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„ $\alpha 5$ -containing nicotinic acetylcholine receptors in animal models of neuropathic pain and their contribution to nicotine-induced analgesia“

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Abstract

Nicotinic acetylcholine receptors (nAChR) are pentameric ligand-gated cation channels made up of 9 α and 3 β subunits that can assemble into a multitude of different homo- or hetero-pentameric receptors. The subunit composition crucially influences the pharmacological and biochemical properties of a given receptor.

The nAChR subtypes mediating pain mechanisms are largely unknown. One of the subunits possibly involved is the $\alpha 5$ subunit. This subunit cannot participate in the formation of a ligand binding site, but requires at least one other α and one other β -subunit to form a functional receptor. The presence of $\alpha 5$ displays a modulatory role, greatly influencing the receptor properties.

In this thesis, I examine the role of $\alpha 5$ -containing nAChRs in mouse animal models of neuropathic pain and in the analgesic response to nicotine.

A chronic constriction injury of the sciatic nerve was performed in WT and $\alpha 5$ -KO mice, in order to induce symptoms of neuropathic pain. Animals were followed with different nociceptive tests over a timecourse and the thermal analgesic response to acute 2 mg/kg subcutaneously applied nicotine was assessed at different timepoints after surgery. In a second set of experiments, WT and $\alpha 5$ -KO mice were intermittently treated with three daily injections of 2 or 4 mg/kg nicotine for 11 days. This produces a tolerance to the drug analgesic effects. These animals were observed over the timecourse in their thermal analgesic responses after the first daily nicotine injection.

After nerve injury, $\alpha 5$ -KO mice showed no significantly different behaviors in the nociception timecourse as compared to WT mice. Thermal analgesia to 2 mg/kg nicotine, at different timepoints after peripheral nerve injury, was not significantly different in $\alpha 5$ -KO as compared to WT animals.

In naive mice, 2 and 4 mg/kg nicotine showed a longer lasting effect in $\alpha 5$ -KO than it did in WT mice. Furthermore, $\alpha 5$ -KO differed from their WT littermates in the development of tolerance after intermittent administration of 2 and 4 mg/kg nicotine.

Our results suggest that the $\alpha 5$ subunit of nAChRs does not play a major role in the animal model of neuropathic pain used. However, the $\alpha 5$ subunit may be involved in analgesic properties of nicotine after application of 2 and 4 mg/kg, as well as in tolerance mechanisms, occurring after chronic, intermittent exposure to nicotine.

Zusammenfassung

Nikotinische Azetylcholinrezeptoren (nAChRs) sind pentamere, Liganden-gesteuerte Kationenkanäle im zentralen und peripheren Nervensystem. Durch Integration und Kombination der bekannten 9α und/oder 3β Untereinheiten entsteht eine Vielzahl möglicher Rezeptorzusammensetzungen, welche die biochemischen wie auch pharmakologischen Eigenschaften des Ionenkanals bestimmt. Verschiedene Zusammensetzungen von nAChRs sind dabei an einer Vielzahl physiologischer Funktionen beteiligt. Die $\alpha 5$ Untereinheit spielt keine Rolle für die Liganden-bindungsstelle von nAChRs, hat aber einen großen Einfluss auf die Eigenschaften des Rezeptors.

Im Rahmen dieser Studie soll eine mögliche Rolle der $\alpha 5$ Untereinheit in Tiermodellen für neuropathischen Schmerz sowie für verschiedene Aspekte der analgetischen Wirkung von Nikotin ermittelt werden.

Zu diesem Zweck wurde das Chronic Constriction Injury (CCI) Schmerzmodell an wildtyp Mäusen sowie an Mäusen mit genetischem knockout des Genes der $\alpha 5$ Untereinheit ($\alpha 5$ -KO) durchgeführt. Die Sensitivität dieser Tiere für verschiedene Modalitäten wurde für 29 Tage nach der Operation ermittelt. Zusätzlich wurde der antinociceptive Effekt von 2 mg/kg Nikotin an verschiedenen Stadien nach Induktion des Schmerzmodells ermittelt. In weiteren Versuchen wurden beide Genotypen 3-mal täglich mit 2 oder 4 mg/kg Nikotin behandelt. Diese Behandlung führt zu einer raschen Entwicklung einer Toleranz gegenüber der analgetischen Wirkung. Die Tiere wurden jeden zweiten Tag und nach der ersten täglichen Nikotin Behandlung bezüglich der analgetischen Wirkung von Nikotin getestet.

Der genetische knockout der $\alpha 5$ Untereinheit hatte keinen signifikanten Einfluss auf die Entwicklung der Sensitivität nach CCI. Auch die antinociceptive Wirkung von 2 mg/kg Nikotin in Tieren mit CCI war vergleichbar für beide Genotypen. Ohne CCI zeigten $\alpha 5$ -KO Tiere eine verlängerte Wirkung von 2 und von 4 mg/kg Nikotin gegenüber ihren WT Geschwistern. Zusätzlich hatten diese $\alpha 5$ -KO Tiere eine reduzierte analgetische Toleranzentwicklung nach wiederholter Nikotin Injektion.

Unsere Ergebnisse legen nahe, dass die $\alpha 5$ Untereinheit nicht am Verlauf neuropathischer Schmerzen nach CCI beteiligt ist. Die $\alpha 5$ Untereinheit scheint jedoch relevant für die Dauer des analgetischen Effektes von Nikotin sowie für die Entwicklung einer Toleranz diesbezüglich.

1. Introduction

1.1. Nociception

Nociception refers to the specific neural processes involved in the encoding and processing of acute noxious stimuli which can be harmful or damaging for an individual. In contrast, the pain experience is defined as “unpleasant and emotional experience with actual or potential tissue damage or described in terms of such damage” (from IASP homepage: www.iasp-pain.org; (Sandkuhler, 2009) and does not only protect from further tissue damage within a present harmful situation, but also ensures survival and proper development of individuals. In extreme conditions, this experience can be missing due to a genetic defect which leads to a lack of learned protective behaviors and in consequence most people suffering from this defect die at an early age. However, more frequently people with chronic pain suffer from dysfunctional pain signaling characterized by spontaneous pain and/or an increased nociceptive sensitivity. This signaling usually exceeds a useful level and an appropriate time to simply protect from tissue damage. These chronic pathological pain conditions are strongly obstructive for daily living and can cause even further psychological discomfort and depression. Thus, the investigation of cellular and molecular mechanisms, occurring during these conditions, is of obvious clinical interest. A more detailed description of these pathological pain conditions and possible mechanisms involved is given below.

1.1.1. Nociceptive Transmission

Peripheral afferent nerve fibers which are responsible for the transmission of nociceptive information are called nociceptors. These nociceptors have a pseudo-unipolar morphology and their cell bodies are located in the dorsal root ganglion. This location and anatomy allows them to innervate peripheral target tissues and to transmit information about mechanical, thermal and/or chemical environment to corresponding spinal segments. Interestingly, nociceptors only respond to stimulus intensities within a determined interval

which is usually considered to reflect potential or acute tissue damage. Thus, nociceptors have the ability to selectively detect and process peripheral stimuli exceeding a noxious threshold.

Two morphologically distinct fiber classes are known to process painful stimuli. One class consists of small and myelinated A δ - and A β -fibers which are responsible for the fast transmission of acute and well-localized pain. The second types of nociceptors are the unmyelinated C-fibers which are responsible for poorly localized pain sensations with a delayed onset. This rough classification can be further subdivided with respect to distinct molecular expression patterns of peptides and proteins (Snider and McMahon, 1998; Dong et al., 2001). Besides this molecular heterogeneity, nociceptors can additionally be subdivided concerning their sensitivity and threshold intensity in the detection of distinct pain modalities (Schmidt et al., 1995; Perl, 2007; Olausson et al., 2008; Basbaum et al., 2009). As a consequence, nociceptors can be specialized for a single noxious modality or they can respond to a wide range of modalities as polymodal nociceptors.

1.1.1.1. Peripheral Nociceptive Stimuli Detection

Nociceptive signals are detected from the environment by specialized receptors. These receptors display characteristic features for the transduction of specific environmental stimuli into neuronal conduction properties. The transduction of environmental temperature is performed by temperature-activated transient receptor potential (TRP) ion channels. These channels are individually specialized for the detection of temperatures in a distinct range comprising noxious as well as innocuous intensities (Patapoutian et al., 2003). The sensory system for the detection of mechanical stimuli consists of a variety of specialized mechanoreceptors. Besides the TRP channel family, members of the acid-sensing ion channels are also relevant for the transduction of mechanical stimuli. Noxious mechanical information is transmitted via high-threshold polymodal or mechanospecific fibers (Perl, 1996).

After environmental stimuli are transduced by sensory receptors, voltage gated ion channels are crucial for the conductance of signals from the peripheral terminals to spinal neurons. A

major role for nociceptive signaling is attributed to various voltage-gated sodium channels (e.g. Nav1.7) as well as potassium and calcium channels. All these channels are essential for the proper generation of action potentials in nociceptors (Basbaum et al., 2009).

1.1.1.2. Spinal Termination of Nociceptors

The major termination site of peripheral afferent neurons is the spinal cord dorsal horn. The cytoarchitecture of the spinal cord grey matter is organized in distinct laminae, which can be separated anatomically as well as electrophysiologically (Basbaum and Jessell, 2000). Nociceptive A δ - and C-fibers both enter the dorsal horn via the dorsolateral fasciculus but terminate in distinct regions. A δ -fibers predominantly terminate in lamina I and in deeper lamina V, whereas C-fibers mainly project to the superficial lamina I and lamina II (substantia gelatinosa). Thus, cells of the superficial laminae are primarily responding to noxious stimulation. These cells further project to the contralateral spinal cord to form the lateral spinothalamic tract. In contrast, lamina V receives input from both nociceptive as well as non-nociceptive A β -fibers. Innocuous, low threshold A β -fibers primarily project in lamina III and lamina IV. This variety of low threshold and high threshold information is further processed by wide dynamic range (WDR) cells. These second order spinal neurons respond nonselectively to high- as well as low threshold primary afferents in relation to the input from noxious and innocuous cells and thus can have a prominent role for hypersensitivity in chronic neuropathic pain states (Woolf, 1983; Sandkuhler, 2009). The main neurotransmitter of nociceptive afferents in the spinal cord dorsal horn is glutamate. However, modulation of synaptic transmission at the spinal level is well known to occur in a complex interplay of neuropeptides (e.g. substance P, calcitonin gene related peptide), neurotrophins (e.g. nerve growth factor, brain-derived neurotrophic factor), cytokines (e.g. fractalkines) and endogenous opiates (e.g. enkephalines, β -endophines) which can be released by primary afferent neurons, spinal interneurons, astrocytes, vascular cells, microglia and mast cells, as well as CD8 and CD4 positive T-cells (Xanthos and Sandkuhler, 2014). The proper signaling of all factors can be disturbed and further contribute to neurogenic inflammation at spinal terminals. Interestingly, nicotinic acetylcholine receptors (nAChRs) are distributed over the

whole grey matter of the spinal cord but their highest density is reported in the dorsal horn (Khan et al., 2003), where most nociceptive fibers first synapse.

1.1.1.3. Supraspinal Processing of Noxious Information

Among others, the WDR and STT (spinothalamic tract) projection neurons transmit noxious information from spinal dorsal horn laminae I and V via the ascending spinothalamic and spinoreticular tracts to supraspinal regions. The first destination and next relay station of these fibers is the thalamus and the reticular formation in the brainstem, respectively. Both regions further innervate cortical structures. The somatosensory cortex is one particularly important final destination for the discriminative aspect of pain. It is important to note that pain is not represented by one specific brain area but rather arises from the parallel participation of a multitude of brain areas (Apkarian et al., 2005). Next to sensory discrimination and evaluation of a painful stimulus, also emotional properties of the nociceptive information are processed. Therefore, other important regions also process the information especially in the anterior cingulate cortex and insular cortex. Additionally, the amygdala and parabrachial nucleus are considered to generate the affective properties of pain experiences (Basbaum et al., 2009). The periaqueductal grey (PAG) and the parabrachial regions in the midbrain are presumably not only innervated by spinomesencephalic ascending projections but are further involved in the brain matrix of pain sensation. That is also the case for the basal ganglia, the habenulo-interpeduncular pathway, and the frontal-, orbital- and the cingulate cortex (Porro et al., 1999; Shelton et al., 2012). Involvement of further brain areas is very likely and contributes to the complex experience of pain which may be influenced by nAChR signaling in the thalamus, hypothalamus, amygdala, hippocampus, basal ganglia cortical structures as well as locus caeruleus, interpeduncular, and raphe nuclei (Gotti et al., 2006).

The main descending, pain modulatory, pathways arise from the PAG and locus caeruleus in the brainstem. PAG projections further relay to serotonergic cells in nuclei raphe magnus of the rostral ventral medulla (RVM), before descending together with noradrenergic projections from locus caeruleus. At the affected spinal level early nociceptive information can be positively and negatively modulated by noradrenaline, 5-hydroxytryptamine (5-HT,

serotonin) or dopamine (Vanegas and Schaible, 2004; Basbaum et al., 2009). Serotonergic projections from the RVM can exert either pain inhibitory actions via 5-HT₁ receptor activation or pain enhancing actions via 5-HT₂ or 5-HT₃ activation at corresponding spinal dorsal horn levels. In contrast, descending noradrenaline projections from the locus caeruleus have only been shown to mediate antinociceptive activity by mainly activating α 2 but also α 1 receptors on primary afferents and inhibitory interneurons in the spinal dorsal horn. Dopaminergic modulation mainly arises from periventricular posterior hypothalamus and depending on local concentrations it displays inhibition of nociceptive signaling by activation of D1 and D3 receptors or pronociceptive effects by activation of D1 receptors, both at spinal cord dorsal horn neurons (Kwon et al., 2013).

1.1.2. Chronic Pain

The nociceptive signal and pain experience helps an organism to judge and prevent contact with situations and stimuli that might be harmful. If tissue damage has occurred, a prolongation of pain signaling can mediate guarding behavior of injured tissue and thus prevent further tissue damage and promote regeneration at the site of injury. However, in some cases, pain signaling can persist much longer than the normal duration of acute tissue damage and healing. This is then referred to as chronic pain (Basbaum et al., 2009). With this definition, chronic pain loses its functionality and becomes a disease itself rather than a symptom of other diseases. The exact definition for the duration of chronic pain is ambivalent and depends on the individual experience of the pain sensation but is often referred to 6 months (from IASP homepage: www.iasp-pain.org). Besides physical injury, the exact causes of chronic pain often comprise other comorbidities with a combination of social, psychological as well as physical abnormalities (Kwon et al., 2013).

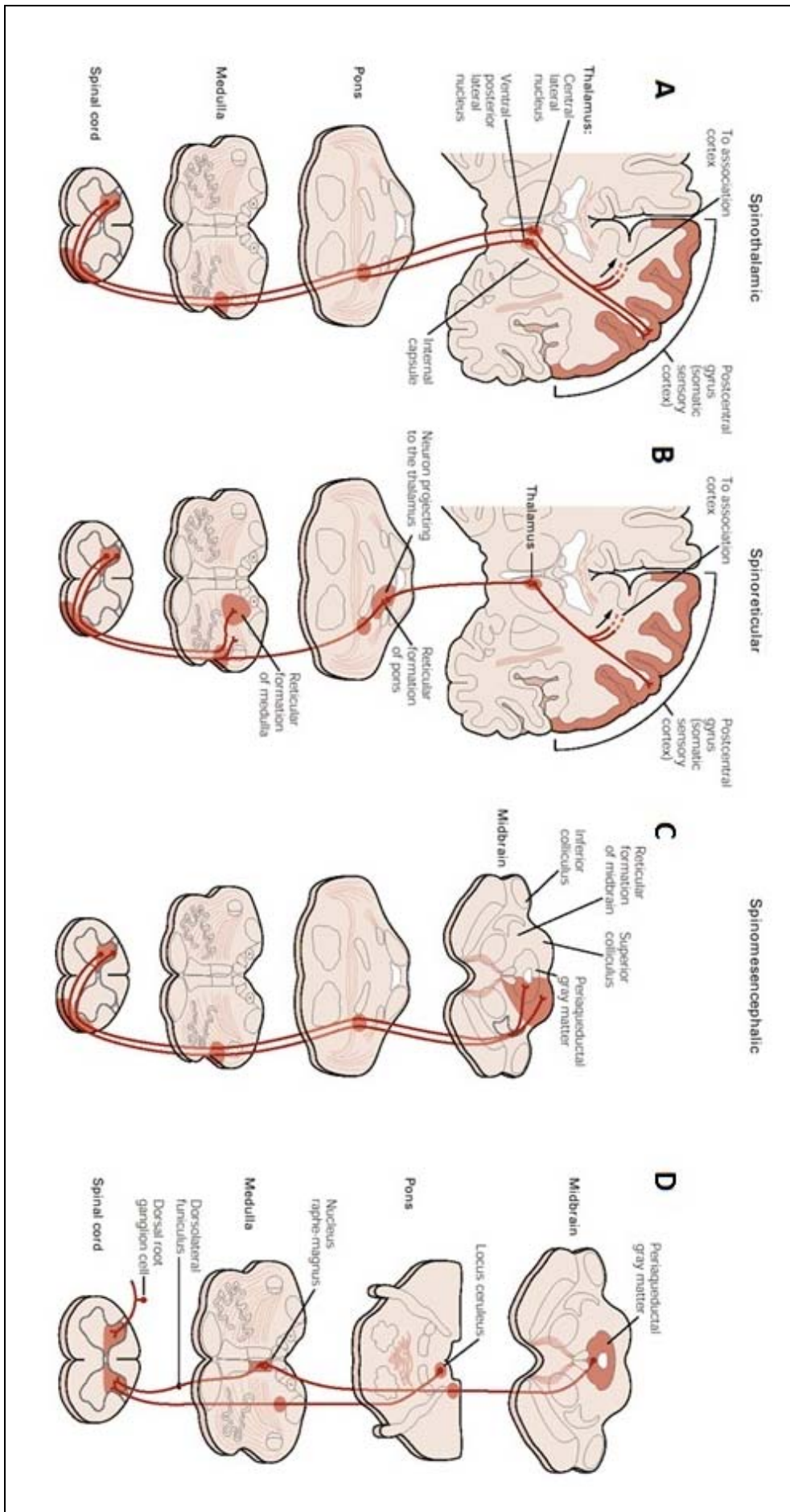


Figure 1: Simplified, schematic illustration of the human nociceptive pathway anatomy. **A:** The spinothalamic tract as main transmission route for nociceptive signals crosses to the contralateral site at a spinal level, before it forms its first synaptic relay station in the thalamus. From this point thalamocortical fibers finally project to distinct cortical structures **B:** The spinoreticular tract follows a similar course as the spinothalamic tract, but shows synaptic terminations at reticular structures at the level of medulla and pons **C:** The spinomesencephalic tract primarily terminates in the periaqueductal grey and superior colliculus of the mammalian midbrain **D:** Descending, pain modulatory fibers predominantly arise from the mammalian brain stem. The illustration is taken and adapted from (Willis, 1985).

