pansorbin, the samples were centrifuged at 2300g for

5 minutes at 4°C. The supernatant was then discarded and the pellet washed twice with IP-High and once with IP-Low at 2300g for 1 minute at 4°C. To re-suspend the pellets, 200µl 1M NaOH was added and the suspensions were then transferred into 5ml Sarstedt tubes including 2 ml scintillation cocktail (Rotszint Eco Plus, ROTH) and subsequently subjected to liquid scintillation counting.

Bicinchoninic acid assays were performed to determine the total concentration of protein of the solved nAChRs, which were stored at – 18 ° C previously. Following the manufacturer's instructions, all protein quantifications were carried out using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

2.8 Statistics

Statistical analyses and plotting was conducted using the software GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Results of the behavioral tests were analyzed using two-way ANOVA followed by a Tukey Multiple Comparison Test post-test. P values lower than 0.05 were considered significant. Data of the IPs were not statistically analysed based on the fact that all the experiments were performed only once (n = 1). All data are presented as mean ± standard error of the mean (SEM).

3 Results

3.1 Effect of nicotine induction on the nociceptive behavior of wild-type and α5 (-/-) mice 4 days post PSNL

First we determined the sensitivity of wild-type and α5 (-/-) PSNL mice for cutaneous heat hyperalgesia. In both genotypes, we tested both hindpaws 2 and 4 days after PSNL. As shown in figure 7, the baseline did not differ between the two genotypes (analysis of the data by two-way ANOVA ensued by a Tukey´s Multiple Comparison post-test).

Subsequently, we injected half of the group with nicotine subcutaneously at day 4 post PSNL, whereas the control group obtained subcutaneously saline injections. Consecutively, wild-type mice and α5 (-/-) mice were tested every 5 minutes, in a 60 minute time course, post subcutaneous nicotine/saline injection.

PWL of the ipsilateral hindpaw of PSNL treated mice

The paw withdrawal latency (PWL) of the ipsilateral hindpaw differed significantly between nicotine and saline injected PSNL-treated animals in both wild-type and α5 (-/-) mice at 5, 10, 15, 20, 25 minutes post injection (****p<0.0001). 30 minutes post the effect was still observable (*p<0.05). 35 and 40 minutes post only wild-type nicotine injected PSNL mice demonstrated a significant difference compared to saline injection (**p<0.01, *p<0.05). After 50 minutes, no nicotine treated animal showed a different behavior compared to the saline control group.

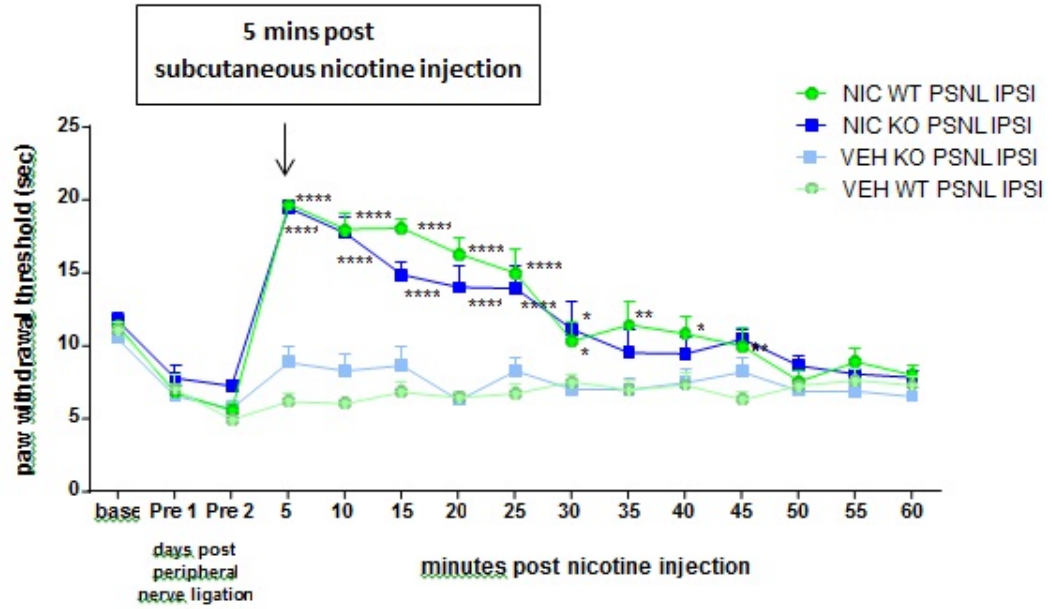

Figure 7: Nociceptive behavior of wild-type and α5 (-/-) mice post PSNL plus effect of subcutaneously applied nicotine at day 4 post PSNL surgery. PSNL operated mice were injected with nicotine or saline 4 days after surgery. Error bars represent the standard error of the mean (n= 8 in all groups of animals, ****p<0.0001, **p<0.01, *p<0.05).

PWL of the ipsilateral hindpaw of SHAM treated mice

In addition, we analysed the nociceptive behavior of the ipsilateral hindpaws of wild-type and α5 (-/-) mice post SHAM surgery before and after subcutaneous nicotine/saline injection. Data was analysed using two-way ANOVA followed by a Tukey´s Multiple Comparison post-test as illustrated in figure 8. The PWL of the ipsilateral hindpaws differed significantly between nicotine and saline injected SHAM-treated animals in both wild-type and α5 (-/-) mice at 5 and 10 minutes post injection (****p<0.0001). 15 and 20 minutes post nicotine injection the PWL of the ipsilateral hindpaws of wild-type SHAM nicotine-treated mice demonstrated a highly significant difference compared to saline treated animals (****p<0, 0001), while α5 (-/-) SHAM mice only differed slightly significant (*p<0.05). After 50 minutes, no nicotine treated animal showed a different behavior compared to the saline control group.