1.1.2.1. Neuropathic Pain

The chronic disease state of maladaptive pain can affect parts of the nervous system, which normally signal pain. This condition is defined as neuropathic pain (Baron, 2000). Neuropathic pain is characterized by an increased pain experience in response to noxious stimuli (hyperalgesia) and/or by a pain experience to stimuli of innocuous intensities (allodynia). The pathophysiological mechanisms responsible for these phenomena can affect peripheral, spinal and supraspinal regions. It is, most likely, not a single mechanism, but rather a combination of secondary changes after nerve injury, which leads to the typical symptoms of neuropathic pain.

1.1.2.1.1. Neuropathic Pain: Peripheral Mechanisms

Neuropathic pain can result from a mechanical injury or a disease affecting the properties of peripheral nerves. Thereby, the chemical environment of primary afferents is changed by infiltration of inflammatory cells (mast cells, macrophages, neutrophils, endothelial cells, etc.) and release of substances such as neurotransmitters, peptides (bradykinin, substance P), lipids (e.g. endocannabinoids), cytokines, chemokines and neutrophins (Basbaum et al., 2009). These and further changes in the chemical milieu of nerve fibers can trigger increased excitability and a lowering of the action potential threshold of peripheral nerves. This is called peripheral sensitization. Further alterations include changes in the expression of voltage gated sodium channels in primary afferents. An accumulation of sodium channels in

C- and A-delta fibers can also be relevant for pathological sensitivity or spontaneous activity after nerve injury (Baron, 2006).

1.1.2.1.2. Neuropathic Pain: Spinal Mechanisms

Mechanisms for the maintenance of chronic pain exist not only at peripheral but also at central nervous sites. Central sensitization is supported by vascular changes, microglia and astrocyte activation, and also an increased release of pro-inflammatory mediators such as cytokines and brain derived neurotrophic factor (BDNF) both of which facilitate increased excitability of pain transmission (Tsuda et al., 2003; Coull et al., 2005; Wieseler-Frank et al., 2005). Central sensitization, leading to hyperexcitability, can take place at spinal as well as supraspinal sites.

In the spinal cord dorsal horn, signals from peripheral nociceptors are transmitted to second order central neurons which release glutamate thereby generating an excitatory postsynaptic current (EPSC). In normal states, this liberation of glutamate into the synaptic cleft activates postsynaptic AMPA receptors. Depending on the amount of excitatory and inhibitory postsynaptic currents (IPSC) arriving at the second order neuron, an action potential may be generated. However, in injured or diseased states, the amount of liberated presynaptic transmitters from nociceptors can be strongly increased. As a consequence, the postsynaptic neuron can be depolarized during simultaneous presence of glutamate. This coincidence activates postsynaptic NMDA receptors. The following calcium influx is sufficient to trigger synaptic strengthening via long term potentiation (LTP). This neuronal based mechanism for amplified synaptic transmission from nociceptor to dorsal horn neuron in turn has been proposed to explain increased sensitivity to noxious stimuli (Drdla and Sandkuhler, 2008).

A further mechanism for central sensitization in chronic pain states affects the inhibitory GABAergic (γ -aminobutyric acid) or glycinergic interneurons in the spinal dorsal horn. The inhibitory action of these cells restricts the excitability of lamina I projection neurons. Thus, these cells have a strong modulatory influence (Melzack and Wall, 1965; Rashid and Ueda, 2002; Rashid et al., 2006). Loss of inhibition is thought to be involved in neuropathic pain.

This idea of disinhibition has been shown in experiments where hyperalgesia is evoked after spinal administration of GABA and glycine receptor antagonists (Sivilotti and Woolf, 1994; Malan et al., 2002; Labrakakis et al., 2009) and a selective loss of inhibitory dorsal horn interneurons after peripheral nerve injury (Moore et al., 2002). Furthermore, normally non-nociceptive A β -fibers can interact with the nociceptive transmission, due to the lack of inhibitory tone. This would be a potential functional mechanism for the generation of allodynia (Basbaum et al., 2009).

1.1.2.1.3. Neuropathic Pain: Supraspinal Mechanisms

The establishment of chronic pain states via neuroplastic changes in structure, function or neurochemical profile can further occur at the supraspinal level. Especially descending pain modulatory structures arising from the brainstem reticular formation have been shown to be involved during persistent pain states (Urban and Gebhart, 1999; Ossipov et al., 2000; Porreca et al., 2002; Zambreanu et al., 2005). Besides these structures, the striatum and the habenula have been functionally associated to pain and analgesia and are thus likely candidates for supraspinal modulations of pain transmission (Barcelo et al., 2012; Shelton et al., 2012). Further anatomical changes associated with neuropathic pain after spinal cord injury have been reported in the thalamus, amygdala, insular cortex and premotor cortex in human magnetic resonance imaging studies (Gustin et al., 2010).

Sensory changes following neuropathic pain in nerve injured animals are associated with selective remodeling of cortical synapses, especially in the primary somatosensory cortex (Kim and Nabekura, 2011). Also altered connectivity in other cortical and subcortical structures as well as reduced hippocampus-prefrontal cortex connections have been described during neuropathy and might be relevant for reported changes in cognitive performances (Cardoso-Cruz et al., 2011; Ren et al., 2011; Mutso et al., 2012).

1.1.2.2. Models of Neuropathic Pain

A peripheral neuropathy is a neuropathic pain disease, which arises from injuries to the peripheral nervous system. For the investigation of underlying mechanisms and development of more effective treatments, animal models that mimic the symptoms of neuropathic pain are used. Since the etiology of neuropathy is not always clear in humans, many different models cover various aspects of nerve injuries. A rough classification can separate inflammatory mediated mechanisms from those due to direct mechanical damage to the nerve. The injury can also cause unnatural firing of primary afferent neurons. Thus, spontaneous pain, allodynia and hyperalgesia can be the consequence. The severity of symptoms can be estimated in behavioral experiments with stimulation of different intensities and modalities. As an example for drug induced neuropathic pain, chemotherapy treatments (e.g. oxaliplatin) can be mentioned, whereas the peripheral sciatic nerve ligation (PSNL) and the spinal nerve ligation (SNL) are examples for a mechanical injury of regular nerve function. The chronic constriction injury (CCI) is sometimes suggested to involve an inflammatory component due to the use of a chromic gut suture and a neuropathic component due to subsequent mechanical compression of the sciatic nerve.

1.1.2.3. Neuropathic Pain Clinical Implications

So far the most commonly used drugs to treat pain are opioids. However, their uses have limitations. Next to their extensive side effects such as nausea, vomiting, drowsiness, respiratory depression, gastrointestinal disturbance, and addictive potential (Woodcock, 2009), opioids still don't manage to counteract conditions of neuropathic pain in all patients (Ollat and Cesaro, 1995; McCormick and Schreiner, 2001). Thus, other drugs are also currently used for therapeutic treatment of neuropathic pain and include off-label use of nonsteroidal anti-inflammatory drugs, some neuroleptics as well as antidepressant drugs. Drugs for a specific action towards neuropathic symptoms are under investigation and include ligands for specific nAChRs (Umana et al., 2013). Treatment options without pharmacological intervention include nerve stimulation, physical therapies and acupuncture.

1.2. Acetylcholine Receptors (AChRs)

The acetylcholine receptors can be divided into two main classes, the muscarinic and the nicotinic acetylcholine receptors (nAChRs). Both can be activated by the endogenous ligand acetylcholine. The muscarinic receptor is a G-protein coupled membrane receptor and binds the natural substance muscarine, a compound isolated from certain mushrooms as *Aminata muscaria*. The receptor can be further subdivided into five subtypes which are all built from seven transmembrane segments (Hulme, 1990; Caulfield and Birdsall, 1998). Muscarinic receptors are not only distributed over a variety of central nervous system structures, but also in parasympathetic neurons and their target organs (Haga, 2013).

The nicotinic acetylcholine receptor is a ligand-gated ion channel and similar to the 5-hydroxytryptamine₃ (5-HT₃), glycine- and GABA A and C receptors, it belongs to the superfamily of Cys-loop receptors. The nAChR consists of five subunits, each of them spanning through the cellular plasma membrane and thereby forming a central pore. When the receptor is activated by ligand binding, the channel becomes permeable to cations such as K⁺, Na⁺ and Ca²⁺, thereby having an excitatory effect on the neuronal synapse. Besides activation by the endogenous ligand acetylcholine, all nAChRs are also activated by nicotine, a well-known plant alkaloid. After binding to the receptor, acetylcholine is metabolized to acetate and choline by acetylcholinesterase within the synaptic cleft. The resulting choline is transported back into the presynaptic terminal where it is further processed to acetylcholine together with acetyl coenzyme A.

In both the central and peripheral nervous systems, a considerable proportion of nAChRs are located on presynaptic terminals. This localization of receptors and their potential to increase intracellular Ca²⁺ concentration can exert a strong modulatory activity on brain synaptic transmission. Activation of these receptors can influence the release of neurotransmitters, including glutamate, serotonin, noradrenaline, dopamine, GABA and acetylcholine itself, all in a positive or negative manner (McGehee et al., 1995; Alkondon et al., 1997; Wonnacott, 1997; Gotti et al., 2006; Albuquerque et al., 2009).

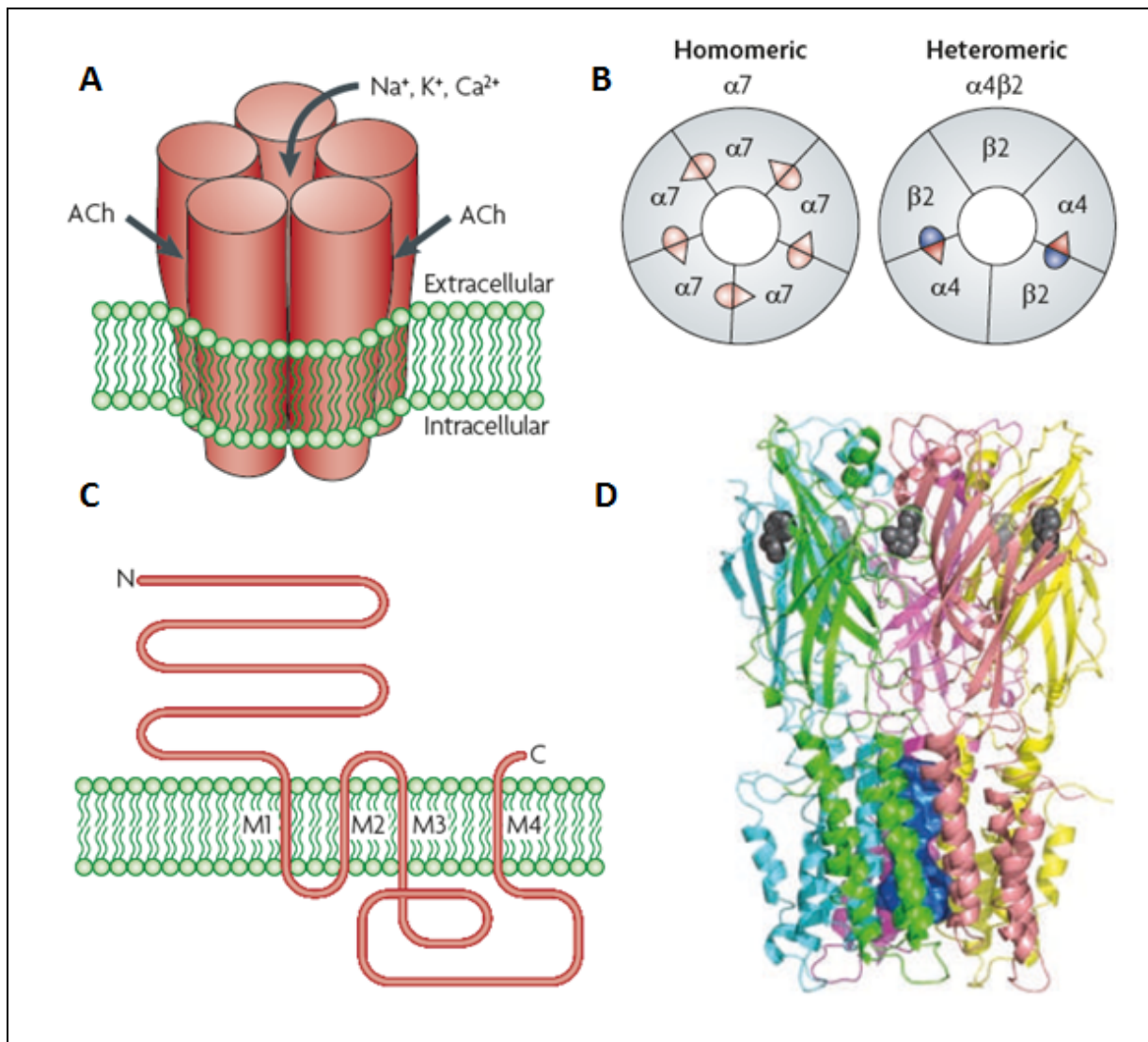


Figure 2: Illustration of nicotinic acetylcholine receptor (nAChR) structures. **A:** nAChRs consist of five subunits, spanning through the plasma membrane. The receptors are permeable for cations, such as sodium, potassium and calcium ions. **B:** The pentamer can be built from five identical subunits (homomeric) or a combination of α and β subunits (heteromeric). Homopentameric receptors have five ligand binding sites between each subunit interface, whereas heteropentameric receptors form only two binding sites between α and β subunit interfaces. In the central nervous system (CNS), the most common homomeric nAChR is composed of five $\alpha 7$ subunits. The $\alpha 4\beta 2$ subtype represents the most abundant heteromeric nAChR in the CNS. Thereby, two $\alpha 4$ and two $\beta 2$ subunits shape the basic structure of the receptor. The fifth subunit completes the receptor without participating in the formation of the ligand binding pockets. The identity of this subunit is variable but can strongly influence the receptor properties (here illustrated as a third $\beta 2$ subunit). **C:** Each subunit consists of four transmembrane domains (M1-M4), an intracellular loop between M3 and M4 as well as an extracellular N- and C-terminus. The acetylcholine binding site is located at these extracellular domains between subunits. **D:** Model of the protein structure of a $\alpha 7$ homopentamer. The ion channel is illustrated in dark blue and five bound ligands are shown in dark grey. The illustrations are taken and adapted from (Changeux, 2010).

1.3. Structure, Distribution and Function of nAChRs

Each subunit of nAChRs is formed by four hydrophobic transmembrane domains (M1 – M4) with a large extracellular NH₂-terminus at M1, containing the receptor family characteristic cysteine-loop, a short extracellular COOH terminus at M4 and a cytoplasmic loop, with varying amino acid sequence and size, between M3 and M4 (Albuquerque et al., 2009). Ligand binding induces rearrangement of the subunits in a manner, which opens the central pore and allows ion influx through the channel.

One subgroup of nAChRs is present at the neuromuscular junction of skeletal muscles and thus plays a role for the neuromuscular transmission. These receptors consist of two α 1, one β 1 and δ as well as either ϵ , γ or δ subunits (Wonnacott and Barik, 2007). However, the major portion of nAChRs is located in the central and peripheral nervous system (Hogg et al., 2003).

Neuronal nAChRs exist in a variety of possible subunit combinations. So far, nine α (α 2 – α 10) and three β (β 2 – β 4) subunits have been identified in neuronal and non-neuronal tissues (Gotti et al., 2009). These subunits can form homopentameric or heteropentameric proteins. A homopentameric receptor uses one type of subunit to integrate five times into the functional receptor. The most common homopentameric nAChR in the mammalian nervous system is the α 7-homopentamer. In contrast to the homopentameric receptors, heteropentameric receptors are composed of a combination of α and β subunits. Hence, pharmacological and biophysical properties of the receptor are highly dependent on the subunit combination and ratios which build the functional receptor (McGehee and Role, 1995; Fucile, 2004; Jensen et al., 2005; Gotti et al., 2007). The subunit organization in distinct areas of the central nervous system (CNS) and the peripheral nervous system (PNS) have been identified by immunoprecipitation and immunopurification studies and were reported by Gotti et al. (2006) in detail. It should be noted that not all theoretically possible subunit combinations for heteropentameric receptors are expressed *in vivo* (Gotti et al., 2006).

In heteropentameric nAChRs, the ligand binding site is located at the interface between α - and β -subunits. A primary site of ligand binding is provided by a cysteine pair in α -subunits. β 2- and β 4-subunits do not possess this cysteine pair and thereby provide a complementary face, thus completing the formation of a ligand binding pocket for the endogenous ligand

acetylcholine. With these restrictions, the foundation of heteropentameric nAChRs is featured by two ligand binding sites and must include two similar α and β subunits. A further supplementary subunit completes the functional receptor. The ligand binding site of homopentameric receptors is located on the interface between each subunit, thus forming five identical binding sites per receptor (Changeux, 2010).

In the PNS, the most frequent heteropentameric nAChR is the $\alpha 3\beta 4$ subtype which is also known as the 'ganglionic type' of nAChRs. In contrast, in the CNS the $\alpha 4\beta 2$ subtype dominates the vast majority of nAChRs and thus is also known as the 'brain type' of nAChRs (Gotti et al., 2009). With incorporation of additional subunits, denoted as * (e.g. $\alpha 4\beta 2^*$), a large variety of functional receptor subtypes is also possible in the PNS and CNS.

The $\alpha 5$ - and the $\beta 3$ -subunits have special roles in the formation of functional nicotinic receptors. These subunits cannot participate in the formation of a ligand binding site with a complementary partner. For this reason, they are known as 'accessory subunits' and can only be incorporated into existing nAChRs (e.g. $\alpha 4\beta 2\alpha 5$) whenever further α as well as a β -subunits are present (Ramirez-Latorre et al., 1996; Gerzanich et al., 1998). The particular characteristics of the $\alpha 5$ subunit are of special interest for our research and are thus explained in further detail below.

1.3.1. Properties of nAChRs

nAChRs which are continuously exposed to their ligand will transform into a temporarily inactive state which is referred to as "desensitization". This inactive state of the receptor will persist after agonist removal and will switch after some time to a resting state which can be activated. The duration of time nAChRs spend in desensitized states, the concentrations sufficient to induce the switch from resting to inactive states, as well as the latency to onset of desensitization after ligand binding all strongly depend on the subunit composition (Giniatullin et al., 2005). Hence, the $\alpha 3\beta 4$ receptors of autonomic ganglia show rather moderate desensitization as compared to the centrally expressed and easily desensitized $\alpha 4\beta 2$ receptors. However, the desensitization of homopentameric $\alpha 7$ receptors takes place in the millisecond range, whereas the $\alpha 4\beta 2$ receptors desensitize slower, in the range of

seconds (Fenster et al., 1997; Quick and Lester, 2002). A further interesting feature of nAChRs is their high affinity desensitization, which is a rather slow process taking place at very low agonist concentrations and lasting up to several minutes. This feature affects mostly $\alpha 4\beta 2$ receptors, rather than $\alpha 7$ or $\alpha 3\beta 4$ nAChRs (Giniatullin et al., 2005). Chronic exposure to nAChR agonists can then lead to more permanent changes in receptor levels via receptor up- or downregulation. It is important to note that a switch between distinct conformational states can also occur without the presence of a ligand but by a process in which agonist presence increases the likelihood of a discrete allosteric receptor transition (Corringer et al., 2000).