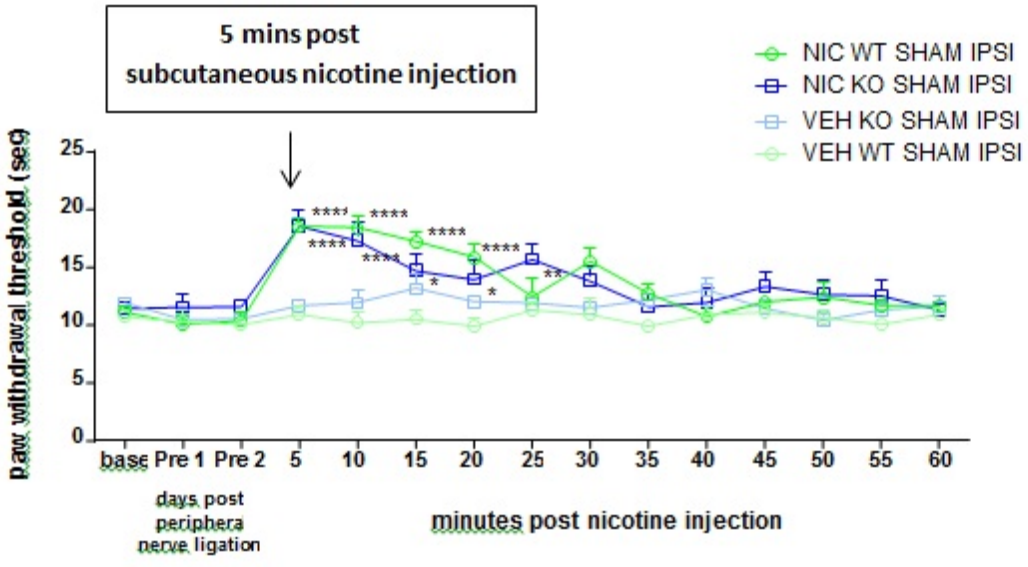

Figure 8: **Nociceptive behavior of wild-type and α5 (-/-) mice post SHAM plus effect of subcutaneous applied nicotine at day 4 post SHAM surgery.** SHAM operated mice were injected with nicotine or saline 4 days after surgery. Error bars represent the standard error of the mean (n= 8 in all groups of animals, ****p<0.0001, **p<0.01, *p<0.05).

Figure 9 demonstrates the PWL of all nicotine-treated mice groups after PSNL/SHAM surgery analysed post injection. The figure shows area under curve analysis of the data shown in Figures 7 and 8, statistically compared using two way ANOVA followed by a Tukey´s Multiple Comparison post-test. Only the PWL of the ipsilateral hindpaws of α5 (-/-) PSNL nicotine-treated mice showed a statistical different response in comparison to the PWL of the contralateral wild-type PSNL nicotine-treated mice hindpaws (**p<0.01)

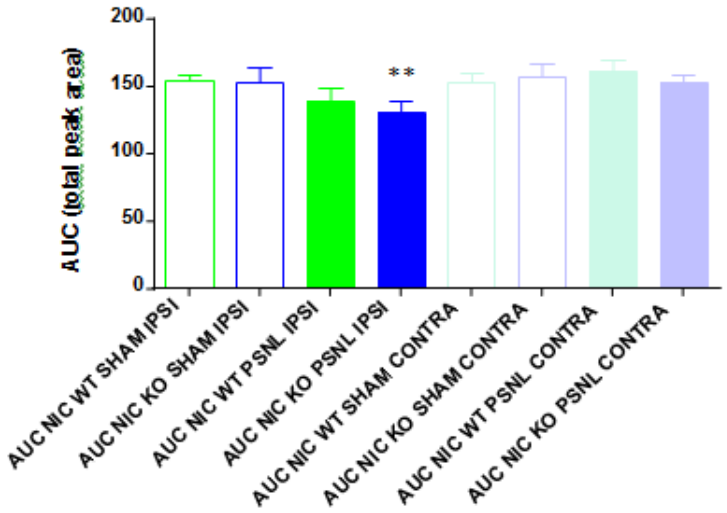

Figure 9: PWL of wild-type and α5 (-/-) mice, either PSNL or SHAM operated and treated with nicotine. Only the PWL of the ipsilateral α5 (-/-) PSNL nicotine-treated mice hindpaws demonstrates a statistical difference in comparison to the contralateral hindpaws. Results were analyzed by the use of a two-way ANOVA with a Tukey´s Multiple Comparison post-test. Error bars show the standard error of the mean (n= 8 in all groups of animals, **p<0.01 compared to contralateral paw).

We also calculated the response of the animals as %MPE: the paw withdrawal thresholds are expressed as the percentage of maximal possible response (%MPE = [(test value - baseline)/ (cut-off time (20s) - control value)] x 100

Figure 10 shows the area under curve analysis of %MPE of all nicotine-treated mice groups after PSNL/SHAM surgery analysed post injection. Data was analysed using two way ANOVA followed by a Tukey´s Multiple Comparison post-test. The PWL between all nicotine-treated mice groups – and all hindpaws tested demonstrated no statistical difference.

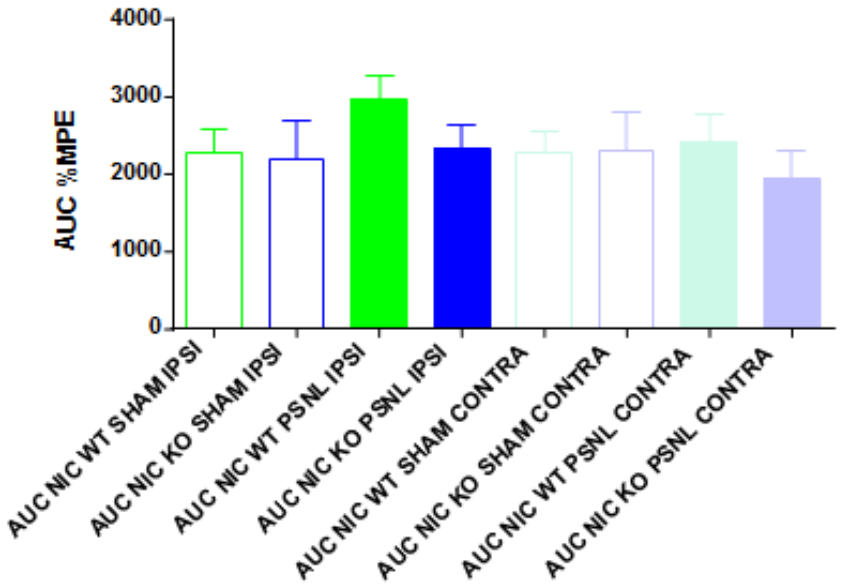

Figure 10: PWL of wild-type and α5 (-/-) mice, either PSNL or SHAM operated and treated with nicotine expressed as %MPE of the AUC. The figure demonstrates results of the experiment comparing the nociceptive behavior of wild-type PSNL/SHAM mice and α5 (-/-) PSNL/SHAM mice post nicotine injection. Data of the results were calculated as % MPE and additionally analyzed as percentage area under curve. No significant differences were obtained by the analysis of the data applying two-way ANOVA ensued by Tukey´s Multiple Comparison post-test. Error bars show the standard error of the mean (n= 8 in all groups of animals).

3.2 Immunoprecipitations with tissue from PSNL and SHAM treated wild-type mice

We performed immunoprecipitations (IP) on dissected tissue from wild-type mice, at days 2 and 7 post PSNL/SHAM induction, to biochemically analyse and compare their nAChR subunit expressions. Receptors were analyzed as described previously (David *et al,* 2010): Briefly, tissue was solubilized with 2% Triton, labeled with [^{3}H]-epibatidine and precipitated with specific antibodies against the nAChR subunits of interest immobilized on protein-A rich pansorbin cells. The subunit selective antibodies used in my study are self-generated and

thoroughly tested on heterologous systems (positive control) plus wild-type and KO mice (negative control) (David *et al.* 2010). We studied spinal cord and specific supraspinal brain regions, all of which have been shown in previous studies (Grady *et al.* 2009; Scholze *et al.* 2012) to contain a relatively high α5 subunit expression. As the binding pocket for nicotinic agonists is built between an α- and a β- subunit, [^{3}H]-epibatidine binds to all heteropentameric nAChR which incorporate α- and β-subunits inadvertent of their specific compositions (Xiao *et al.* 1998; Xiao and Kellar 2004).

3.2.1 Is there an early effect of PSNL induction on the α5 nAChR subunit expression in the spinal cord and or supraspinal regions of wild-type mice?

First, we wanted to test if the introduction of PSNL had an early effect on the α5 nAChR subunit expression levels in the spinal cord of wild-type mice. This experiment was based on the fact that in an earlier study (Vincler and Eisenach 2004), the induction of neuropathy (SNL) had been shown to upregulate the α5 nAChR subunit in the dorsal horn of the spinal cord on the ispilateral side to the injury. As β3 constitutes only an accessory subunit (Exley *et al.* 2012) every nAChR comprises a β2, a β4 or both subunits. Hence, nAChR precipitated with a combination of antibodies against β2 and β4 equal the absolute number of receptors that can be found in the spinal cord. Consequently, the result of IP combined with a mix of anti- β2 and anti- β4 antibodies resembled the 100% reference. As shown in a previous study (Grössl *et al.* 2013) the total number of nAChR in the spinal cord equals 43.7 ± 1.16 fmol/mg, while only a small percentage of receptors contained the accessory subunit α5 at 0.9 fmol/mg.

As shown in figure 11, we could generally confirm these findings, when analyzing the spinal cord of our animals. Both in the thoracal part as well as in the L3-L6 region of the spinal cord, the α5-subunit was hardly detectable and unchanged between PSNL treated animals and controls. The total number of receptors was found to be between 30-40 fmol/mg (n=1) in all spinal cord samples and therefore very close to the experiments from Grössl et al.

We also analysed higher brain areas, such as thalamus, hippocampus, habenula, interpeduncular nucleus and striatum, all brain regions, which have been shown to be involved in pain processing. In the interpeduncular nucleus, I detected a total number of receptors of 120 fmol/mg (n=1), 10% of which contain the α5-subunit. This is in contrast to (Grady *et al.* 2009), who determined 360 fmol/ mg as an overall amount of nAChRs in the interpeduncular nucleus of adult wild-type mice. Again, an upregulation of the α5 nAChR subunit could not be demonstrated at any time point tested post PSNL, compared to interpeduncular nuclei taken post SHAM surgery (Table 2).

In the habenula I detected a total number of 130 fmol/mg receptors (n=1), which is much less, than previously found in experiments from our working group (Scholze *et al.*, 2012), and also less then reported by Grady *et al.*, 2009. The level of β2-containing nAChRs was quite

similar to previous findings, however I only detected one quarter of β4 containing nAChRs. α5-levels were low, but clearly detectable, and unchanged in response to peripheral nerve ligation at any time points analysed.

In the hippocampus I detected an overall amount of nAChRs of around 15-41 fmol/mg (n=1). A small percentage of receptors was determined to contain the accessory subunit α5 at 1-3 fmol/mg. The total number of receptors seems to increase between days 2 and 7 post injury, however there seems to be a similar trend in the SHAM treated animals. This experiment has only been performed once, so it will have to be reproduced and results have to be statistically analysed in order to make a scientifical statement.

In the striatum I detected a total number of 40- 69 fmol/mg (n=1) receptors. Again the anti-α5 antibody precipitated 1-5 fmol/mg. This time the total number of receptors seems to decrease between days 2 and 7 post injury, with a similar trend in the SHAM treated animals. But again, this experiment has only been performed once, and will have to be reproduced.