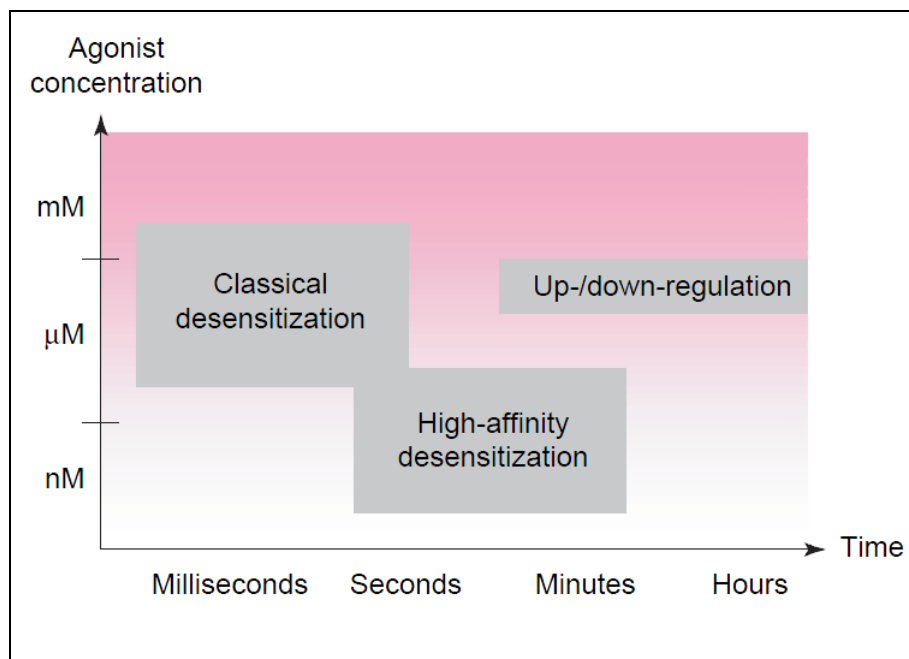


Figure 3: Mechanisms of ligand-induced receptor response modulation. The classical desensitization of nAChRs is induced by micromolar to millimolar agonist concentrations at the receptor site. Beyond that, lower agonist concentrations can induce a desensitized state of the receptor even without activating the receptor. For $\alpha 4\beta 2$ receptors, the active concentration of nicotine to induce high-affinity desensitization can be reduced about 1000 times compared to concentrations necessary to induce receptor activation. A chronic exposure to nicotinic receptor agonists can lead to longer-lasting regulatory changes regarding receptor levels. The onset, degree and persistence of modulatory receptor mechanisms strongly depend on the affected nAChR subtype. The illustration is taken from (Giniatullin et al., 2005).

1.3.2. Properties and Distribution of $\alpha 5$ -containing nAChRs

The presence of the $\alpha 5$ subunit in heterologously expressed cell systems can affect channel conductance, desensitization, and the pharmacological properties of a given receptor subtype. Thus, injection of $\alpha 5$ subunit mRNA together with $\alpha 4$ - and $\beta 2$ -subunit mRNA into *Xenopus* oocytes leads to increased desensitization rates as well as larger maximum currents of expressed nAChRs as compared to the same receptor subunit combination without incorporation of the $\alpha 5$ subunit (Ramirez-Latorre et al., 1996; Gerzanich et al., 1998). The permeability for Ca^{2+} is also increased in $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR subtypes, if the $\alpha 5$ subunit is integrated (Gerzanich et al., 1998).

The endogenous expression of $\alpha 5$ in peripheral sympathetic and parasympathetic ganglia and its modulatory qualities further make this subunit an interesting component for the control of autonomic organ function (De Biasi, 2002; Wang et al., 2002). In human non-neuronal tissues, the $\alpha 5$ subunit mRNA is also detected in testis, thymus and the gastrointestinal tract, whereas liver, spleen and kidney are reported as non-expressing organs (Flora et al., 2000).

In superior cervical ganglion (SCG) cells of wildtype (WT) C57BL/6 mice, the $\alpha 3\beta 4$ subtype is predominant. Within this tissue, the $\alpha 5$ subunit exclusively incorporates into this receptor subtype and thereby the resulting $\alpha 3\beta 4\alpha 5$ combination represents about 24% of all $\alpha 3\beta 4$ receptors in SCG cells (David et al., 2010). Using mice with genetic deletions of specific subunits, single-channel recordings of distinct $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$ and $\alpha 3\beta 4\beta 2$ receptors in SCG cell cultures show unaltered receptor conductances for $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ receptors. In contrast, $\alpha 3\beta 4\alpha 5$ receptors showed increased channel opening times and also increased burst durations as compared to the receptors lacking the $\alpha 5$ subunit (Ciuraszkiewicz et al., 2013).

In contrast to the peripheral distribution of $\alpha 5$ subunit in the mouse SCG, in rat central nervous tissue such as hippocampus, striatum, cerebral cortex and thalamus, the $\alpha 5$ subunit is mainly found in nuclei also expressing the $\alpha 4$ subunit and predominantly incorporated in $\alpha 4\beta 2\alpha 5$ receptors (Wada et al., 1990). In these tissues, the $\alpha 5$ subunit is suggested to display a regulatory role for the receptor with results showing that its presence results in resistance to receptor up-regulation after chronic exposure to nicotine *in vivo* (Mao et al., 2008). In the

rodent habenulo-interpeduncular system, the $\alpha 5$ subunit is additionally reported to co-assemble with the $\beta 4$ subunit which increases the likelihood to be integrated in $\alpha 3\beta 4$ -containing receptors in this tissue (Grady et al., 2009; Scholze et al., 2012). The impact of $\alpha 5$ -containing receptors in the habenulo-interpeduncular system and for animal behavior in general is discussed below.

1.4. nAChRs as Therapeutic Targets

Due to the broad distribution of nAChRs in the mammalian nervous system, these receptors influence a variety of neuronal circuits and processes. This includes sensory signal transmission, mood regulation, reward and various cognitive processes (Changeux, 2010; Miwa et al., 2011). These receptors have become an important target for drug development and novel therapies. Distinct subunit combinations may facilitate the ability to selectively target specific receptor subtypes for a concrete therapeutic effect. Several natural toxins, which bind to the nAChR have been identified so far. The most famous besides nicotine is epibatidine which is extracted from the skin of the dart-poison frog *Epipedobates tricolor*. Furthermore, an array of synthetic subtype-selective ligands with potential therapeutic applications has been designed to targeted specific circuits. These compounds differ in regards to their subtype-selectivity and affinity, as well as functional potency, all of which can be relevant for their therapeutic efficacy (Wonnacott and Barik, 2007). Another possible approach to target nAChRs for the therapeutic purposes is the use of positive allosteric modulators of nAChRs. These substances can modulate the response of the receptor evoked by an agonist by either enhancing the receptor function or reducing the required energy to shift the receptor from resting to active state, or by reducing receptor desensitization and allowing the receptor to remain active for a longer time (Umana et al., 2013).

1.4.1. Nicotine

Nicotine is a plant alkaloid ingredient of the nightshade plant family (Solanaceae) and is especially found in the leaves of the tobacco plant (*Nicotinia tabacum*). There, as in some

other plant family members (e.g. potatoes and tomatoes), it is thought to act as a natural insecticide (Soloway, 1976). Ever since it became common to chew or smoke the leaves of *Nicotinia* a considerable proportion of the world population has become addicted to nicotine. In modern times, nicotine has become of certain scientific and clinical interest not only because of the consequences of tobacco consumption but also due to a wide variety of physiological effects. Positive effects of nicotine were described for basic attentional tasks and cognitive performances in healthy individuals as well as patients who suffer from ADHS, schizophrenia or Alzheimer's disease (Demeter and Sarter, 2013).

1.4.1.1. Nicotine Pharmacology

Due to the broad distribution of nAChR's in the mammalian organism, the pharmacological effects of nicotine in mammals are highly complex. Nicotine easily penetrates the blood-brain barrier and is a nonselective agonist at all nAChRs but particular for the $\alpha 4\beta 2$ subtype (see figure 4). Some of the known physiological effects of nicotine include influence on heart rate, blood pressure, respiration, muscle contraction, locomotion, body temperature, cognitive functions, hormone secretion, and the processing of pain. Genetic background exerts a strong influence in the physiological effects of nicotine (Aschhoff et al., 1999; Stolerma et al., 1999). However, the exact physiological and pharmacological effects of nicotine further depend on the age, sex, the basal physiological condition, as well as the route, dose and regime of administrations (Damaj et al., 2007). The most potent nicotine stereoisomer after subcutaneous (s.c.) injection is (-)-nicotine followed by (+/-)-Iso-nicotine, (+/-)-nor-nicotine, and (+)-nicotine (Damaj et al., 1998).

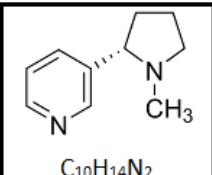
	(-)-Nicotine	$\alpha 4\beta 2$	$\alpha 7$	$\alpha 3\beta 4$
	Binding affinity K_i (nM)	0.6 - 10	400 - 15,000	290 - 476
	Functional potency EC_{50} (μ M)	0.35 - 5	49 - 113	8.1 - 110

Figure 4: Chemical structure and functional properties of (-)-nicotine. Binding affinity (K_i) as well as functional potency (EC_{50}) of (-)-nicotine are both lowest for $\alpha 4\beta 2$ -containing receptors followed by $\alpha 3\beta 4$ -containing receptors and $\alpha 7$ -homopentameric receptors. The chemical structure of (-)-nicotine is taken from (<http://www.sigmaaldrich.com/catalog/product/fluka/36733?lang=de>

®ion=AT). Values for binding affinity and functional potency of (-)-nicotine are taken from (Wonnacott and Barik, 2007).

1.4.1.2. Nicotine Pharmacokinetics

Nicotine is not degraded by acetylcholinesterases and therefore remains at the synapse site for a much longer duration than acetylcholine. Nicotine can efficiently cross the plasma membrane and thus accumulates within the cell and also interacts with intracellular nAChRs (Lester et al., 2009; Miwa et al., 2011). The majority of nicotine is metabolized in the liver. Many different metabolic routes have been identified so far, with the major one being transformation of nicotine to cotinine (70-80% in humans; (Benowitz and Jacob, 1994). The main enzyme which is responsible for the metabolic inactivation of nicotine is CYP2A6 in humans and CYP2A5 in mice. The amino acid sequences of both enzymes match to 84% (Siu and Tyndale, 2007). CYP2A5 and CYP2A6 belong to the monooxygenase superfamily of cytochrome P450 enzymes which often catalyze oxidation of organic substances. The metabolism of nicotine to cotinine involves a second step which is performed by a cytoplasmic aldehyde oxidase. This enzyme converts the intermediate substrate of the first reaction, nicotine- $\Delta^{1'(5')}$ -iminium ion, to cotinine. Most terminal metabolites of nicotine and/or cotinine are finally excreted in the urine.

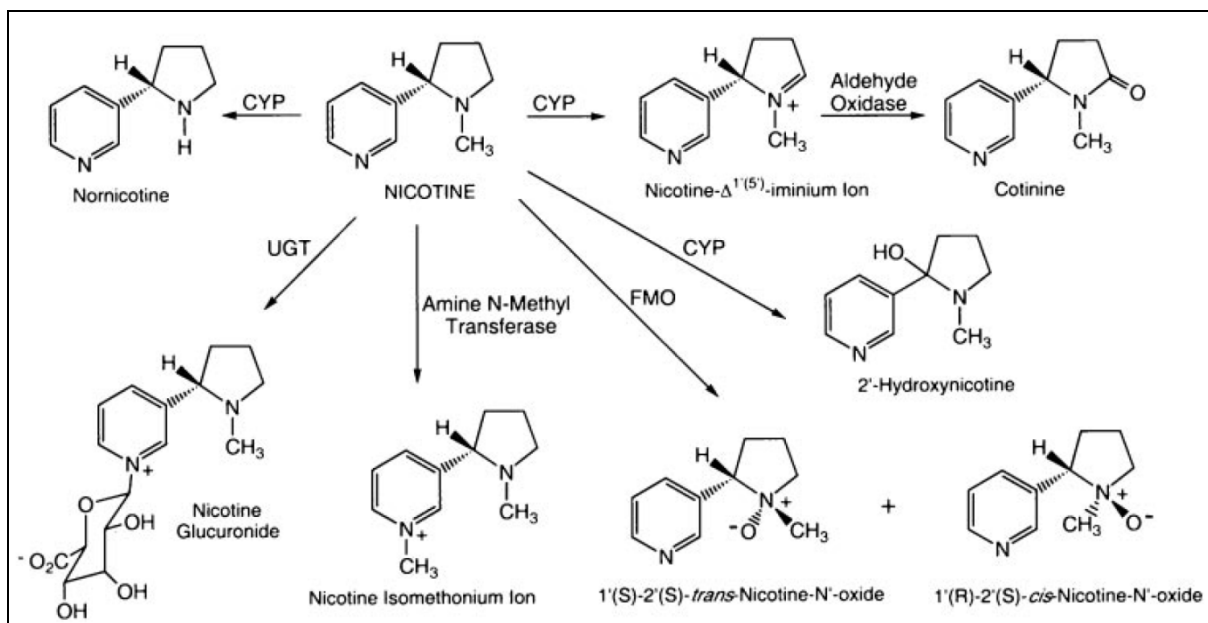


Figure 5: Schematic illustration of the primary routes of nicotine metabolism and its resulting metabolites. CYP: cytochrome P450; UGT: uridine diphosphate-glucuronosyltransferase; FMO: flavin-containing monooxygenase; the illustration is taken from (Hukkanen et al., 2005).

The metabolism of nicotine is highly relevant for the pharmacological action of nicotine. Damaj et. al (2007) could demonstrate a dose dependent prolongation of nicotine's analgesic and hypothermic effects in mice after pretreatment with methoxsalen (9-methoxyfurol[3,2-g][1]benzopyran-7-one), an inhibitor of the human CYP2A6. Furthermore, genetic variations in the amino acid sequence of CYP2A6 can have distinct consequences for the metabolism of nicotine in humans and therewith also for the pharmacological effects of nicotine.

An example of the impact of nicotine metabolism is illustrated by DBA/2 and C57Bl/6 mice, two widely used strains, which differ in their behavioral response to nicotine and their CYP2A5 enzyme structure (Lindberg et al., 1992), do not differ in their CYP2A6 metabolism of nicotine, but in their metabolism of cotinine (Siu and Tyndale, 2007). As cotinine crosses the blood-brain barrier (Lockman et al., 2005) and has the feasibility to bind nicotinic receptors (Vainio and Tuominen, 2001), some behavioral discrepancies between these two strains might be attributed to cotinine pharmacology or an interference of cotinine with the nicotine pharmacology rather than nicotine pharmacology alone. Even if the metabolite in

general is less potent than nicotine *in vivo*, cotinine is reported to partially desensitize nAChRs and further to display effects on cognitive functions with a possible anti-psychotic and cytoprotective activity (Buccafusco et al., 2007). However, the concrete pharmacological properties of cotinine have to be further investigated for unambiguous statements in this direction.

In humans, nicotine shows a half-life of 100 - 150 minutes, whereas half of the cotinine is metabolized after 770 - 1130 minutes (Hukkanen et al., 2005). The half-life of nicotine in mice ranges between 7 - 10 minutes (Petersen et al., 1984; Siu and Tyndale, 2007) and is thereby similar in DBA/2 and C57BL/6 mice (8.6 ± 0.4 and 9.2 ± 1.6 minutes respectively; (Siu and Tyndale, 2007). In contrast to that, the half-life of cotinine in these two strains differs statistically significant (51.0 ± 4.1 for DBA/2 and 23.7 ± 2.0 minutes for C57BL/6 mice; (Siu and Tyndale, 2007). The peak effects of nicotine for physiological functions are reported to take place between 5 and 15 minutes after nicotine administration (Galeote et al., 2006; Jackson et al., 2010).

1.5. nAChRs in Pain Transmission

Since the early 20th century, nAChRs have been under investigation. The first description of analgesic properties of tobacco leaves during syphilis-induced pain conditions is actually dated back to the 16th century (Umana et al., 2013). After more detailed studies in this field during the 20th century, this effect was recognized to be due to nicotine (Davis et al., 1932; Fertig et al., 1986). The nonselective antagonist of neuronal nAChRs, mecamylamine, but not atropine, a muscarinic receptor antagonist, was later found to attenuate the antinociceptive effect of nicotine (Sahley and Berntson, 1979; Tripathi et al., 1982). Tripathi et al. characterized the complex mechanism for the analgesic action of nicotine which involves a variety of different nAChR subtypes at different neuronal localizations and with different net outcomes for the pain experience (Tripathi et al., 1982).

In recent years, clinical and preclinical tests have confirmed analgesic and antinociceptive properties of nicotine, but also the toxicity of this alkaloid which is responsible for the range of side effects from nausea and increased heart rate up to the powerful addictive properties

(Henningfield et al., 1985; Flood and Daniel, 2004; Hong et al., 2008). In non-smoking patients nasal spray or transdermal patches of nicotine can relieve increased pain sensitivity which seems to be less effective in smoking patients (Habib et al., 2008; Yagoubian et al., 2011). Still to this date, the mechanism for the development of tolerance for nicotine antinociceptive effects after chronic administration is not fully understood (Cepeda-Benito et al., 1998; Colombo et al., 2013). Since the analgesic properties of epibatidine were described in 1994 (Sullivan et al., 1994), the development of nAChR agonists with analgesic properties has been promoted (Khan et al., 1998; Decker and Meyer, 1999). In this context, not only anti- but also pronociceptive properties of nAChR agonists have been described. However, the pronociceptive activity of ligands depends on the administration protocol and the affected brain areas (Hamann and Martin, 1992; Josiah and Vincler, 2006).

In order to separately target the antinociceptive effects and to exclude undesirable side effects, the distinct subunit composition of nAChRs which are most relevant for this effect must be identified.