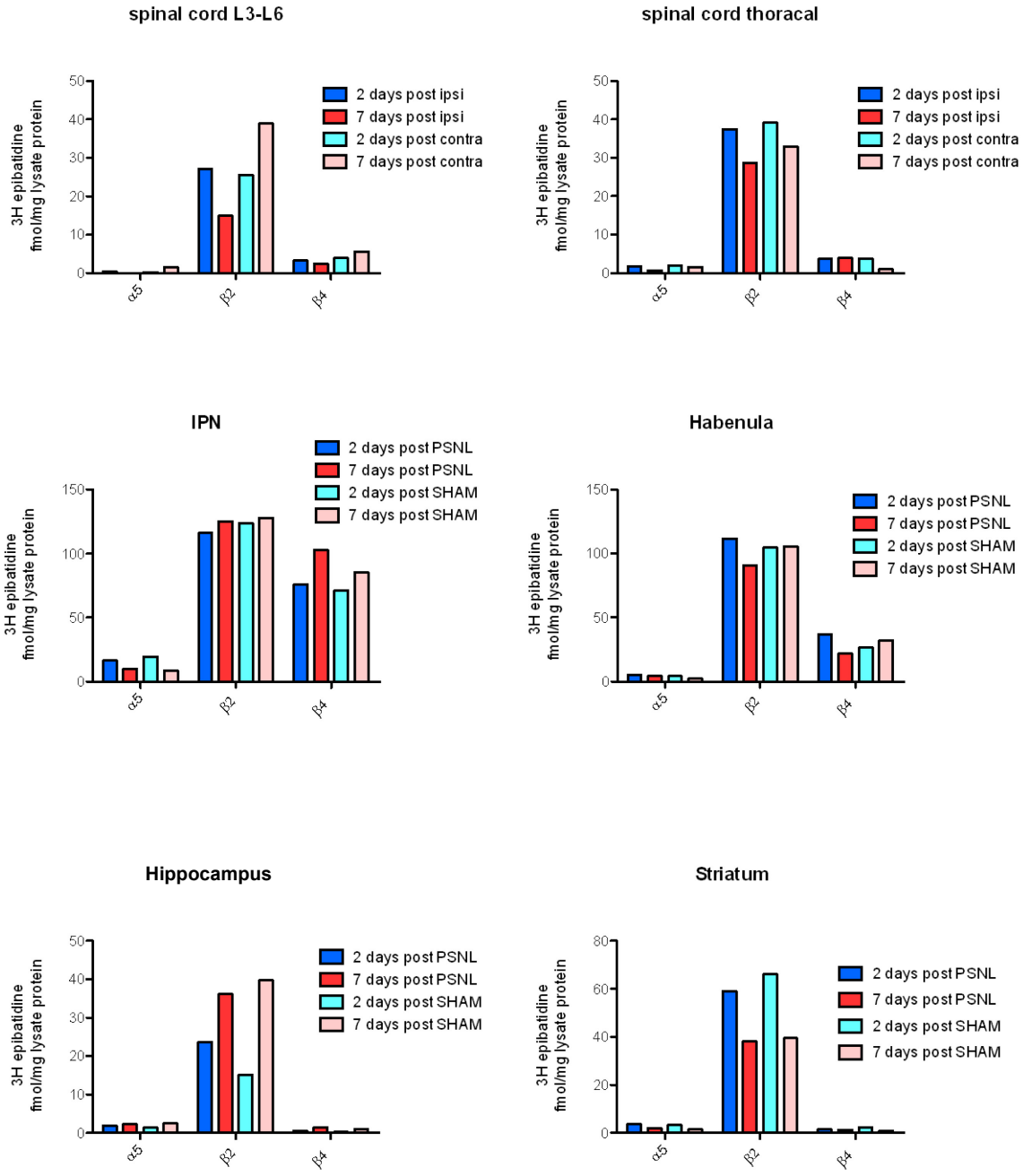

Figure 11: β2, β4 and α5 nAChR subunit expressions in spinal cord and supraspinal regions 2, 7 days post PSNL/SHAM surgery. nAChRs were labeled with [^{3}H]-epibatidine (1nM) followed by addition of a specific antibody, β2, β4 and α5. Non-specific binding was measured via addition of excessive nicotine (300µM) and subtracted from the total value to receive specific binding of [^{3}H]-epibatidine. The number of precipitated receptors was measured in fmol and related to protein levels of the samples in mg. All experimentns have been performed once.

4 Discussion

Systemic administration of nicotinic receptor agonists has long been known to induce mechanical, chemical and thermal analgesia (Damaj *et al.* 1998; Bannon *et al.* 1998). In addition, nicotinic acetylcholine receptors are found in spinal cord dorsal horn and various brain areas associated with pain, supporting their importance in pain mechanisms. The subtype(s) of nicotinic acetylcholine receptors, which are involved in the analgetic response to nicotine, as well as in maintaining neuropathic pain have not been unveiled up to date. One subunit suggested in playing an important role is α5 at the spinal and supraspinal level.

In order to further elucidate the influence of the α5 subunit on nociceptive behavior, I performed PSNL surgeries on wild-type and α5 (-/-) mice to subsequently assess their nociceptive behavior at day 2 and 4 post injury and further to analyze their response to subcutaneous nicotine injection. Finally, we examined the α5 nAChR subunit expression in specific spinal cord and in particular supraspinal regions, at various days post PSNL induction.

4.1 Wild-type and α5 (-/-) mice show no difference 4 days post PSNL to subcutaneous nicotine injection

I could not demonstrate a diminished nicotine-induced antinociceptive activity in α5 (-/-) mice 4 days post PSNL surgery, when compared to wild-type nicotine-treated mice. This is in contrast to a recent publication (Jackson *et al.* 2010), who showed a decline in nicotine-induced spinal and supraspinal antinociceptive activity in naïveα5 (-/-) mice, as assessed by the tail-flick and hot-plate tests, respectively. This could be explained by the fact, that we tested our animals with Hargreaves and acetone tests, but never used tail-flick or hot-plate tests. In addition, Jackson et al only tested naïve wild-type and α5 (-/-) mice, while I investigated the animals post PSNL surgery. Furthermore, in Jackson's report, all animals were analyzed 5 minutes post nicotine injection, while I tested my animals every 5 minutes, in a 60 minute time course, post subcutaneous nicotine injection.

4.2 Wild-type and α5 (-/-) mice show different nicotine sensitivity 4 days post SHAM surgery

In addition to PSNL treated mice, I also tested the antinociceptive activity in wild-type and α5 (-/-) mice at day 4 post SHAM surgery. Again I used the Hargreaves test every 5 minutes, in a 60 minute time course post subcutaneous nicotine injection. Surprisingly, this time I could show a significant difference in the antinociceptive activity of nicotine between wild-type SHAM mice and α5 (-/-) SHAM mice at 15 and 20 minutes post injection. These findings indicate that α5 (-/-) mice, which do not have a peripheral ligation and should therefore, resemble the behavior of naïveα5 (-/-) mice, are less sensitive to the initial effects of nicotine in antinociception, as demonstrated before (Jackson *et al.* 2010).

4.3 PSNL induction does not influence spinal and supraspinal α5 nAChR subunit expression

Previous investigations demonstrated by immunohistochemical stainings, that the expression of the α5 nAChR subunit is enhanced in the outer lamina of the spinal cord dorsal horn ipsilateral to injury as a result of spinal nerve ligation (SNL) (Vincler and Eisenach 2004). We tried to assess this question in a more quantitative manner, by analyzing the spinal cord of PSNL treated mice with our well-established immunoprecipitation technique. Surprisingly, we could not demonstrate a steady, consistent up-regulation of the α5 nAChR subunit due to peripheral nerve ligation. In addition to spinal cord, we also tested various surpaspinal regions, but again we could not see any changes in the levels of α5 after PSNL.

The main differences of our experimental setup compared to the setup from Vincler and Eisenach (Vincler and Eisenach 2004) were the usage of mice instead of rats, further the usage of PSNL as an animal model of neuropathic pain rather than SNL and moreover, analysis of the nAChRs in spinal and supraspinal regions via IP instead of using immunohistochemistry. Therefore, the difference of our results to those from the study mentioned before could be based on the species differences in nicotinic receptors. The role of the β4 subunit for example has previously been shown to be incompatible between subunit expression patterns in rat and mouse (Marks *et al.* 1992). Further, a separate subpopulation of only β2-containing nAChRs on mouse noradrenergic terminals has been proposed, whereas in the rat, all nAChRs seem to contain a β4 subunit. And moreover substantial species differences between mice and rats in both the pharmacology and developmental regulation of nAChR subtypes that modulate hippocampal [^{3}H]Norepinephrine release (Azam and McIntosh 2006).

I performed PSNL surgeries on mice instead of SNL surgeries which were done in the prior study from Vincler and Eisenach (Vincler and Eisenach 2004). The experimental procedure during the PSNL model, involves the ligation of the ipsilateral sciatic nerve at the high-thigh level, so that 1/3 to 1/2 is truncated in the ligature (Seltzer *et al.* 1990). In the SNL model however, L5 and L6 spinal nerves are unilaterally and tightly ligated at a location distal to the dorsal root ganglia (Kim and Chung 1992). In comparison to the PSNL, the ligation side and the extent (e.g. complete ligation) are more consistent in SNL. Nevertheless, the onset of pain in both models is within hours to 1 day and the duration of pain is over weeks; however it may vary between different studies and among tests (Lee *et al.* 1998; Kim *et al.* 1997). Therefore, the alleviated pain and nAChR changes observed in the study from Vincler and Eisenach (Vincler and Eisenach 2004) might have been due to their use of the SNL model.

Moreover, in comparison to our setup, the data from the previous study (Vincler and Eisenach 2004) mentioned was observed via immunoreactivity, we in contrast used immunoprecipitations. In IP-studies the lysates of spinal cord from 2-3 animals are pooled and analyzed. With our technique, we cannot perform anatomical characterizations, as done in the study from Vincler and Eisenach (Vincler and Eisenach 2004), where the expression of the nAChRs in the spinal cord was analysed via immunohistochemistry. It might be possible,

that small changes, wich might have occurred for example at lamina 1 or 2 of the dorsal horn spinal cord only, could not be detected with our method.

However, probably the most important difference in our setup, compared to the experiment setup of Vincler and Eisenach (Vincler and Eisenach 2004), was the use of self-generated antibodies, which were all targeted against the cytoplasmic loop region of mouse nAChR subunits as previously published for anti-α5, anti-β2, and anti- β4 (David *et al.* 2010). The discrepancy between our results and the results observed in the study mentioned earlier may also be due to the fact, that they used commercially available antibodies, whose specificity has been questioned and shown to be insufficient (Moser *et al.* 2007). We are therefore currently attempting to generate an anti- α5 antibody which can be used for immunostaining in order to replicate the results demonstrated by Vincler and Eisenach (Vincler and Eisenach 2004).

In order to further investigate this topic, we are currently trying to perform in situ hybridization with which we will be able to analyze the entire spinal cord instead of only studying the superficial laminae, as it was done in the study previously mentioned (Vincler and Eisenach 2004). Additionly, experiments with a different peripheral nerve ligation model are currently peformed on α5 (-/-) mice and wild-type mice. The chronic construction model (CCI) (Bennett and Xie 1988) is a more severe, long lasting nerve injury, possibly showing more severe differences between wild-type and α5 (-/-) mice, which might be detected easier.

5 Reference List

Aceto M. D., Bagley R. S., Dewey W. L., Fu T. C. and Martin B. R. (1986) The spinal cord as a major site for the antinociceptive action of nicotine in the rat. *Neuropharmacology* 25, 1031-1036.

Albuquerque E. X., Pereira E. F., Alkondon M. and Rogers S. W. (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev.* 89, 73-120.

Auvenshine R. C. (2000) Acute vs. chronic pain. *Tex. Dent. J.* 117, 14-20.

Azam L. and McIntosh J. M. (2006) Characterization of nicotinic acetylcholine receptors that modulate nicotine-evoked [3H]norepinephrine release from mouse hippocampal synaptosomes. *Mol. Pharmacol.* 70, 967-976.

Badio B. and Daly J. W. (1994) Epibatidine, a potent analgetic and nicotinic agonist. *Mol. Pharmacol.* 45, 563-569.

Bannon A. W., Decker M. W., Holladay M. W., Curzon P., Donnelly-Roberts D., Puttfarcken P. S., Bitner R. S., Diaz A., Dickenson A. H., Porsolt R. D., Williams M. and Arneric S. P. (1998) Broad-spectrum, non-opioid analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors. *Science* 279, 77-81.

Barcelo A. C., Filippini B. and Pazo J. H. (2012) The striatum and pain modulation. *Cell Mol. Neurobiol.* 32, 1-12.

Baron R. (2009) Neuropathic pain: a clinical perspective. *Handb. Exp. Pharmacol.* 3-30.

Bennett G. J. and Xie Y. K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33, 87-107.

Campbell J. N. and Meyer R. A. (2006) Mechanisms of neuropathic pain. *Neuron* 52, 77-92.

Carr D. B. and Reuben S. (2005) More on current issues in pain management for the primary care practitioner. Acute pain: a multi-modal management approach. *J. Pain Palliat. Care Pharmacother.* 19, 69-70.

Carr D. B. and Sendil D. (2002) Current approaches to analgesic drug delivery for chronic pain. *Technol. Health Care* 10, 227-235.

Castro-Lopes J. M., Tavares I. and Coimbra A. (1993) GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. *Brain Res.* 620, 287-291.

Champtiaux N., Gotti C., Cordero-Erausquin M., David D. J., Przybylski C., Lena C., Clementi F., Moretti M., Rossi F. M., Le N. N., McIntosh J. M., Gardier A. M. and Changeux J. P. (2003)

Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J. Neurosci.* 23, 7820-7829.

Coggeshall R. E., Lekan H. A., Doubell T. P., Allchorne A. and Woolf C. J. (1997) Central changes in primary afferent fibers following peripheral nerve lesions. *Neuroscience* 77, 1115-1122.

D'Mello R. and Dickenson A. H. (2008) Spinal cord mechanisms of pain. *Br. J. Anaesth.* 101, 8-16.

Damaj M. I., Fei-Yin M., Dukat M., Glassco W., Glennon R. A. and Martin B. R. (1998) Antinociceptive responses to nicotinic acetylcholine receptor ligands after systemic and intrathecal administration in mice. *J. Pharmacol. Exp. Ther.* 284, 1058-1065.