1.5.1. nAChR Subunit Composition and Involvement in Pain Transmission

The analgesic properties of several nAChR agonists per se is well established so far but a significant knowledge about the neuronal circuits involved, underlying cellular mechanisms and the integration of nAChRs is missing. In the following sections, we will concentrate on the specific nAChR subtypes, their contribution to nicotine induced analgesia, and possible sites of action.

1.5.1.1. $\alpha 4\beta 2$ -containing nAChR in Pain Transmission

After the discovery of nicotine analgesic properties, an investigation into the underlying mechanisms in the contribution of distinct nAChR subtypes was initiated. The profile of antinociceptive properties and pharmacological potencies of several nAChR ligands enabled assumptions about the individual nAChRs subtype contributions (Damaj et al., 1998; Khan et al., 1998; Decker and Meyer, 1999).

The involvement of $\alpha 4\beta 2$ subunits in nociceptive transmission mechanisms have been suggested since the initial research on nicotinic receptor analgesia. A more distinct investigation of these subunits for the analgesic action of nicotine was performed in 1999 by Marubio and colleagues after the generation of genetically modified mice lacking the $\alpha 4$ ($\alpha 4$ -KO) or $\beta 2$ ($\beta 2$ -KO) subunit. The researchers could demonstrate a highly reduced antinociceptive effect of nicotine in both genotypes as compared to their WT littermates (Marubio et al., 1999). This was further validated with the use of dihydro- β -erythroidine (DH β E) since this $\alpha 4\beta 2$ subtype-selective antagonist was demonstrated to inhibit the antinociceptive effect of nicotine when co-injected in mice with a nerve injury (Rashid et al., 2006). The same results were obtained after co-injection of nicotine with mecamylamine but not after injection together with the $\alpha 7$ -homopentamer antagonist methyllycaconitine (MLA). Additionally, the same study showed that GABA_A antagonists bicuculline and picrotoxin also significantly reduced the analgesic effect of nicotine. This effect was related to the ability of nAChRs to stimulate the release of GABA and other neurotransmitters (Sher et al., 2004; Jensen et al., 2005). These results were further confirmed in whole-cell patch-clamp studies on substantia gelatinosa neurons. Mecamylamine and DH β E strongly reduced the frequency of miniature inhibitory postsynaptic currents of GABAergic and glycinergic neurons, whereas excitatory postsynaptic currents remained unaffected (Rashid et al., 2006). These results strongly indicate for a tonic inhibitory tone of GABAergic and glycinergic neurons on nociceptive transmission in the substantia gelatinosa, which is regulated by presynaptic $\alpha 4\beta 2$ nAChRs. Administration of nAChR agonists could increase this inhibitory tone, by increasing GABA and glycine neurotransmitter release, and thereby execute their inhibitory effect on nociceptive transmission (Genzen and McGehee, 2005). On the other hand, a decreased activity of $\alpha 4\beta 2$ receptors on GABA and glycine terminals might be one possible spinal mechanism for the induction of neuropathic pain after nerve injury (Rashid et al., 2006).

Follow-up studies confirmed the idea of a $\alpha 4\beta 2$ -mediated spinal inhibitory transmission using electrophysiological recordings in spinal cord slices and concluded the receptor subtype to be necessary but not sufficient for the generation of antinociception (Gao et al., 2010). The interplay between GABA mediated inhibition of nociceptive transmission under nAChR control was further investigated in behavioral studies using $\beta 2$ -KO mice. A reduced basal mechanical nociceptive threshold was demonstrated in these animals which could be

reversed with administration of the GABA agonist muscimol. In contrast, the GABA antagonist bicuculline reduced mechanical thresholds in WT animals, but had no effect in $\beta 2$ -KO animals (Yalcin et al., 2011).

Hence, spinal $\alpha 4\beta 2$ nAChRs on spinal inhibitory GABAergic and glycinergic neurons as well as on serotonergic and noradrenergic inhibitory descending transmission seem to be most relevant for the modulation of pain signaling (Rueter et al., 2000; Kiyosawa et al., 2001; Li and Eisenach, 2002; Cordero-Erausquin et al., 2004; Abdin et al., 2006). Furthermore, $\alpha 4\beta 2$ nAChRs are additionally present on supraspinal sites which may play a crucial role for inhibitory pain transmission and the analgesic action of $\alpha 4\beta 2$ ligands, namely the nucleus raphe magnus (NRM), dorsal raphe (DR), rostral ventromedial medulla (RVM), locus caeruleus (LC), periaqueductal grey (PAG; (Bitner et al., 1998; Cucchiaro et al., 2005; Gotti et al., 2006; Galindo-Charles et al., 2008; Nirogi et al., 2013).

1.5.1.2. Other nAChRs in Pain Transmission

So far, strong evidence supports the idea that $\alpha 4\beta 2$ nAChRs mediate the antinociceptive effects of receptors ligands. However, further investigations indicate additional contribution of other nAChR subtypes, such as $\alpha 3\beta 4$, $\alpha 3\beta 2$ and $\alpha 7$ nAChRs (Takeda et al., 2003; Young et al., 2008). For example, mice lacking the $\beta 4$ subunit show reduced nicotine-mediated thermal analgesia after s.c. administration of higher doses of nicotine (3 mg/kg), but are similar to their WT littermates after lower (1 mg/kg) doses (Semenova et al., 2012). The high rate of expression of $\alpha 3\beta 4$ nAChRs in peripheral ganglia suggests that these receptors can also be involved in sensory nociceptive transmission.

In human spinal cord tissue, $\alpha 3\beta 4$ receptors are found on unmyelinated C-fibers in electrophysiological and immunohistochemical studies (Lang et al., 2003). Also the $\alpha 5$ subunit could incorporate into those $\alpha 3\beta 4$ receptors. Whether the activation of these receptors is either pro- or antinociceptive is not well understood and thus requires further investigation (Umana et al., 2013).

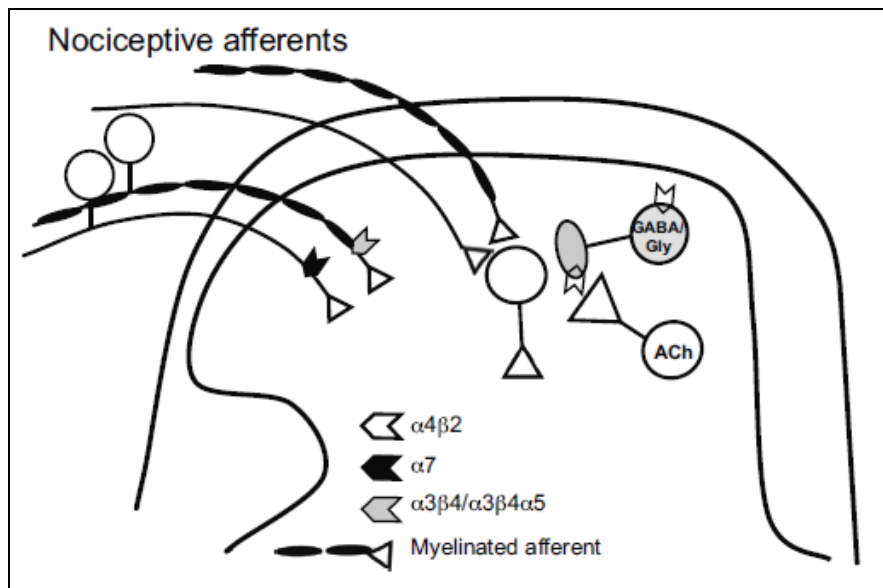


Figure 6: Schematic illustration of a few identified nAChR localizations in ascending nociceptive transmission. The illustration shows some well- identified nAChR expression sites. Receptors located on nociceptive afferents in the spinal dorsal horn can enhance nociceptive transmission. On the other hand, receptors located on GABAergic or glycinergic interneurons within the spinal dorsal horn most likely influence ascending nociceptive transmission by modulating the inhibitory tone generated by spinal interneurons. A further involvement of nAChRs in supraspinal and in descending, pain modulatory fibers is described and illustrated in Umana et al. (2013). These include $\alpha 4\beta 2$ -containing as well as $\alpha 7$ -homopentameric receptors and most likely affect serotonergic pain modulatory transmission. The exact site of action of nAChRs on descending pain modulation and the projection pattern of fibers affected by nAChR modulation is still an ongoing investigation. The illustration is taken from (Umana et al., 2013).

1.6. Nicotine Tolerance

Tolerance describes the effect in which the amount of a given drug needs to be increased after repetitive use, in order to reach the same magnitude of effect that was seen after the first application. Tolerance to nicotine treatment is known for several physiological effects of nicotine and for both acute and chronic administration paradigms. This includes the antinociceptive and analgesic effects of nicotine where tolerance has been demonstrated after acute (Damaj et al., 1996) and chronic exposure to the drug (Galeote et al., 2006; Brett et al., 2007; Schroeder et al., 2011).

1.6.1. Acute Nicotine Tolerance

Acute tolerance is a physiological short-term adaptation to the effect of a drug which can be explained by desensitization or inactivation of the ligand-specific receptors. In behavioral tests, acute tolerance to nicotine has been described for locomotor impairments in rats (Stolerman et al., 1974) and for nicotine-induced seizures (Miner and Collins, 1988). The maximum acute tolerance to the antinociceptive effects of subcutaneously applied nicotine has been reported to occur between 0.5 and 1 hour after nicotine pretreatment in a dose dependent manner and with 4 mg/kg as the most efficient dose (Damaj et al., 1996). The acute tolerance to antinociceptive effects is lost after 6 hours. The same study also described an acute tolerance effect to nicotine-induced hypothermia and motor impairment.

1.6.2. Chronic Nicotine Tolerance

Chronic tolerance appears after continuous infusion or long-term repeated application of a receptor drug agonist. Most studies investigating the chronic analgesic tolerance effects of nicotine have used a regulated continuous infusion of the drug using osmotic minipumps. In rats, continuous, systemic nicotine leads to increased mechanical hypersensitivity in a dose-dependent manner (Josiah and Vincler, 2006). Furthermore, hypersensitivity following peripheral nerve injury is further increased before and after surgery in animals which receive a chronic nicotine infusion as compared to animals with saline infusions (Josiah and Vincler, 2006; Brett et al., 2007). Interestingly, Josiah and Vincler could demonstrate that co-administration of nicotine together with the $\alpha 4\beta 2$ -selective antagonist dihydro- β -erythroidine (DH β E) prevents this tolerance development towards hypersensitivity. In contrast, the NMDA receptor antagonist MK-801 is not sufficient to block nicotine-induced hypersensitivity. A disruption of nicotine antinociception tolerance after six days of chronic but intermittent drug treatment has been previously described when a co-treatment with mecamylamine is administered as well (McCallum et al., 1999). Chronic treatment with mecamylamine alone does not affect the antinociceptive effect of nicotine. There is also evidence for an involvement of mu-opioid receptors in the development of tolerance to nicotine-induced antinociception since mice lacking these receptors show a more rapid tolerance response (Galeote et al., 2006). In contrast, the GLT-1 activator of glutamate

transport, ceftriaxone, slightly elevates the antinociceptive effect but blocks the development of nicotine-induced tolerance to antinociception (Schroeder et al., 2011).

1.6.3. Mechanisms of Nicotine Tolerance

The molecular mechanisms underlying behavioral tolerance for nAChR-mediated effects most likely underlie intrinsic receptor posttranslational changes (Colombo et al., 2013). In 1983, an upregulation of nAChRs was first described in ligand binding assays in several brain regions (e.g. cortex, midbrain, hindbrain, hippocampus, hypothalamus) after chronic treatment with nicotine (Marks et al., 1983; Schwartz and Kellar, 1983). The degree of receptor upregulation in nAChRs seems to strongly depend on the brain region, cellular identity in the specific region, receptor subtype combinations, and also cellular localization of the affected receptors (Nashmi et al., 2007; Colombo et al., 2013). Particularly the $\alpha 4\beta 2$ receptor subtype has been implicated to be extensively upregulated in chronic nicotine studies, whereas the $\alpha 3\beta 4$ and the $\alpha 7$ subtypes seem to be less affected by upregulation mechanisms (Olale et al., 1997; Nguyen et al., 2003; Doura et al., 2008).

In 2008, Mao et al. showed an increase in $\alpha 4\beta 2$ receptors in the hippocampus, striatum, thalamus, and cerebral cortex after 14 days of continuous nicotine infusion. Interestingly, the upregulation of these receptors was prevented if the $\alpha 5$ subunit was integrated (Mao et al., 2008). The resistance towards upregulation *in vivo* underlines the regulatory properties of the $\alpha 5$ subunit and makes it an interesting candidate for the regulation of nicotine-mediated behavioral tolerance.

As mentioned before, the upregulation of receptors relies on mechanisms occurring after translation, as the amount of mRNA for specific nAChR subunit was not found to be changed in mouse brain after chronic nicotine exposure (Marks et al., 1992; Pauly et al., 1996). Furthermore, using heterologous expression systems, the characteristics of upregulation per se were described to be cell-autonomous and as a receptor intrinsic mechanism in nAChRs (Nashmi and Lester, 2007).

In a review of the literature, many possible mechanisms leading to upregulated receptor states after chronic nicotine exposure have been reported including decreased degradation

with increased cellular trafficking (Peng et al., 1994; Wang et al., 1998), membrane integration and maturation (Kuryatov et al., 2005; Sallette et al., 2005; Corringer et al., 2006; Richards et al., 2011), as well as changes in subunit stoichiometry (Nashmi et al., 2003) and conformation affinity (Buisson and Bertrand, 2001; Vallejo et al., 2005). As mentioned before, nicotine can also act intracellularly. However, these multiple processes, responsible for receptor upregulation, do not mutually exclude each other and most likely occur selectively depending on the exact exposure paradigm, the receptor subunit composition, and the affected cell-type (Miwa et al., 2011; Govind et al., 2012). Even though a chronic nicotine exposure seems to increase total nAChR number, the amount of receptors in activatable states and thus the possibility of activation itself might be decreased, resulting in physiological tolerance (Marks et al., 1993). The persistent inactivation of nAChR acting on antinociceptive transmission might further affect inhibitory, pain modulatory circuits. This hypothesis of disinhibition might most likely occur at supraspinal (locus caeruleus, nucleus raphe magnus) or spinal (GABAergic and glycinergic interneurons, descending inhibitory fibers) loci (Josiah and Vincler, 2006).

Furthermore, the selective organization of receptor upregulation after chronic nicotine exposure will result in modulated synaptic actions of specific nAChRs, changing e.g. the amount of presynaptic nAChR induced transmitter release. This might further induce secondary adaptations in the synapse, changing neuronal interactions and excitability (Miwa et al., 2011). These secondary neuronal modifications might also be involved in the mechanisms resulting in tolerance after chronic nicotine administration.

1.7. $\alpha 5$ -containing nAChRs in Pain Transmission

As mentioned previously, nAChRs were described to be expressed on primary afferent fibers entering the spinal dorsal horn (Khan et al., 1996), on spinal inhibitory interneurons (Cordero-Erausquin et al., 2000; Khan et al., 2001) as well as on descending monoaminergic fibers (Cordero-Erausquin and Changeux, 2001). Interestingly, the $\alpha 5$ subunit can be found in functional nAChRs at all of these sites. Due to its modulatory and regulatory properties for the receptor, this subunit is of special interest for the investigation of physiological changes in pain transmission and the analgesic actions of nicotine. The expression of nAChRs on pre-

or post-synaptic regions may be affected by cellular and molecular reorganizations occurring after peripheral nerve injury. Additionally, exogenous agonist action can be altered in these receptors, due to $\alpha 5$ subunit integration. All these will be discussed in the following sections.

1.7.1. $\alpha 5$ -containing nAChRs in Neuropathic Pain

In the rat spinal cord dorsal horn, the $\alpha 5$ subunit of nAChRs was reported in a series of studies to be most abundantly expressed on lamina I and II fibers (Vincler and Eisenach, 2004). Following peripheral nerve injury, the amount of $\alpha 5$ subunit expression in these fibers was highly increased, as demonstrated with immunocytochemistry directed against the $\alpha 5$ subunit before and after surgery. This change in the $\alpha 5$ subunit density profile only affected the ipsilateral site, whereas the contralateral site was not affected (see figure 7; (Vincler and Eisenach, 2005). Interestingly, the immunoreactivity for other subunits ($\alpha 4$, $\alpha 7$, $\beta 2$, $\beta 3$, $\beta 4$) was much less or not affected in the same study. Another study reported a significant upregulation of the $\alpha 5$ subunit in the contralateral site after nerve injury as well (Young et al., 2008). It has to be noted that the results for the immunocytochemical localization studies of the $\alpha 5$ subunit may be criticized since the antibodies which are commercially available and which were used in these studies have been previously shown to display unspecific binding (Moser et al., 2007). However, knockdown of the $\alpha 5$ subunit after treatment with an antisense oligonucleotide for the $\alpha 5$ subunit reduced the subunit upregulation which was observed after spinal nerve ligation (Vincler and Eisenach, 2005). Antisense treatment for the $\alpha 5$ subunit, in addition, significantly reversed the reduced paw withdrawal threshold, which was observed after nerve injury.

1.7.2. $\alpha 5$ -containing nAChRs in Nicotine-induced Analgesia

Besides neuropathic pain, receptors containing the $\alpha 5$ subunit are also believed to be involved in the antinociceptive effects of nAChR agonists. In order to investigate this hypothesis, Jackson and colleagues tested mice with homozygous and mice with heterozygous deletions of the $\alpha 5$ subunit gene for their sensitivity towards nicotine induced analgesia. Their results show a great reduction in the effect for $\alpha 5$ -KO animals for any dose

tested (see figure 7; (Jackson et al., 2010). Furthermore, the group described a gene-dosage effect, because the heterozygous animals show a reduced effect of nicotine which was still stronger than that observed for homozygous $\alpha 5$ -KO animals. The loss of nicotine-induced antinociception was specific for nicotine because a subcutaneous treatment with 8 mg/kg morphine sulfate showed analgesic effects for any group tested. The reduced analgesic efficacy for nAChR agonists in $\alpha 5$ -KO mice was reversed by subcutaneous injections of 70 mg/kg metanictotine and for 10 mg/kg epibatidine (Jackson et al., 2010). Besides the acute analgesic effect, hyperalgesia, which can also be induced by chronic nicotine infusion, is unchanged in $\alpha 5$ -KO animals as compared to their WT littermates (Jackson et al., 2008).