David R., Ciuraszkiewicz A., Simeone X., Orr-Urtreger A., Papke R. L., McIntosh J. M., Huck S. and Scholze P. (2010) Biochemical and functional properties of distinct nicotinic acetylcholine receptors in the superior cervical ganglion of mice with targeted deletions of nAChR subunit genes. *Eur. J. Neurosci.* 31, 978-993.

Decosterd I. and Woolf C. J. (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87, 149-158.

Derbyshire S. W., Jones A. K., Gyulai F., Clark S., Townsend D. and Firestone L. L. (1997) Pain processing during three levels of noxious stimulation produces differential patterns of central activity. *Pain* 73, 431-445.

Dickenson A. H., Bee L. A. and Suzuki R. (2005) Pains, gains, and midbrains. *Proc. Natl. Acad. Sci. U. S. A* 102, 17885-17886.

Exley R., McIntosh J. M., Marks M. J., Maskos U. and Cragg S. J. (2012) Striatal alpha5 nicotinic receptor subunit regulates dopamine transmission in dorsal striatum. *J. Neurosci.* 32, 2352-2356.

Flores C. M., Rogers S. W., Pabreza L. A., Wolfe B. B. and Kellar K. J. (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. *Mol. Pharmacol.* 41, 31-37.

Fowler C. D., Arends M. A. and Kenny P. J. (2008) Subtypes of nicotinic acetylcholine receptors in nicotine reward, dependence, and withdrawal: evidence from genetically modified mice. *Behav. Pharmacol.* 19, 461-484.

Fowler C. D., Lu Q., Johnson P. M., Marks M. J. and Kenny P. J. (2011) Habenular alpha5 nicotinic receptor subunit signalling controls nicotine intake. *Nature* 471, 597-601.

Fucile S., Barabino B., Palma E., Grassi F., Limatola C., Mileo A. M., Alema S., Ballivet M. and Eusebi F. (1997) Alpha 5 subunit forms functional alpha 3 beta 4 alpha 5 nAChRs in transfected human cells. *Neuroreport* 8, 2433-2436.

Fukuoka T., Tokunaga A., Kondo E., Miki K., Tachibana T. and Noguchi K. (1998) Change in mRNAs for neuropeptides and the GABA(A) receptor in dorsal root ganglion neurons in a rat experimental neuropathic pain model. *Pain* 78, 13-26.

Gao B., Hierl M., Clarkin K., Juan T., Nguyen H., Valk M., Deng H., Guo W., Lehto S. G., Matson D., McDermott J. S., Knop J., Gaida K., Cao L., Waldon D., Albrecht B. K., Boezio A. A., Copeland K. W., Harmange J. C., Springer S. K., Malmberg A. B. and McDonough S. I. (2010) Pharmacological effects of nonselective and subtype-selective nicotinic acetylcholine receptor agonists in animal models of persistent pain. *Pain* 149, 33-49.

Gerzanich V., Wang F., Kuryatov A. and Lindstrom J. (1998) alpha 5 Subunit alters desensitization, pharmacology, Ca++ permeability and Ca++ modulation of human neuronal alpha 3 nicotinic receptors. *J. Pharmacol. Exp. Ther.* 286, 311-320.

Girod R., Crabtree G., Ernstrom G., Ramirez-Latorre J., McGehee D., Turner J. and Role L. (1999) Heteromeric complexes of alpha 5 and/or alpha 7 subunits. Effects of calcium and potential role in nicotine-induced presynaptic facilitation. *Ann. N. Y. Acad. Sci.* 868, 578-590.

Gotti C., Clementi F., Fornari A., Gaimarri A., Guiducci S., Manfredi I., Moretti M., Pedrazzi P., Pucci L. and Zoli M. (2009) Structural and functional diversity of native brain neuronal nicotinic receptors. *Biochem. Pharmacol.* 78, 703-711.

Gotti C., Moretti M., Clementi F., Riganti L., McIntosh J. M., Collins A. C., Marks M. J. and Whiteaker P. (2005) Expression of nigrostriatal alpha 6-containing nicotinic acetylcholine receptors is selectively reduced, but not eliminated, by beta 3 subunit gene deletion. *Mol. Pharmacol.* 67, 2007-2015.

Gotti C., Zoli M. and Clementi F. (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol. Sci.* 27, 482-491.

Grady S. R., Moretti M., Zoli M., Marks M. J., Zanardi A., Pucci L., Clementi F. and Gotti C. (2009) Rodent habenulo-interpeduncular pathway expresses a large variety of uncommon nAChR subtypes, but only the alpha3beta4* and alpha3beta3beta4* subtypes mediate acetylcholine release. *J. Neurosci.* 29, 2272-2282.

Grady S. R., Salminen O., McIntosh J. M., Marks M. J. and Collins A. C. (2010) Mouse striatal dopamine nerve terminals express alpha4alpha5beta2 and two stoichiometric forms of alpha4beta2*-nicotinic acetylcholine receptors. *J. Mol. Neurosci.* 40, 91-95.

Grössl F., Huck S. and Scholze P. (2013) Subunit composition of nAChR in the rodent spinal cord. *manuscript in preparation*.

Gustin S. M., Wrigley P. J., Siddall P. J. and Henderson L. A. (2010) Brain anatomy changes associated with persistent neuropathic pain following spinal cord injury. *Cereb. Cortex* 20, 1409-1419.

Hargreaves K., Dubner R., Brown F., Flores C. and Joris J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32, 77-88.

Hay C. and de B. J. (1997) Carrageenan-induced hyperalgesia is associated with increased cyclo-oxygenase-2 expression in spinal cord. *Neuroreport* 8, 1249-1251.

Herber D. L., Severance E. G., Cuevas J., Morgan D. and Gordon M. N. (2004) Biochemical and histochemical evidence of nonspecific binding of alpha7nAChR antibodies to mouse brain tissue. *J. Histochem. Cytochem.* 52, 1367-1376.

Hogg R. C., Raggenbass M. and Bertrand D. (2003) Nicotinic acetylcholine receptors: from structure to brain function. *Rev. Physiol Biochem. Pharmacol.* 147, 1-46.

Jackson K. J., Marks M. J., Vann R. E., Chen X., Gamage T. F., Warner J. A. and Damaj M. I. (2010) Role of alpha5 nicotinic acetylcholine receptors in pharmacological and behavioral effects of nicotine in mice. *J. Pharmacol. Exp. Ther.* 334, 137-146.

Jackson K. J., Martin B. R., Changeux J. P. and Damaj M. I. (2008) Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. *J. Pharmacol. Exp. Ther.* 325, 302-312.

Kedmi M., Beaudet A. L. and Orr-Urtreger A. (2004) Mice lacking neuronal nicotinic acetylcholine receptor beta4-subunit and mice lacking both alpha5- and beta4-subunits are highly resistant to nicotine-induced seizures. *Physiol Genomics* 17, 221-229.

Khan I., Osaka H., Stanislaus S., Calvo R. M., Deerinck T., Yaksh T. L. and Taylor P. (2003) Nicotinic acetylcholine receptor distribution in relation to spinal neurotransmission pathways. *J. Comp Neurol.* 467, 44-59.

Khan I. M., Buerkle H., Taylor P. and Yaksh T. L. (1998) Nociceptive and antinociceptive responses to intrathecally administered nicotinic agonists. *Neuropharmacology* 37, 1515-1525.

Khan I. M., Stanislaus S., Zhang L., Taylor P. and Yaksh T. L. (2001) A-85380 and epibatidine each interact with disparate spinal nicotinic receptor subtypes to achieve analgesia and nociception. *J. Pharmacol. Exp. Ther.* 297, 230-239.

Kim K. J., Yoon Y. W. and Chung J. M. (1997) Comparison of three rodent neuropathic pain models. *Exp. Brain Res.* 113, 200-206.

Koerber H. R., Mirnics K., Kavookjian A. M. and Light A. R. (1999) Ultrastructural analysis of ectopic synaptic boutons arising from peripherally regenerated primary afferent fibers. *J. Neurophysiol.* 81, 1636-1644.

Kohama I., Ishikawa K. and Kocsis J. D. (2000) Synaptic reorganization in the substantia gelatinosa after peripheral nerve neuroma formation: aberrant innervation of lamina II neurons by Abeta afferents. *J. Neurosci.* 20, 1538-1549.

Kuryatov A., Onksen J. and Lindstrom J. (2008) Roles of accessory subunits in alpha4beta2(*) nicotinic receptors. *Mol. Pharmacol.* 74, 132-143.

Lee B. H., Yoon Y. W., Chung K. and Chung J. M. (1998) Comparison of sympathetic sprouting in sensory ganglia in three animal models of neuropathic pain. *Exp. Brain Res.* 120, 432-438.

Malmberg A. B. and Basbaum A. I. (1998) Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain* 76, 215-222.

Mao D., Perry D. C., Yasuda R. P., Wolfe B. B. and Kellar K. J. (2008) The alpha4beta2alpha5 nicotinic cholinergic receptor in rat brain is resistant to up-regulation by nicotine in vivo. *J. Neurochem.* 104, 446-456.

Marks M. J., Pauly J. R., Gross S. D., Deneris E. S., Hermans-Borgmeyer I., Heinemann S. F. and Collins A. C. (1992) Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *J. Neurosci.* 12, 2765-2784.

Marubio L. M., del M. A.-J., Cordero-Erausquin M., Lena C., Le N. N., de Kerchove d. A., Huchet M., Damaj M. I. and Changeux J. P. (1999) Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. *Nature* 398, 805-810.

Matsumoto M., Xie W., Inoue M. and Ueda H. (2007) Evidence for the tonic inhibition of spinal pain by nicotinic cholinergic transmission through primary afferents. *Mol. Pain* 3, 41.

McCarson K. E. and Krause J. E. (1994) NK-1 and NK-3 type tachykinin receptor mRNA expression in the rat spinal cord dorsal horn is increased during adjuvant or formalin-induced nociception. *J. Neurosci.* 14, 712-720.

Moroni M., Vijayan R., Carbone A., Zwart R., Biggin P. C. and Bermudez I. (2008) Non-agonist-binding subunit interfaces confer distinct functional signatures to the alternate stoichiometries of the alpha4beta2 nicotinic receptor: an alpha4-alpha4 interface is required for Zn2+ potentiation. *J. Neurosci.* 28, 6884-6894.

Moroni M., Zwart R., Sher E., Cassels B. K. and Bermudez I. (2006) alpha4beta2 nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Mol. Pharmacol.* 70, 755-768.

Moser N., Mechawar N., Jones I., Gochberg-Sarver A., Orr-Urtreger A., Plomann M., Salas R., Molles B., Marubio L., Roth U., Maskos U., Winzer-Serhan U., Bourgeois J. P., Le Sourd A. M., De Biasi M., Schr+Âder H., Lindstrom J., Maelicke A., Changeux J. P. and Wevers A. (2007) Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *Journal of Neurochemistry* 102, 479-492.

Mutso A. A., Radzicki D., Baliki M. N., Huang L., Banisadr G., Centeno M. V., Radulovic J., Martina M., Miller R. J. and Apkarian A. V. (2012) Abnormalities in hippocampal functioning with persistent pain. *J. Neurosci.* 32, 5747-5756.

Nelson M. E. and Lindstrom J. (1999) Single channel properties of human alpha3 AChRs: impact of beta2, beta4 and alpha5 subunits. *J. Physiol* 516 (Pt 3), 657-678.

Noguchi K. and Ruda M. A. (1992) Gene regulation in an ascending nociceptive pathway: inflammation-induced increase in preprotachykinin mRNA in rat lamina I spinal projection neurons. *J. Neurosci.* 12, 2563-2572.

Ossipov M. H., Lai J., Malan T. P., Jr. and Porreca F. (2000) Spinal and supraspinal mechanisms of neuropathic pain. *Ann. N. Y. Acad. Sci.* 909, 12-24.

Payne R. (1986) Neuropathic Pain Syndromes, with Special Reference to Causalgia and Reflex Sympathetic Dystrophy. *The Clinical Journal of Pain* 2.

Perry D. C., Xiao Y., Nguyen H. N., Musachio J. L., Davila-Garcia M. I. and Kellar K. J. (2002) Measuring nicotinic receptors with characteristics of alpha4beta2, alpha3beta2 and alpha3beta4 subtypes in rat tissues by autoradiography. *J. Neurochem.* 82, 468-481.

Ramirez-Latorre J., Yu C. R., Qu X., Perin F., Karlin A. and Role L. (1996) Functional contributions of alpha5 subunit to neuronal acetylcholine receptor channels. *Nature* 380, 347-351.