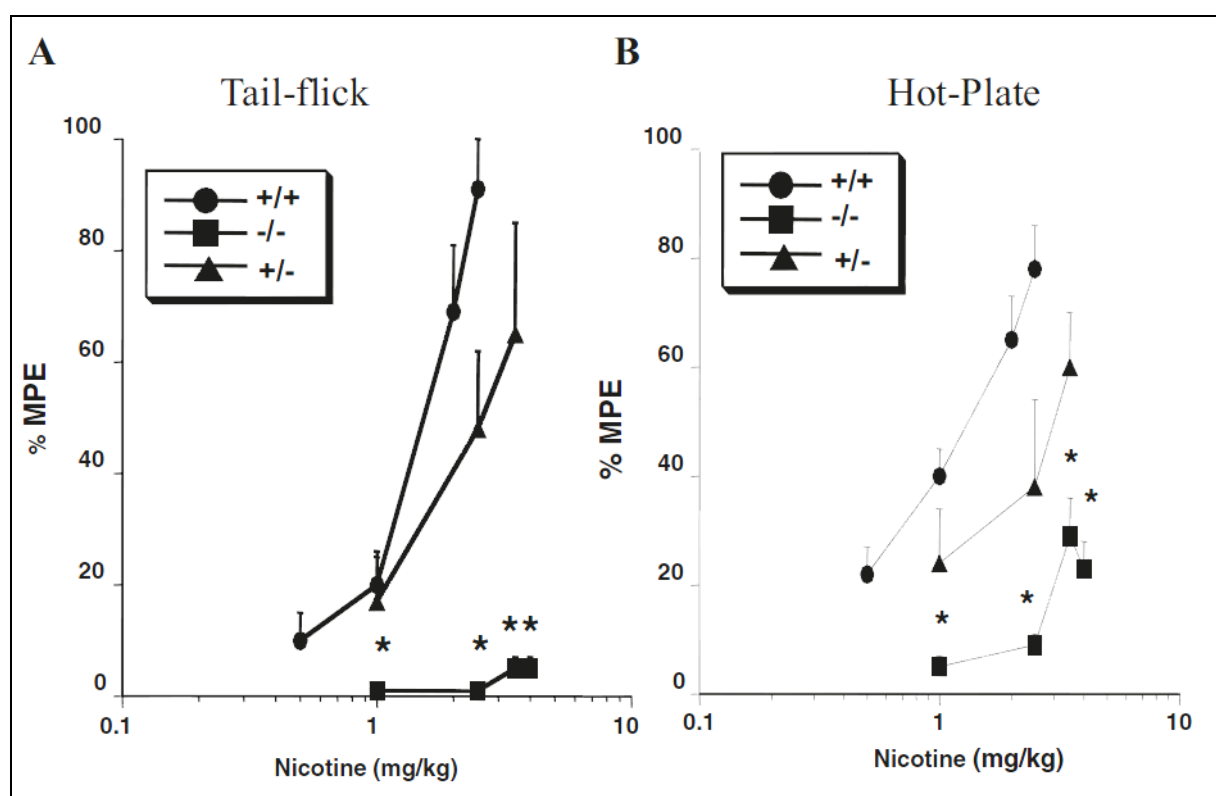


Figure 7: Dose-response curves for the antinociceptive effect of subcutaneously applied nicotine in $\alpha 5$ -KO and WT mice. Animals with homozygous deletions (- / -) were tested parallel with their heterozygous (+ / -) and wildtype (+ / +) littermates. Animals were tested for heat sensitivity 5 minutes after nicotine injection on either the Tail-flick (A) or the Hot-Plate (B) test. In wildtype as well as in homozygous mice, nicotine has a significant analgesic effect in both tests and at any dose tested. Homozygous animals in contrast show a highly reduced sensitivity towards the analgesic effects of acute nicotine, if compared to their wildtype littermates and at any dose tested. A gene-dosage effect is believed to occur, because both groups with genetic implications show a reduced sensitivity in both behavior tests compared to wildtype animals. The antinociceptive response was calculated as percentage of maximal possible effect (%MPE) as follows: %MPE = [(test value - baseline)/(cut-off time - baseline)] x 100; baseline represents the value before nicotine injection and cut-off was adjusted to 10 seconds and 40 seconds for Tail-flick and Hot-Plate test, respectively. *, p

> 0.05 tested against the wildtype group. Each point includes minimal six mice and shows the mean response +/- SEM. The figure is taken from (Jackson et al., 2010).

1.7.3. Functional implications for the mutations of the $\alpha 5$ Subunit Gene

Genetic deletions of the $\alpha 5$ subunit affect the *CHRNA5* gene on human chromosome 15. A polymorphism in this gene is associated with an increased nicotine intake in humans and thus with an increased risk for development of nicotine dependence (Wang et al., 2009).

Mice with deletions for the $\alpha 5$ subunit of nAChRs ($\alpha 5$ -KO) do not show any obvious physical or neurological deficits. They grow to normal size without impaired thermo-regulation, pupil size, or resting heart rate. Furthermore, the animals develop conventional levels of mRNA for $\alpha 4$, $\alpha 6$, $\alpha 7$, $\beta 2$, and $\beta 4$ in an anatomically normally developed brain. No statistically significant differences could be observed between $\alpha 5$ -KO and WT animals in a battery of behavioral tests including neurological screening for sensory and motor function, several open field tasks, as well as baseline nociceptive responses (Wang et al., 2002; Salas et al., 2003). Mice lacking the $\alpha 5$ subunit are however reported to show greater resistance against seizures (Kedmi et al., 2004), as well as decreased sensitivity for the locomotor impairments and hypothermia induced by nicotine (Salas et al., 2003; Jackson et al., 2010).

Within the rodent habenulo-interpeduncular system, the gene encoding for the $\alpha 5$ subunit is related to nicotine addictive behavior (Berrettini et al., 2008; Bierut et al., 2008). $\alpha 5$ -KO mice show increased nicotine consumption (Fowler et al. 2011) as well as reduced somatic signs of nicotine withdrawal (Salas et al. 2009). These effects have been attributed to the habenulo-interpeduncular system since the re-expression of $\alpha 5$ subunits in the medial habenula of knockout animals using a lentivirus vector carrying the $\alpha 5$ subunit gene normalized nicotine intake (Fowler et al., 2011). Neuronal projections from the habenula to other brain regions (e.g. raphe nuclei, ventral tegmental area, substantia nigra, interpeduncular nucleus) further implicate this region in the regulation of emotional stress, anxiety and pain behaviors (Hikosaka, 2010).

Chronic nicotine infusion for 15 days followed by treatment with the nicotinic antagonist mecamylamine induced comparable anxiety-related responses in $\alpha 5$ -KO animals as to their WT littermates, although reduced somatic nicotine withdrawal signs (Jackson et al., 2008). When tested in the two-lever drug discrimination and conditioned place preference tests, discriminative effects of nicotine as well as nicotine reward effects were unchanged in animals with genetic deletions in the $\alpha 5$ subunit gene (Jackson et al., 2010). Beyond nicotine addiction and reward, $\alpha 5$ -containing receptors are also involved in pain transmission and in pathways mediating the analgesic effects of nicotine.

1.8. Aim of the Study

The aim of the current study was to characterize the nociceptive responses of $\alpha 5$ -KO mice during neuropathic pain. Furthermore, we were interested in a possible involvement of $\alpha 5$ subunit containing nAChRs in the antinociceptive effects of nicotine in naive as well as nerve injured animals. Finally, due to the potential regulatory influence of the $\alpha 5$ subunit on nAChRs, we were interested if $\alpha 5$ subunit containing nAChRs play a role in the development of tolerance to the analgesic effects of nicotine.

2. Methods

2.1. Animals

Male, adult C57BL/6 mice were housed in a temperature (20-24°C) and humidity (40-60%) controlled animal facility with a light/dark cycle of 12/12 hours. All animals had ad libitum access to dry food and tap water. Single animals were housed in cages of 225x167mm (Type II), whereas groups from 2 - 6 animals were housed in cages of 325x170mm (Type IIL), all with soft bedding. All experiments were performed during the animal's light cycle and at an age of 2-5 months. Animals with deletions of the gene for the nAChR $\alpha 5$ subunit ($\alpha 5$ -KO) and their wildtype (WT) littermates were used (Salas et al., 2003). $\alpha 5$ -KO animals backcrossed for at least 7 generations to the C57BL/6 background were provided by Avi Orr-Urtreger (Sourasky Medical Center, Tel Aviv). Homozygous $\alpha 5$ -KO, wildtype and heterozygotes littermates were obtained by crossing heterozygotes parent animals. Animals were genotyped at weaning. $\alpha 5$ -KO and wildtype animals were housed and raised in shared cages, whereas heterozygotes mice were used for further breeding.

2.2. Chronic Constriction Injury

The CCI model of peripheral neuropathic pain was developed by Bennett and Xie (1988). The initial neuropathy in this model is not evoked by a direct injury to the nerve, but rather by an infiltration of immune cells and inflammatory agents, which are essential for the generation of neuropathic pain in this model. The inflammatory component additionally leads to a swelling of the nerve, which is immediately restricted by the ligations. For these two components of the model it is essential to use a chromic gut suture, as it stimulates the immune response at the site of ligation (Bennett and Xie, 1988; Eliav et al., 1999), and to pay special attention for the tightness of the suture around the nerve. As mentioned before, the ligation should not be the immediate cause of the nerve injury. The model is then characterized by the inflammatory component in the beginning, which is displaced by a

neuropathic component with ongoing time. If performed correctly, this leads to a chronic sensitization of the affected hind paw for mechanical and thermal stimulation.

2.2.1. SHAM Surgeries

In addition to nerve injuries, SHAM surgeries were performed as a control experiment, in order to exclude an effect of the surgery procedure (e.g. anesthesia) itself. For a SHAM surgery the sciatic nerve is freed from surrounding muscle tissue, but otherwise left intact.

2.2.2. Surgery Protocol

All surgeries were performed in a designated surgery room. The used surgery protocol for CCI was modified from its first description in 1988, in order to apply the procedure to mice (Minett et al., 2011).

1. Surgery Preparation

Before surgery, all surfaces in the proximity of the work space and all tools, which were needed for surgery, were disinfected with 70% ethanol. Tools were further kept in ethanol during the surgery. Furthermore, both sutures, PROLENE® suture (*Ethicon Prolene®: Polypropylen suture; 7-0 [0.5 Ph.Eur]; ACC 8.9 mm tip; Johnson-Johnson Inc., U.S.*) for muscle and skin and chromic gut suture (*CATGUT®: chrom absorbable surgical suture; USP 6/0 EP 1 with round boodied ½ circle, 15 mm tip; smiAG, Belgium*) for nerve ligation, were kept in sterile saline (*0.9% sodium chloride solution; pH-value: 4.5 - 7; Fresenius Kabi Austria GmbH*), in order to protect them from drying. The chromic gut suture was prepared as pieces of four to five centimeters in length. Finally, clean individual cages of 225x167mm (Type II) were prepared with soft bedding and easily accessible food.

2. Anesthesia

The animal was put into the induction chamber (*mouse anesthetic induction chamber; Rothacher Medical GmbH, Switzerland*) which was connected to the oxygen and nitrous oxide gas line. The gas flow was adjusted to about 3L. In order to initiate narcosis, 2.5 - 3% of Isoflurane (*Forane®: inhalation anesthetic; active ingredient: isoflurane [99.9%]; Abbott House, U.K.*) was added as anesthetic to the gas flow (*Anesthetic Vaporizer: Isoflurane Vapor 19.3; Drägerwerk AG Lübeck, Germany*). After animal lost consciousness, the amount of Isoflurane was reduced to 1.5%. Subsequently, the animal was taken from the induction chamber to the surgery surface. Anesthesia was maintained via a respiratory mask. As animals might react to the narcosis and the Isoflurane individually different, the concentration of Isoflurane was adjusted when necessary.

3. Dissection and Ligation

The anesthetized animal was then fixed to the surgery surface. Therefore the effected hind leg was positioned carefully at an angle of about 90° with respect to the backbone. For fixation, the paw was fixed to the surgery surface with Leukosilk® (*BSN medical GmbH, Germany*). For support of the upcoming dissection procedure, a folded piece of tissue (*Ashine® Brauncel® sterilized, high-bleached surgical cellulose swabs; B. Braun Petzold GmbH, Germany*) was put beneath the affected thigh. The thigh was shaved and subsequently disinfected with 70% ethanol.

From this point, steps were performed with help of a binocular microscope (*Olympus SZ51 Stereo Microscope*). An incision of about 10 mm was performed starting at the sciatic notch caudal to the hip bone. The incision was made with help of a scalpel or scissors in parallel to the femur axis. The freed muscle was then further spread along a small layer of adipose tissue with use of two forceps. SHAM surgeries were only performed until this step. After spreading of the muscle, the procedure of SHAM surgery was continued with closing the wound and recovery as described below (see step 6 and 7). For the nerve injury model, residual surrounding tissue of the nerve was removed carefully with help of two sharp forceps. In the next step, the nerve

was slightly lifted by use of curved, blunt-tipped forceps underneath the nerve. Stretching or squeezing of the nerve was avoided.

The sciatic nerve was then accessible to place three sutures of chromic gut underneath it. The sutures were tied with two single loop knots to ligations surrounding the nerve. Special attention was given for the tightness of ligations around the nerve, in order to avoid full compression or transection of the nerve.

4. Wound Closure

The ligated nerve was carefully replaced to its initially surrounding tissue. Next, the muscle tissue was sutured with one ligation, whereas the skin was sutured with three ligations using PROLENE® suture for both. In the last steps, the closed wound was cleaned with ethanol and further covered with Furacin® balm (Furacin®: *Sol; 0.2%; ointment; active ingredient: nitrofurazone; ingredients: 0.2g nitrofurazone [in 100g], Macrogol 300, Macrogol 1500, acetic acid 99%; Riemser Arzneimittel AG, Germany*).

5. Recovery

After these steps, the gas flow as well as Isoflurane flow was shut off and the animal was allowed to recover. The animal was placed in a single cage with soft bedding and easily accessible food, in order to avoid further stress for the animals. The position of the animal was chosen to allow steady breathing and a smooth awakening.

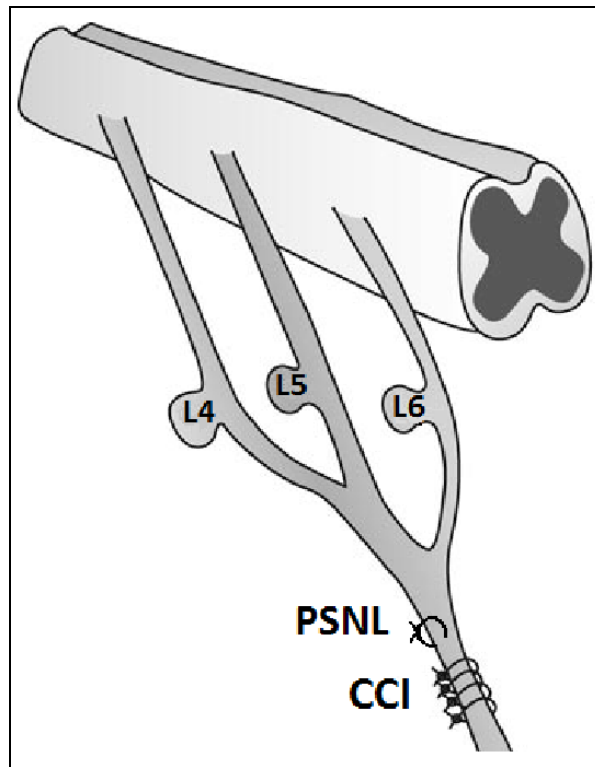


Figure 8: Schematic illustration of a chronic constriction injury (CCI) and a peripheral sciatic nerve ligation (PSNL). The CCI implies three loose ligations of the sciatic nerve with a chromic gut suture, whereas a PSNL is performed with a single tight ligation of about $\frac{1}{2}$ of the sciatic nerve diameter. L4-L6: spinal nerves entering lumbar spinal segments 4 - 6; the illustration is taken and adapted from (Minett et al., 2011).

2.3. Behavioral Experiments

All behavioral experiments were performed in the same temperature and humidity controlled behavior test rooms, between 8am and 6pm under consistent lighting conditions. Habituation to the testing room, experimental equipment, as well as experimental operator and experimental procedure was begun at least three days before any experiment started. The behavioral test equipment was cleaned with soapy water and 70% ethanol before and after each test session. The experimental operator was blinded to the genotype of the animals and treatment groups were assigned randomly. Furthermore, experimental groups were tested in parallel with control groups. All behavioral experiments were in accordance and previously approved by the Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (BMWF).

2.3.1. Methods for Pain Sensitivity Testing after Nerve Injury

In order to clearly define nociceptive responses in an animal after nerve injury, it is essential to apply a range of tests, which cover a range of possible noxious stimulus modalities. For this purpose, we performed three different tests for each animal: heat sensitivity with the Hargreaves test, cold sensitivity with the acetone evaporation test and mechanical sensitivity with the von Frey test. All these tests were performed on the same day, with animals inside red, individual plexi glass cylinders of 740mm in diameter and 1530mm in height. The red cylinder is thought to limit visual stimulation from the surrounding and from neighboring animals. Each experimental day was started with habituation to the experimental conditions for at least one hour. Behavior experiments were started when the majority of animals showed decreased exploratory behavior. If most animals were still not calm enough for testing, the habituation was prolonged for about 30 minutes or until testing was feasible. Furthermore, each experimental day was started with the von Frey test and followed by the acetone evaporation test, both performed on the same wire grid. As soon these tests were completed, animals were habituated to the experimental set up of the Hargreaves test for at least one hour. One daily set of experiments usually included eight animals. Tests were first performed on the contralateral paw of the first animal in order and were continued in sequence, before starting experiments on the ipsilateral paw. Animals were only tested if awake but calm and not distracted or grooming.

2.3.1.1. Mechanical Sensitivity (von Frey Test)

The von Frey test is used for the assessment of mechanical or tactile perception threshold in animals and humans since the 1890s. The test is performed by use of an array of calibrated monofilaments with determined, increasing diameter. These filaments can be used to provoke a consistently accurate force to a selected target region. For proper use, the hair has to be consistently applied to the stimulated region in a perpendicular manner. Thereby the force should be gently increased by the experimental operator to allow the filament to bend. This position is then held for a determined duration. If no reaction of the stimulated

individual can be observed, the subsequent stronger force in the array of filaments can be used. Details about the characteristics of used von Frey filaments are given in table 1.

For our purpose, the repeated measures von Frey test was performed with help of Aesthesio® Precision Tactile Sensory Evaluator filaments (*DanMic Global LLC, U.S.*), always starting with the lowest force of 0.008 grams. The exact stimulation site was the area between the walking pads of the mouse hind paw. The duration of a single stimulation was set to 5 seconds. Stimulation with a distinct force was repeated five times each with a minimum of 60 seconds interval before the following stimulation was started. The number of withdrawal responses was recorded and if mechanical threshold was not achieved (defined as 3 out of 5 responses) the next force in order was used to repeat the procedure. An upper cut-off at 2.0 grams was used. Filaments above this threshold would lift the hind paw before bending and thus not allow a reliable stimulation. During the test procedure, animals were placed on a wire grid in individual compartments. This allowed easy access to the hind paw from underneath the animal's position.

Table 1: Characteristics of the series of used von Frey filaments

Color	Evaluator Size	Catalog Item Number	Target Force (grams)	Target Force* (milliNewtons)	Theoretical Pressure LBS/Sq. Inch	Theoretical Pressure Grams/Sq. mm
Green	1.65	514001	0.008	0.08	3.59	2.53
	2.36	514002	0.02	0.20	6.23	4.39
	2.44	514003	0.04	0.40	7.01	4.93
	2.83	514004	0.07	0.70	7.85	5.53
Blue	3.22	514005	0.16	1.6	12.5	8.77
	3.61	514006	0.40	3.9	22.9	16.1
Purple	3.84	514007	0.60	5.9	26.1	18.4
	4.08	514008	1.0	9.8	34.6	24.4
	4.17	514009	1.4	13.7	39.6	27.9
	4.31	514010	2.0	19.6	39.0	27.4

For experiments, stimulation was started with the lowest force (0.008 grams). The target force was only increased, if the former force was not sufficient to evoke 3 withdrawal responses out of 5 stimulations. Upper cut-off was determined for 2.0 grams. The table is taken and adapted from Aesthesio® Precision Tactile Sensory Evaluator official User Manual (<http://www.danmicglobal.com/AesthesioUserManual012013.pdf>)

2.3.1.2. Thermal Sensitivity - Cold (Acetone Evaporation Test)

In order to assess the sensitivity to cool stimulation the Acetone evaporation test was performed. At room temperature, application of acetone to a surface leads to a rapid evaporation of the liquid which induces a cooling effect. This drop in temperature is approximately 10°C. If applied to the skin, it can give valid information about the cold sensitivity of an animal in the 10° to 15°C range, which is usually considered to be innocuous (Minett et al., 2011).

The application is performed with a pipette using a drop of 40µL, targeted at the ventral part of the plantar heel from underneath the animal. Therefore, the animal is placed in an individual plexi glass cylinder on a wire grid (same as for the von Frey test). The skin cooling in mice usually causes the animal to show withdrawal behaviors. If these animals suffer from neuropathic pain, these responses will be elongated or occur more frequently than in healthy or SHAM animals. The summed duration of observed withdrawal behaviors is recorded with a stop watch. Recording time lasted for 2 minutes and the test was repeated three times for each hind paw, with an at least 5 minutes interval between each stimulation. The average of these three values was calculated and interpreted as the mean withdrawal response duration for the specific hind paw.

2.3.1.3. Thermal Sensitivity - Heat (Hargreaves Test)

The Hargreaves test was developed in 1988 by Hargreaves et al. and since then serves as a test for the investigation of withdrawal thresholds to noxious heat stimulation. A calibrated radiant heat source is used to stimulate the plantar surface of the hind paw. The animals are

placed in individual compartments standing on a glass plate. From underneath the animal, the radiant heat source can be targeted via a mirror to the desired site of stimulation. The animals should be calm and it is essential that both hind paws touch the glass plate completely. A lifted or folded paw can disturb a consistent heating process (Hirata et al., 1990). A lifted paw on the opposite site can influence the ability of the animal to lift the stimulated paw properly.

The stimulation starts simultaneously with a timer and these are stopped immediately after a withdrawal behavior occurs. The intensity of our radiant heat source was adjusted to 100 – 110 mW/cm², which resulted in a basal paw withdrawal latency of about 10 seconds. In order to assess noxious heat threshold before and after nerve injury, each paw was stimulated three times and the mean of the resulting three withdrawal latencies was calculated. Between stimulations an interval of at least two minutes was allowed, before stimulating the same animal again. If a withdrawal response was distracted by some other behaviors such as grooming or a change of position, this specific observation was rejected and repeated later. In order to avoid tissue damage after stimulation, a cut-off value of 20 seconds was used.

We also used the Hargreaves test (*Hargreaves analgesiometer apparatus, Stoelting Co, U.S.*) for investigating the analgesic effect of nicotine in the very same animals. Nicotine was injected subcutaneously after the mean paw withdrawal latency for that day after surgery was determined. Following injection, the withdrawal latency for each paw was assessed with a single stimulation every five minutes, starting five minutes after injection and performing the last stimulation 60 minutes after injection. During the test procedure, the glass surface was carefully cleaned with tissue and ethanol anytime if necessary to reduce the impact of urine and feces.

2.3.2. Methods for Testing the Analgesic Activity of Nicotine

2.3.2.1. Nicotine and Nicotine Injections

Nicotine (IUPAC Name: ([-]-1-Methyl-2-[3-pyridyl]-pyrrolidine)) was purchased from Sigma-Aldrich Co. LLC. as liquid free base and stored at room temperature. Further properties of the used substance are listed in table 2. The drug was dissolved in sterile saline (*0.9% sodium chloride solution; pH-value: 4.5 - 7; Fresenius Kabi Austria GmbH*) in order to reach the desired concentration for experiments. Nicotine injections were performed subcutaneously (s.c.) caudal to the animal's neck by gently lifting the skin after gently restraining the animal with a towel. Before injection, the animals were weighed and the amount of drug was adjusted to the specific weight of each animal. For drug injections, sterile, fine dosage, disposable syringes Inject®-F with 1 mL volume and 0.01 mL graduation were used together with a sterile, disposable, Sterican® hypodermic-needle of 0.30 x 12 mm (both ordered from *B.Braun Melsungen AG, Germany*).

Table 2: Physical Properties of (-)-Nicotine

Hill Notation	Molecular Weight	Density (20 °C)	Aggregate State (room temperature)	Purity
C ₁₀ H ₁₄ N ₂	162.23 g/mol	1.010 g/ml	liquid	≥ 98%

Nicotine was order from Sigma-Aldrich Co. LLC. as liquid free base and stored at room temperature. Further information about the chemical: IUPAC Name: ([-]-1-Methyl-2-[3-pyridyl]-pyrrolidine); Beilstein Registry Number: 82109; EC Number: 200-193-3; CAS Number: 54-11-5; MDL Number: MFCD00006369)

2.3.2.2. Analgesic Action of Nicotine in Nerve Injured Animals (Hargreaves Test)

The Hargreaves test was used to determine the analgesic action of nicotine in nerve injured animals. The advantage of the Hargreaves test is that the experimenter can distinguish

between ipsilateral nerve injured and contralateral uninjured hind paws. For more details about the Hargreaves test itself see chapter 2.3.1.3. Nicotine experiments in nerve injured animals were performed 4, 11 and 29 days after nerve injury. The basal sensitivity to mechanical, cold and heat stimulation for that day prior to nicotine was also measured. This paw withdrawal latency after radiant heat stimulation of the specific day was used as baseline reference for each animal, in order to judge the antinociceptive and analgesic activity of nicotine. The contralateral paw was tested first, 5 minutes after injection and was then followed by the ipsilateral paw. This procedure was repeated every 5 minutes for 60 minutes in total.

2.3.2.3. Acute, Analgesic Action of Nicotine (Hot Plate Test)

The hot plate technique for the determination of noxious heat sensitivity was first described by Woolfe and Macdonald in 1944 (first publication: (Macdonald et al., 1946). For this test, a temperature controlled metal surface is set to a specific temperature. The time an animal spends on this surface before showing a nociceptive behavior gives information about the heat threshold of that animal. We used a Hot Plate (*Bio-CHP Cold Hot Plate; Bioseb – In vivo Research Instruments*; plate dimensions: 165x165mm) which was set to 55°C (+/- 0.5°C). The surface was framed by a high plexi glass barrier, in order to restrict the animal from stepping away or jumping out of the heated plate. The animal was placed on the 55°C plate and a timer was activated simultaneously. The animal was observed on the plate until the first sign of nociception could be seen, at which point the timer was stopped and the animal was put back into its home cage. The recorded time was interpreted as paw withdrawal latency. For the baseline measurement, the test was repeated three times with a five minute interval and the mean was used as paw withdrawal latency. In order to assess the analgesic action of nicotine, the test was started five minutes after injection. This was repeated with a five minute interval for 25 minutes. Thereafter, two more tests were performed 35 and 45 minutes after injection.

The development of tolerance to the analgesic action of nicotine is well documented for the acute (Damaj et al., 1996) and the chronic (Galeote et al., 2006; Brett et al., 2007; Schroeder et al., 2011) exposure to the drug. In order to investigate the tolerance effect after chronic

exposure, we performed three daily s.c. injections for 11 days. After the first injection in the morning at about 10 am, the following two injections were performed with a 4 hour interval. Behavior was measured on the Hot Plate test after the first daily injection in the morning and was repeated every second day for 11 days. The experimental procedure for the Hot Plate test was the same as described before. The experiment was performed with the same animals after they were tested for their acute analgesic response.

2.3.3. Overview of Experimental Structure

1. Timecourse Nerve Injury (CCI)

Animals: $\alpha 5$ -KO and WT mice; Tests: Hargreaves test (*heat sensitivity*), Acetone evaporation test (*cold sensitivity*), von Frey test (*mechanical sensitivity*); Timecourse: 2, 4, 7, 9, 11, 15, 22 and 29 days postsurgery

2. Nicotine Acute Effect

Animals: $\alpha 5$ -KO and WT mice; Drugs: nicotine injections s.c. 2mg/kg or 4mg/kg; Tests: Hot-Plate test (*heat sensitivity*); Timecourse: 5 - 45 minutes postinjection

3. Nicotine in Nerve Injury (CCI)

Animals: $\alpha 5$ -KO and WT mice; Drugs: nicotine injections s.c. 2mg/kg; Tests: Hargreaves test (*heat sensitivity*); Timecourse: 5 - 60 minutes postinjection and 4, 11, 29 days postsurgery

4. Nicotine Tolerance

Animals: $\alpha 5$ -KO and WT mice; Drugs: nicotine injections s.c. 2mg/kg or 4mg/kg; Tests: Hot-Plate test (*heat sensitivity*); Timecourse: 5 - 45 minutes postinjection - repeated nicotine injections 3 times daily for 11 days in total, behavior test every second day after first injection

2.4. Tissue Dissection and Biochemical Investigation

After behavior testing was completed, selected central nervous tissue was dissected from animals, which received a nerve injury before. The criterion for collected tissue was an expression of the $\alpha 5$ subunit and/or an involvement in pain transmission.

For nervous tissue collection, animals were anesthetized with CO₂ before decapitation with big scissors just at the transition of head to the spinal cord. To reach the brain, the skull was cut and removed with small scissors and subsequently the brain was freed from its cranial cavity with a surgical scoop. The brain was kept moist with a few drops of cooled, calcium free tyrode's solution (150mM NaCl, 4mM KCl, 2.0mM MgCl₂, 10mM glucose, and 10mM HEPES, pH 7.4) in a culture dish. In the following the interpeduncular nucleus, habenula, striatum, hippocampus and thalamus were dissected with fine forceps, a scalpel and microscissors in the named sequence. Subsequently, the spinal cord was removed from the vertebral canal and the lumbar regions 3 - 6 (L3 - L6) were separated from residual thoracic segments. Finally, the two spinal parts were further divided in contra- and ipsilateral parts. After dissection, each nervous tissue part was immediately put in Eppendorf® polypropylene tubes filled with calcium free tyrode's solution on ice. After dissection, the tubes with discrete nervous tissue parts were centrifuged for 20 seconds short spin and the supernatant tyrode's solution was carefully removed with a vacuum pump. Immediately after this step, the tissue was flash frozen with liquid nitrogen and stored for further use at - 80 °C.

In order to investigate the amount of the nAChR $\alpha 5$ subunit within the named tissue, we used immunoprecipitation studies. These experiments were performed in WT animals and 4, 10 or 30 days after CCI or SHAM surgery. These animals were performed during the diploma thesis of Bogdan Ianosi and are described in detail in his thesis.

2.5. Statistical Analyses

Statistical analyses and graphing of data obtained from behavior experiments were processed with GraphPad Prism version 6.01 (*GraphPad Software, Inc., U.S.*). All results shown were analyzed with a Two-Way ANOVA followed by the Bonferroni multiple

comparisons tests. Data are expressed as means \pm standard error of mean (SEM). Significance was set to p-values ≤ 0.05 (confidence interval of 95%) indicated by symbols in the graphs. Trends toward significance and stronger levels of significance are not depicted. the Area under the curve (AUC) was calculated for nicotine experiments considering negative as well as positive peaks from baseline responses.

2.6. Supplementary Information

For an extra set of experiments we used male, adult C57BL/6 mice with parents that are homozygous for one nAChR subunit deletion. These deletions affected the $\alpha 5$, $\beta 2$ or $\beta 4$ subunit genes ($\alpha 5$ -KO*, $\beta 2$ -KO or $\beta 4$ -KO, respectively). $\beta 2$ -KO animals (Picciotto et al., 1995) were provided by J.P. Changeux (Pasteur Institute, Paris) and $\alpha 5$ -KO* as well as $\beta 4$ -KO (Kedmi et al., 2004) animals were provided by Avi Orr-Urtreger (Sourasky Medical Center, Tel Aviv). All groups were backcrossed to the B57Bl/6 background for at least 6 generations after germ line transmission. As a control, an independent group of wildtype (WT*) animals was used. In order to obtain further generations from purchased knock-out animals, homozygotes mice for a specific subunit were further crossed in our animal facilities. These homozygotes animals were housed in cages with their littermates for the first 14 days. Animals older than 14 days were mixed together randomly with animals of the other genotypes. All animals lived in shared cages (Type IIL) of 5-6 animals. These animals were tested for their acute analgesic response to nicotine and for their tolerance development to the analgesic action of nicotine. The tests were performed on the Hot Plate and the exact test procedure fits to the procedure described for $\alpha 5$ -KO and WT littermates (see chapter 2.3.2.3. and 2.3.2.4.).

3. Results

The $\alpha 5$ subunit of nAChRs was described to be involved in pain transmission (Khan et al., 2001; Vincler and Eisenach, 2004; Vincler and Eisenach, 2005; Young et al., 2008) and to be relevant for the analgesic action of nicotine (Jackson et al., 2010). We tested $\alpha 5$ -KO and WT mice littermates concerning their sensitivity to different modalities after peripheral nerve injury. Furthermore, we tested the animals concerning the analgesic action of acute and chronic nicotine as well as the acute action of nicotine in nerve injured animals.

3.1. Paw withdrawal latency after Nerve Injury

There were no major differences in baseline nociception between $\alpha 5$ -KO and WT mice for either heat, cold, or mechanical sensitivities. A significant effect (p -value ≤ 0.05) of interaction, time, and groups was seen for all modalities tested with two-way ANOVA measures of ipsilateral paws, which was followed by a posthoc comparison of tested timepoints after surgery to baseline.

3.1.1. Heat Sensitivity (Hargreaves Test)

The Hargreaves test was used to examine the heat sensitivity of a distinct paw in animals which received a CCI or a SHAM surgery (figure 9). Before surgery all groups show a comparable paw withdrawal latency of about 11 seconds for heat stimulation. After surgery an immediate and significant drop in paw withdrawal latency from baseline can be observed 2 days after surgery in all animals that received a nerve injury. The drop in paw withdrawal latency reaches a peak effect between 4 and 11 days postsurgery. After 11 days a gradual increase in paw withdrawal latency can be observed for the subsequent days. However, paw withdrawal latency stays significantly increased for the complete time of behavioral testing

in animals with CCI (*Two-way ANOVA analyses Time Factor: $F [8, 512] = 15.40$; $p\text{-value} < 0.0001$*). Animals after SHAM surgery do not show any significant change in paw withdrawal latency at any time point after the surgery. The same observation is true for the contralateral paw of all groups tested. Differences between the genotypes ($\alpha 5$ -KO and WT) at any timepoint could not be observed with posthoc analysis comparing groups.

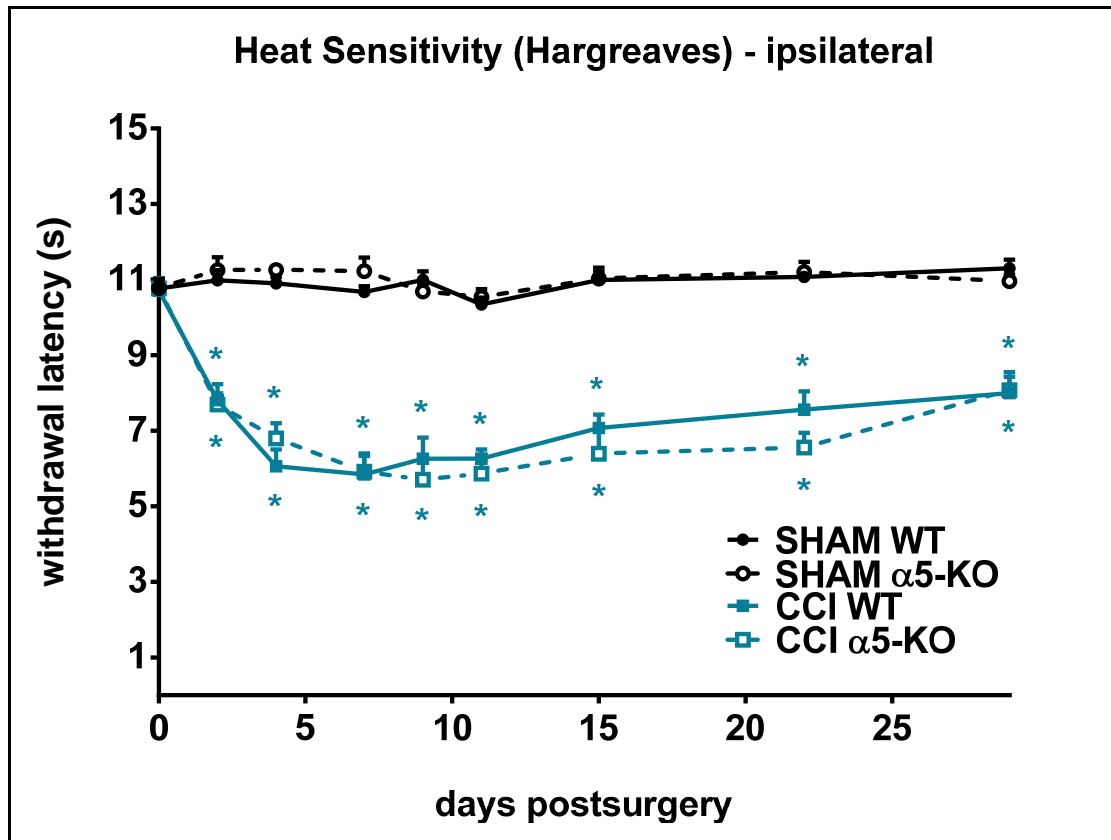


Figure 9: Heat sensitivity measured by the Hargreaves test in $\alpha 5$ -KO and WT animals after CCI or SHAM surgery. The paw withdrawal latency to thermal heat stimuli is greatly reduced following CCI. Animals which received a SHAM surgery do not show any decreases in their paw withdrawal latency. Within the CCI or SHAM group no statistically significant difference can be seen between genotypes in the response to thermal heat stimulation. Comparisons are shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for $p\text{-values} \leq 0.05$ and error bars show standard error of mean (SEM; upper stars: WT; lower stars: KO); $n=9$ for each group.