Rashid M. H., Furue H., Yoshimura M. and Ueda H. (2006) Tonic inhibitory role of alpha4beta2 subtype of nicotinic acetylcholine receptors on nociceptive transmission in the spinal cord in mice. *Pain* 125, 125-135.

Salas R., Orr-Urtreger A., Broide R. S., Beaudet A., Paylor R. and De B. M. (2003) The nicotinic acetylcholine receptor subunit alpha 5 mediates short-term effects of nicotine in vivo. *Mol. Pharmacol.* 63, 1059-1066.

Salas R., Sturm R., Boulter J. and De B. M. (2009) Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. *J. Neurosci.* 29, 3014-3018.

Salminen O., Murphy K. L., McIntosh J. M., Drago J., Marks M. J., Collins A. C. and Grady S. R. (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol. Pharmacol.* 65, 1526-1535.

Sartorius N., Baghai T. C., Baldwin D. S., Barrett B., Brand U., Fleischhacker W., Goodwin G., Grunze H., Knapp M., Leonard B. E., Lieberman J., Nakane Y., Pinder R. M., Schatzberg A. F., Svestka J., Baumann P., Ghalib K., Markowitz J. C., Padberg F., Fink M., Furukawa T., Fountoulakis K. N., Jensen P., Kanba S. and Riecher-Rossler A. (2007) Antidepressant medications and other treatments of depressive disorders: a CINP Task Force report based on a review of evidence. *Int. J. Neuropsychopharmacol.* 10 Suppl 1, S1-207.

Scholze P., Ciuraszkiewicz A., Groessl F., Orr-Urtreger A., McIntosh J. M. and Huck S. (2011) alpha4beta2 nicotinic acetylcholine receptors in the early postnatal mouse superior cervical ganglion. *Dev. Neurobiol.* 71, 390-399.

Scholze P., Koth G., Orr-Urtreger A. and Huck S. (2012) Subunit composition of alpha5-containing nicotinic receptors in the rodent habenula. *J. Neurochem.* 121, 551-560.

Seltzer Z., Dubner R. and Shir Y. (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 43, 205-218.

Shelton L., Becerra L. and Borsook D. (2012) Unmasking the mysteries of the habenula in pain and analgesia. *Prog. Neurobiol.* 96, 208-219.

Skoubis P. D., Hradil V., Chin C. L., Luo Y., Fox G. B. and McGaraughty S. (2006) Mapping brain activity following administration of a nicotinic acetylcholine receptor agonist, ABT-594, using functional magnetic resonance imaging in awake rats. *Neuroscience* 137, 583-591.

Tapia L., Kuryatov A. and Lindstrom J. (2007) Ca2+ permeability of the (alpha4)3(beta2)2 stoichiometry greatly exceeds that of (alpha4)2(beta2)3 human acetylcholine receptors. *Mol. Pharmacol.* 71, 769-776.

Ueda M., Iida Y., Tominaga A., Yoneyama T., Ogawa M., Magata Y., Nishimura H., Kuge Y. and Saji H. (2010) Nicotinic acetylcholine receptors expressed in the ventralposterolateral thalamic nucleus play an important role in anti-allodynic effects. *Br. J. Pharmacol.* 159, 1201-1210.

Ueda M., Iida Y., Yoneyama T., Kawai T., Ogawa M., Magata Y. and Saji H. (2011) In vivo relationship between thalamic nicotinic acetylcholine receptor occupancy rates and antiallodynic effects in a rat model of neuropathic pain: persistent agonist binding inhibits the expression of antiallodynic effects. *Synapse* 65, 77-83.

Vincler M. and Eisenach J. C. (2004) Plasticity of spinal nicotinic acetylcholine receptors following spinal nerve ligation. *Neurosci. Res.* 48, 139-145.

Vincler M. A. and Eisenach J. C. (2005) Knock down of the alpha 5 nicotinic acetylcholine receptor in spinal nerve-ligated rats alleviates mechanical allodynia. *Pharmacol. Biochem. Behav.* 80, 135-143.

von Hehn C. A., Baron R. and Woolf C. J. (2012) Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron* 73, 638-652.

Wang N., Orr-Urtreger A., Chapman J., Rabinowitz R., Nachman R. and Korczyn A. D. (2002) Autonomic function in mice lacking +¦5 neuronal nicotinic acetylcholine receptor subunit. *The Journal of Physiology* 542, 347-354.

Wonnacott S. (1997) Presynaptic nicotinic ACh receptors. *Trends Neurosci.* 20, 92-98.

Woolf C. J. and Costigan M. (1999) Transcriptional and posttranslational plasticity and the generation of inflammatory pain. *Proc. Natl. Acad. Sci. U. S. A* 96, 7723-7730.

Woolf C. J. and Ma Q. (2007) Nociceptors--noxious stimulus detectors. *Neuron* 55, 353-364.

Woolf C. J. and Mannion R. J. (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* 353, 1959-1964.

Woolf C. J. and Salter M. W. (2000) Neuronal plasticity: increasing the gain in pain. *Science* 288, 1765-1769.

Woolf C. J., Shortland P. and Coggeshall R. E. (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355, 75-78.

Xiao Y. and Kellar K. J. (2004) The Comparative Pharmacology and Up-Regulation of Rat Neuronal Nicotinic Receptor Subtype Binding Sites Stably Expressed in Transfected Mammalian Cells. *Journal of Pharmacology and Experimental Therapeutics* 310, 98-107.

Xiao Y., Meyer E. L., Thompson J. M., Surin A., Wroblewski J. and Kellar K. J. (1998) Rat a3/b4 Subtype of Neuronal Nicotinic Acetylcholine Receptor Stably Expressed in a Transfected Cell Line: Pharmacology of Ligand Binding and Function. *Molecular Pharmacology* 54, 322-333.

Yang L., Zhang F. X., Huang F., Lu Y. J., Li G. D., Bao L., Xiao H. S. and Zhang X. (2004) Peripheral nerve injury induces trans-synaptic modification of channels, receptors and signal pathways in rat dorsal spinal cord. *European Journal of Neuroscience* 19, 871-883.

Young T., Wittenauer S., McIntosh J. M. and Vincler M. (2008) Spinal alpha3beta2* nicotinic acetylcholine receptors tonically inhibit the transmission of nociceptive mechanical stimuli. *Brain Res.* 1229, 118-124.

Zoli M., Moretti M., Zanardi A., McIntosh J. M., Clementi F. and Gotti C. (2002) Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *J. Neurosci.* 22, 8785-8789.

6 Curriculum vitae

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