3.1.2. Cold Sensitivity (Acetone Evaporation Test)

The acetone evaporation test was used to assess the sensitivity to cold stimuli (figure 10). The duration of nociceptive behaviors after hind paw stimulation with acetone was measured. Before CCI or SHAM surgery, most animals showed nociception behaviors directed to the stimulated hind paw for about 2 to 3 seconds. After CCI, all animals show a significantly increased duration of nociception behaviors for at least 15 days (*Two-way ANOVA analyses Time Factor: $F [8, 520] = 8.658$; $p\text{-value} < 0.0001$*). WT and $\alpha 5$ -KO animals with CCI both do not show a significantly increased duration of nociception behaviors 29 days after surgery. Both groups with SHAM surgery show a gradual decrease in nociception behavior duration after surgery. This decrease in sensitivity becomes significant after 29 days for WT animals, whereas $\alpha 5$ -KO animals show a significantly decreased duration of nociception behaviors starting 7 days after surgery. For the contralateral paw, animals with SHAM surgery show a similar decrease in sensitivity, whereas nerve injured animals do not show any change in sensitivity for the contralateral paw for 29 days. However, significant differences in the duration of nociception behaviors between the genotypes tested could not be detected at any timepoint after surgery using posthoc analysis for comparing groups.

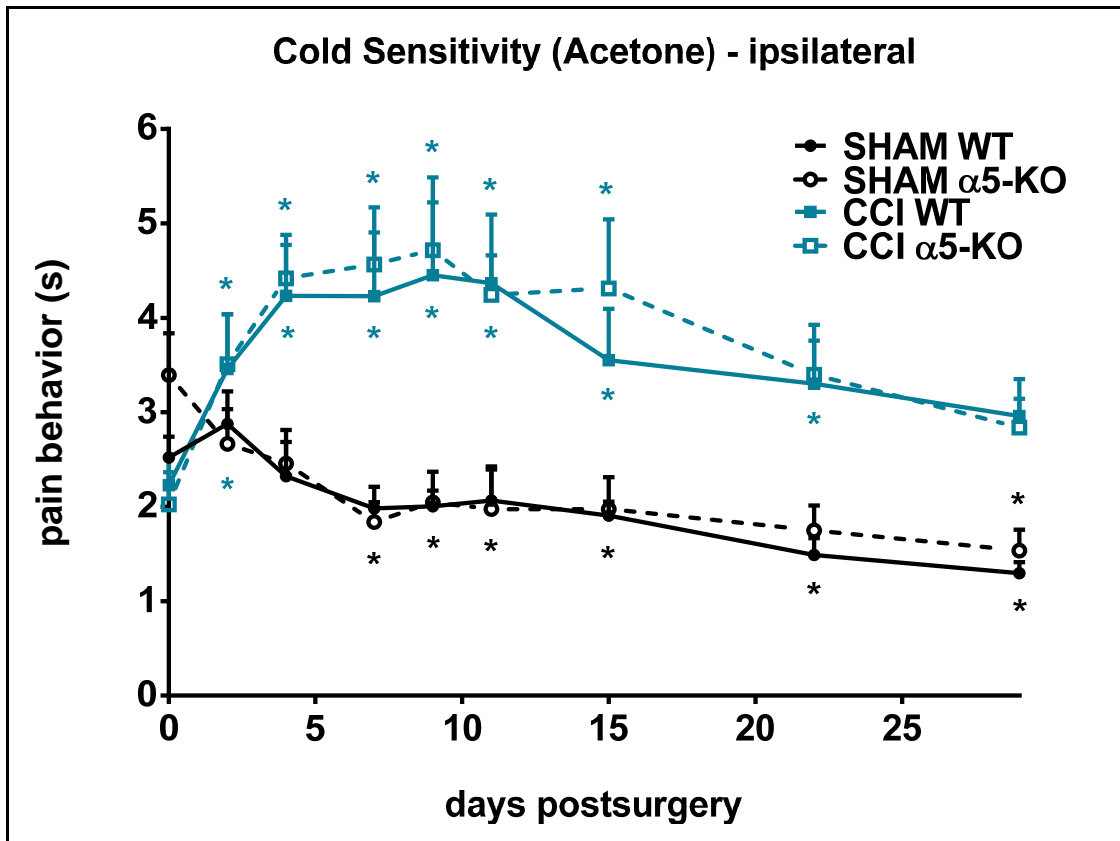


Figure 10: Cold sensitivity tested with the Acetone test in $\alpha 5$ -KO and WT animals after CCI or SHAM surgery. The duration of nociceptive behaviors is greatly elevated following CCI. Animals which received a SHAM surgery show a slight reduction of nociceptive behaviors with time. Within the CCI or SHAM group no statistically significant difference can be seen between genotypes in response to cold stimulation. Comparisons are shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: WT; lower stars: KO); $n=9$ for each group.

3.1.3. Mechanical Sensitivity (von Frey Test)

The von Frey test was used to investigate mechanical sensitivity in $\alpha 5$ -KO and WT mice after CCI or SHAM surgery (figure 11). Before surgery all groups show an average mechanical threshold between 0.6 and 0.8 grams. After SHAM surgery, the sensitivity of ipsilateral hindpaws consistently stays in this range for 29 days. Following CCI, the mechanical threshold for the ipsilateral paw decreases significantly 4 days after surgery for $\alpha 5$ -KO and 2 days after surgery for WT animals and stays significantly decreased for 29 days (*Two-way ANOVA analyses Time Factor: $F [8, 512] = 2.965$; p -value = 0.003*). The contralateral paws did

not show significant shifts from baseline responses. Further posthoc comparison between groups did not show significant differences between genotypes at any timepoint after surgery.

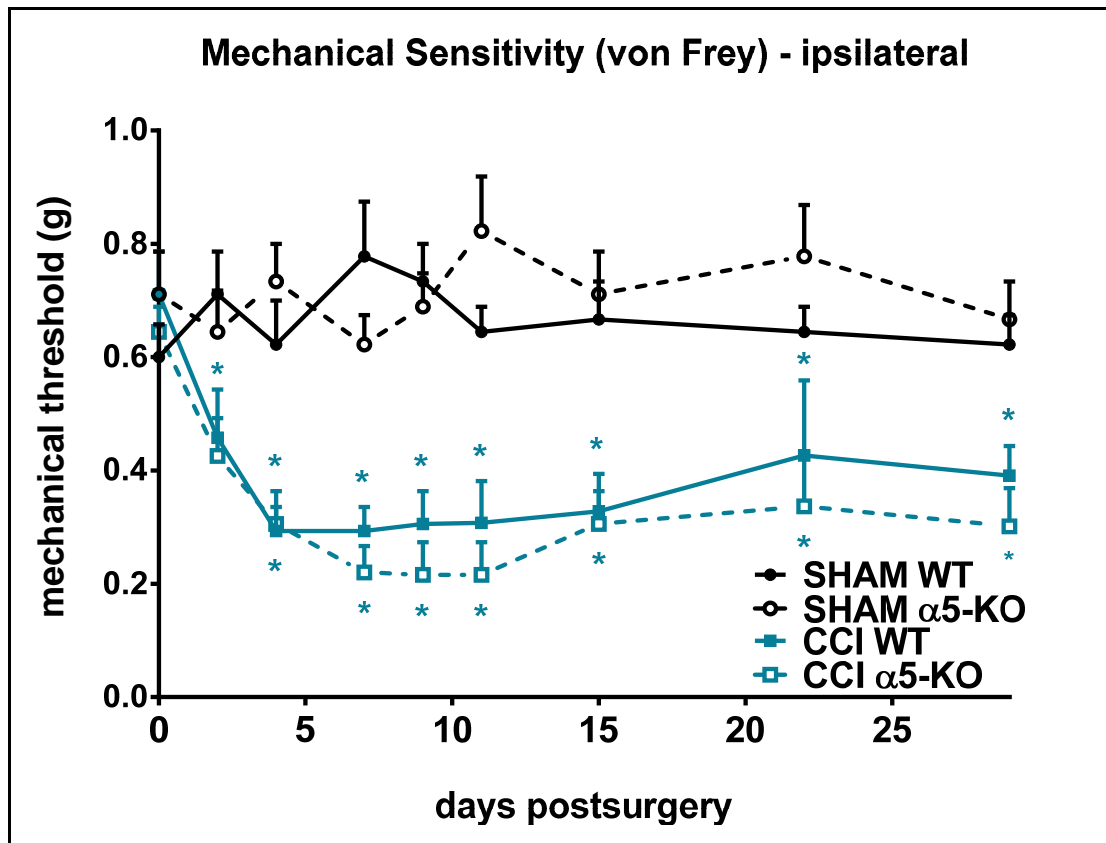


Figure 11: Mechanical sensitivity tested with von Frey monofilaments in $\alpha 5$ -KO and WT animals after CCI or SHAM surgery. The mechanical threshold is greatly reduced following CCI. Animals which received a SHAM surgery do not show any significant change in their mechanical threshold. Within the CCI or SHAM group no statistically significant difference can be seen between genotypes in response to mechanical stimulation. Comparisons are shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: WT; lower stars: KO); $n=9$ for each group.

3.2. Nicotine

The analgesic response to nicotine was assessed in naive animals, in animals which suffer from CCI and after chronic treatment with nicotine. In all tests nicotine was injected

subcutaneously (s.c.) and the subsequent tests for thermal heat sensitivity started 5 minutes after injection.

3.2.1. Acute, Analgesic Action of Nicotine (Hot Plate Test)

The acute analgesic action of nicotine was evaluated for 2 mg/kg (figure 12) and for 4 mg/kg (figure 13) doses of nicotine. After injection, mice were tested for 45 minutes with the Hot Plate test. Two-way ANOVA revealed significant time (*Time Factor: $F [7, 98] = 9.294$; $p\text{-value} < 0.0001$*) and interaction factors (*Interaction Factor: $F [7, 98] = 2,739$; $p\text{-value} = 0.0122$*) for 2 mg/kg and for time (*Time Factor: $F [7, 98] = 11.08$; $p\text{-value} < 0.0001$*) after 4 mg/kg injection. All animals show significantly increased withdrawal latencies 5 minutes after 2 mg/kg or 4 mg/kg nicotine administration as compared to baseline with posthoc comparisons. The analgesic effect 5 minutes after injection is not show statistically different between WT and $\alpha 5$ -KO mice for either dose tested (2 mg/kg with SEM: 10.78 +/- 1.58 s for WT and 13.45 +/- 1.12 s for $\alpha 5$ -KO; 4 mg/kg with SEM: 19.92 +/- 3.78 for WT and 23.84 +/- 4.76 for $\alpha 5$ -KO). In WT animals, the significant effect as compared to baseline is lost 10 minutes and 15 minutes after 2 mg/kg and 4 mg/kg injection, respectively. In contrast, $\alpha 5$ -KO mice show an increased duration of significantly increased withdrawal latencies. After 2 mg/kg injection of nicotine $\alpha 5$ -KO mice show significantly increased withdrawal latencies for 20 minutes and after 4 mg/kg the increase in withdrawal latencies stays significant for 25 minutes.

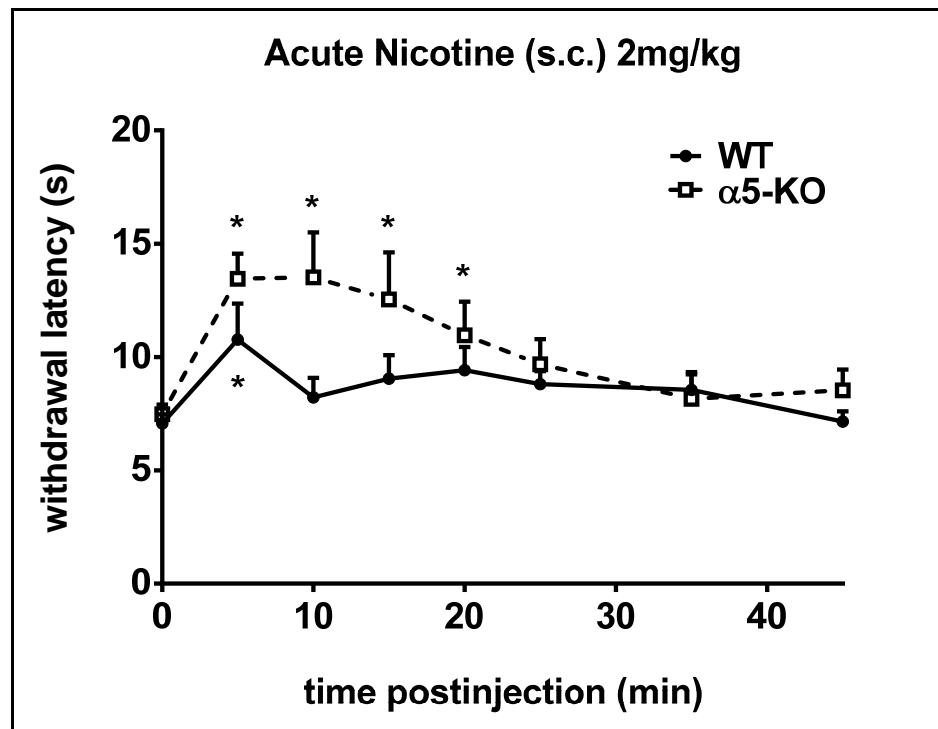


Figure 12: Acute, thermal analgesic action of 2 mg/kg nicotine in $\alpha 5$ -KO and WT littermates. After nicotine injection both groups show increased paw withdrawal latency. The significant increase lasts for 20 minutes in $\alpha 5$ -KO animals, whereas this returns to baseline in WT mice as early as after 10 minutes. Comparisons are shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: KO; lower stars: WT); $n=8$ for each group.

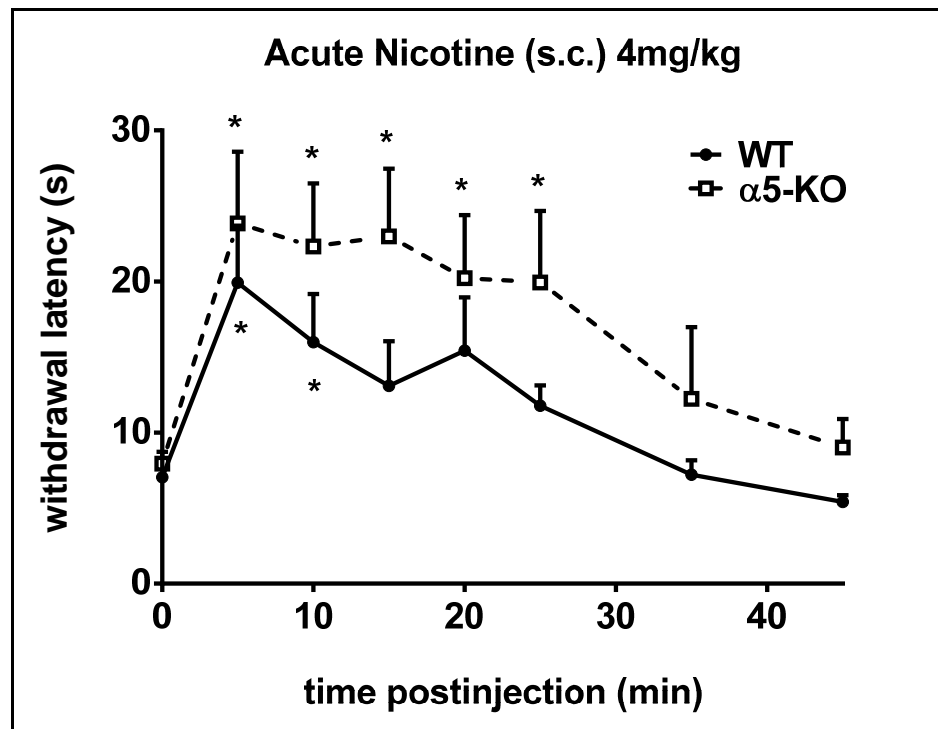


Figure 13: Acute, thermal analgesic action of 2 mg/kg nicotine in $\alpha 5$ -KO and WT littermates. After nicotine injection both groups show increased paw withdrawal latency. The significant increase lasts for 20 minutes in $\alpha 5$ -KO animals, whereas this returns to baseline in WT mice as early as 10 minutes. Comparisons are shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: KO; lower stars: WT); $n=9$ for WT and $n=7$ for $\alpha 5$ -KO.

3.2.2. Antinociceptive Action of Nicotine in Nerve Injured Animals

The thermal analgesic response after application of 2 mg/kg nicotine in animals with CCI was performed with the Hargreaves test 4 (figure 14), 11 (figure 15) and 29 (figure 16) days after surgery.

3.2.2.1. Analgesic Action of Nicotine 4 days postsurgery

Two-way ANOVA analyses of ipsilateral paws show significance for time (*Time Factor*: $F [12, 384] = 28.29$; p -value < 0.0001) and group (*Group Factor*: $F [3, 32] = 10.73$; p -value < 0.0001). All animals which received nicotine 4 days after CCI showed an immediate increase in

withdrawal latencies 5 minutes after injection, reflecting the analgesic effect of nicotine. The duration of statistically significant antinociception 4 days after surgery differs slightly in relation to surgery treatment and genotype. In animals with nerve injury the analgesic effect lasts at least 30 minutes, in WT animals with CCI it even lasts for 40 minutes. Also WT mice with SHAM surgery show significantly increased withdrawal latencies for 30 minutes after nicotine injection. Only in KO animals with SHAM surgery the effect is lost after 15 minutes, but shows up again 25 minutes after injection. The cut-off was hit in 8/9 cases for SHAM WT, 7/9 cases for SHAM KO, 9/9 cases for CCI WT and 5/9 cases for CCI KO animals for the ipsilateral paw and at some point during the test interval of 60 minutes after injection. The AUC net area does not show statistically significant differences when comparing WT and $\alpha 5$ -KO mice (figure 17).

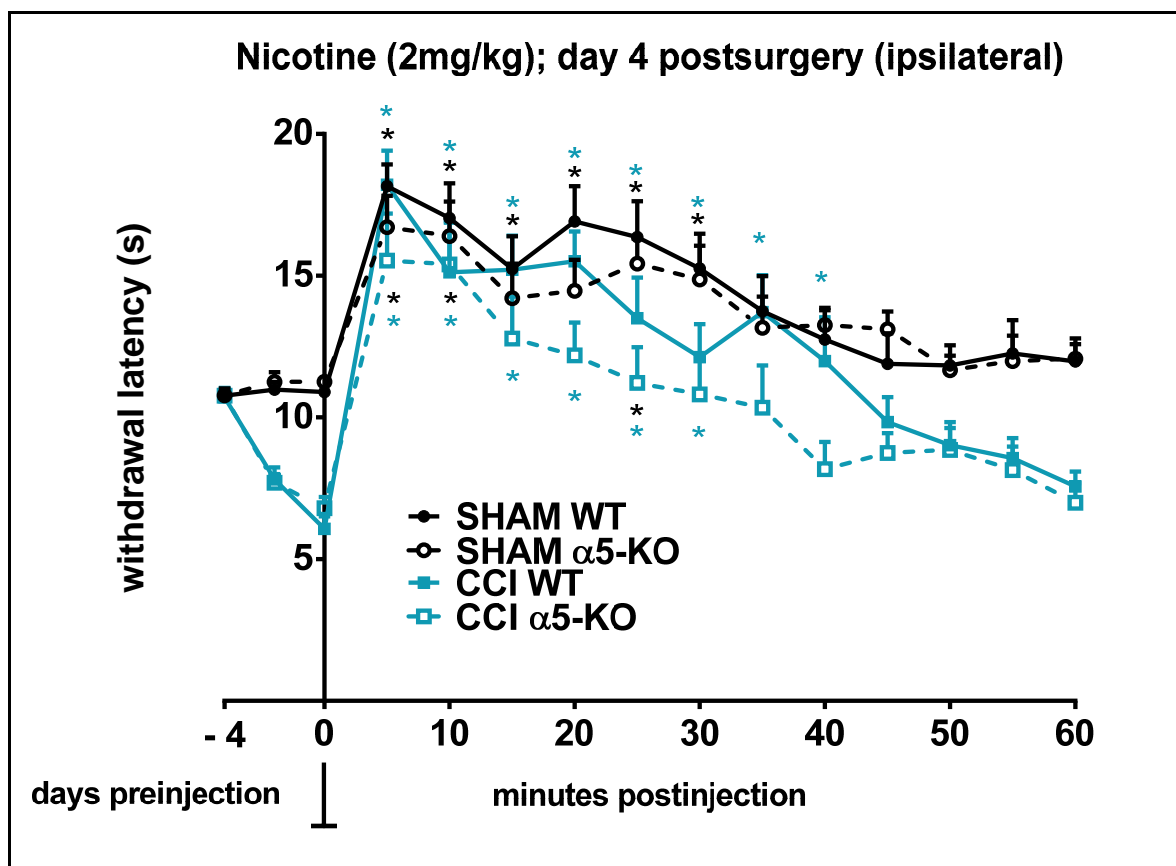


Figure 14: Paw withdrawal latency of $\alpha 5$ -KO and WT mice to heat stimulation on the Hargreaves test after subcutaneous injection of 2 mg/kg nicotine at 4 days after surgery. All groups show a strong increase in their paw withdrawal latency after nicotine injection. After 45 minutes the paw withdrawal latency of all groups is back to baseline. The duration of elevated paw withdrawal latency differs slightly with respect to the genotype and surgery treatment. In order to avoid tissue damage after stimulation, a cut-off value of 20 seconds was used. Days preinjection comprise 4 and 2 days before injection was performed. Surgery for CCI was performed 4 days preinjection. Comparisons are

shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: WT; lower stars: KO); $n=9$ for each group.

3.2.2.2. Analgesic Action of Nicotine 11 days postsurgery

Two-way ANOVA of ipsilateral paws revealed significance in time (*Time Factor*: $F [12, 384] = 18.30$; p -value < 0.0001) and group factors (*Group Factor*: $F [3, 32] = 7.243$; p -value = 0.0008). All mice with CCI show significantly increased withdrawal latencies 5 minutes after injection of nicotine and 11 days after surgery. In WT animals this effect stays significant for 55 minutes, whereas $\alpha 5$ -KO animals lose the antinociceptive effect 35 minutes after injection. In contrast, WT animals with SHAM surgery show a significant analgesic effect starting 10 minutes after injection and lasting for the first 20 minutes postinjection. Increases in withdrawal latency were observed in SHAM animals with $\alpha 5$ -KO for the first 15 minutes and 25, 30 and 40 minutes after injection. Cut-off was hit at some point during the 60 minutes test phase after nicotine injection in 4/9 cases for SHAM WT, 6/9 cases for SHAM KO, 4/9 cases for CCI WT and 4/9 cases for CCI KO mice. The AUC net area does not show any statistically significant differences for the overall effect of nicotine on the paw withdrawal latency in WT or $\alpha 5$ -KO mice (figure 17).

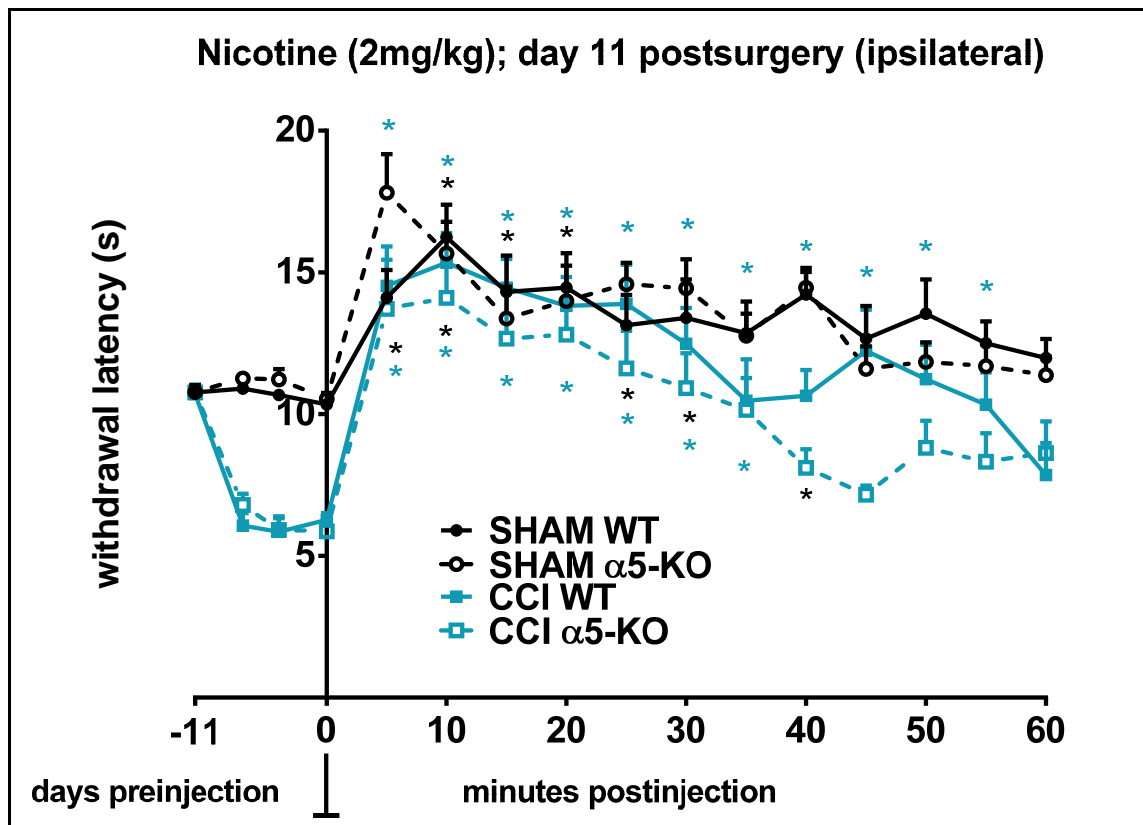


Figure 15: Paw withdrawal latency of $\alpha 5$ -KO and WT mice to heat stimulation on the Hargreaves test after subcutaneous injection of 2 mg/kg nicotine at 11 days after surgery. All groups show a strong increase in their withdrawal latency after nicotine injection. After 45 minutes, the paw withdrawal latency of all groups is back to baseline. The duration of elevated paw withdrawal latency differs slightly with respect to the genotype and surgery treatment. In order to avoid tissue damage after stimulation, a cut-off value of 20 seconds was used. Days preinjection comprise 4, 7 and 11 days before injection was performed. Surgery for CCI was performed 11 days preinjection. Comparisons are shown to the baseline value ($x=0$) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: WT; lower stars: KO); $n=9$ for each group.

3.2.2.3. Analgesic Action of Nicotine 29 days postsurgery

Two-way ANOVA analyses of the ipsilateral paws show significance for time (*Time Factor: F [12, 384] = 18.17; p -value < 0.0001*). The paw withdrawal latency is significantly increased from baseline 5 minutes post nicotine injection in all groups tested at 29 days after surgery. In WT animals after SHAM surgery, this effect lasts for 10 minutes. One further significant peak can be observed 30 minutes after injection, which is probably thought to be an artifact. In WT animals after CCI, the antinociceptive effect lasts for 30 minutes and is also seen at 40

minutes after injection. KO animals with CCI show significantly increased withdrawal latency for 25 minutes and additionally at 35 minutes after injection. SHAM animals with $\alpha 5$ -KO show an antinociceptive response to nicotine for 35 minutes. Cut-off was hit in 6/9 cases for SHAM WT, in 7/9 cases for SHAM KO, in 5/9 cases for CCI WT and in 5/9 cases for CCI KO mice at some point during the 60 minute test phase. No statistically significant differences in AUC net areas could be observed 29 days after surgery concerning the genotypes (figure 17).

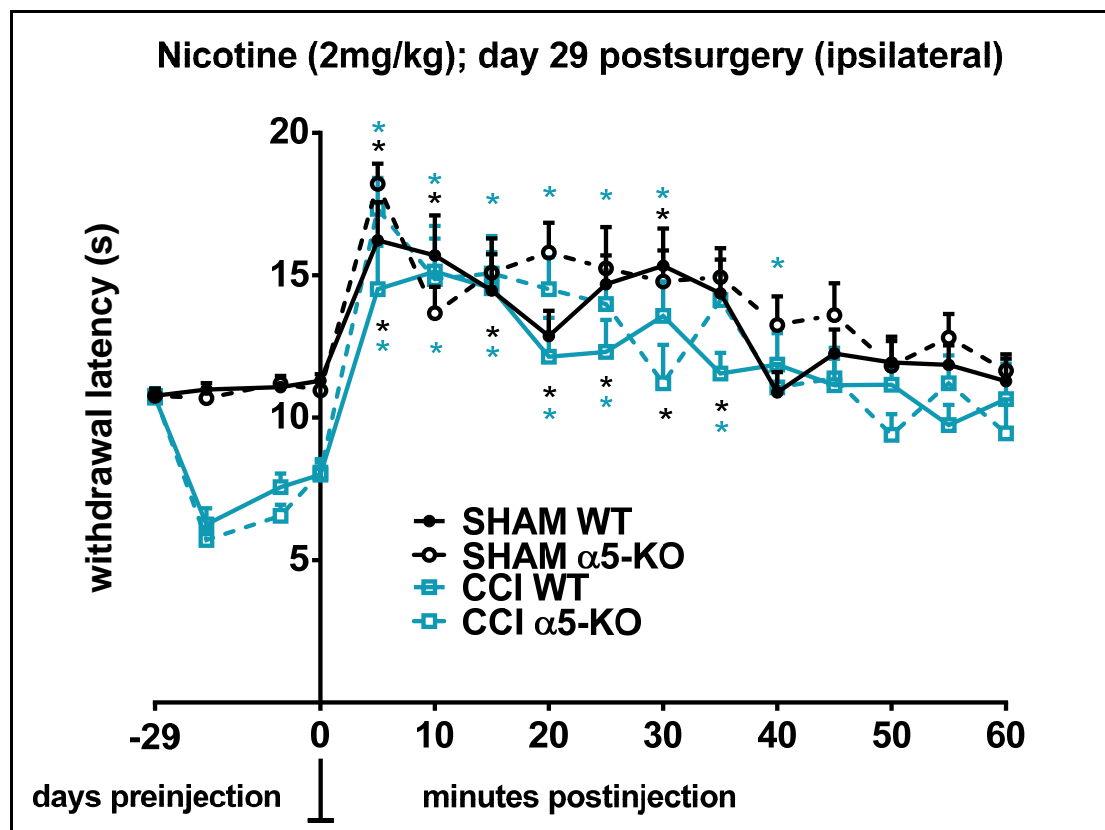


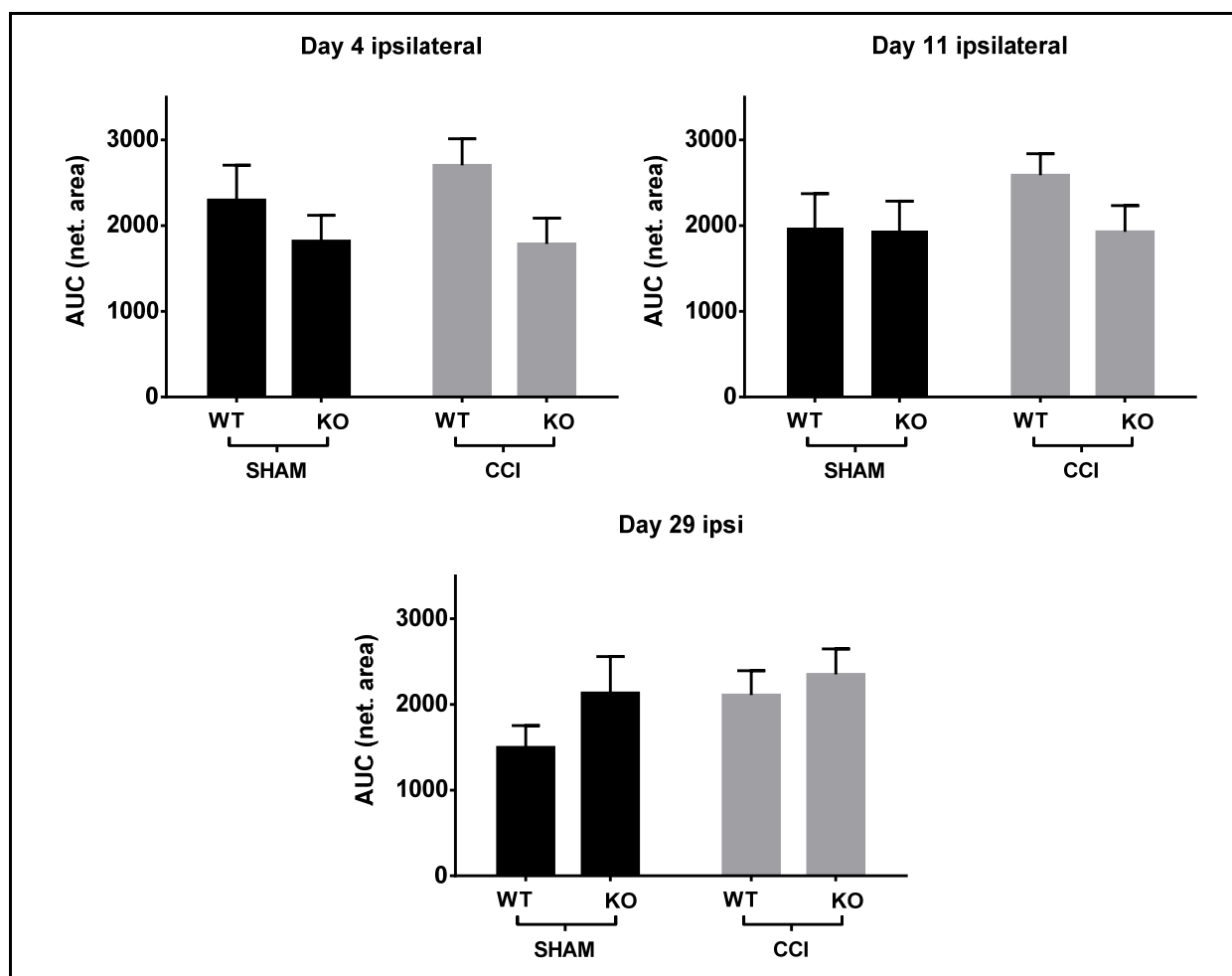
Figure 16: Paw withdrawal latency of $\alpha 5$ -KO and WT mice to heat stimulation on the Hargreaves test after subcutaneous injection of 2 mg/kg nicotine at 29 days after surgery. All groups show a strong increase in their paw withdrawal latency after nicotine injection. After 45 minutes the paw withdrawal latency of all groups is back to baseline. The duration of elevated paw withdrawal latency differs slightly with respect to the genotype and surgery treatment. In order to avoid tissue damage after stimulation, a cut-off value of 20 seconds was used. Days preinjection comprise 9, 22 and 29 days before injection was performed. Surgery for CCI was performed 29 days preinjection. Comparisons are shown to the baseline value ($x=0$) and are performed with a two-way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p -values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: WT; lower stars: KO); $n=9$ for each group.

Table 3: Measurement for the effect of nicotine on the contralateral paw after CCI or SHAM surgery and 2 mg/kg nicotine administration

Surgery / Genotype	Timepoints (minutes) of significantly elevated paw withdrawal latency after nicotine injection
SHAM WT	Day 4: 5 - 15; 25 - 30 11: 5 - 35 29: 5 - 10; 20 - 25
SHAM α 5-KO	Day 4: 5 - 25 11: 20 29: 5; 15-20
CCI WT	Day 4: 5 - 35 11: 5 - 35 29: 5 - 15; 25
CCI α 5-KO	Day 4: 5 - 15; 25 11: 5 - 10; 25 29: 5 - 25

Analyses refer to the baseline value of the specific day and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p-values ≤ 0.05 . n=9 for each group.

Figure 17: Area Under Curve (AUC) of the analgesic nicotine effect at the ipsilateral paw for 60 minutes after injection in mice with SHAM or CCI surgery



Area Under Curve (AUC) was calculated for 60 minutes after nicotine injection with respect to individual baseline values and as net area considering positive (exceeding baseline value) as well as negative values (falling below baseline value). Analyses are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p-values ≤ 0.05 . n=9 for each group.

3.2.3. Tolerance to the analgesic action of nicotine after chronic nicotine

The heat sensitivity of $\alpha 5$ -KO and WT mice was assessed with the Hot Plate test every second day after the first of three daily nicotine injections. This experiment was performed with 2 mg/kg (figure 18) or 4 mg/kg (figure 19) of nicotine.

3.2.3.1. Repeated administration of 2 mg/kg nicotine

The results for the first day of heat sensitivity after nicotine treatment with 2 mg/kg have already been described in section 3.2.1. Interestingly, the analgesic action of nicotine, which can be seen in $\alpha 5$ -KO and in WT mice on the first day of chronic nicotine treatment, is completely lost in WT animals on the third day of nicotine treatment and thereafter. This suggests that tolerance has developed. In WT animals the paw withdrawal latency was not significantly increased as compared to baseline any time after the first day of treatment. In contrast to that, $\alpha 5$ -KO mice still show significantly increased withdrawal latencies compared to baseline on treatment days 3 (*Time Factor*: $F [7, 98] = 4.422$; $p\text{-value} = 0.0003$), 7 (*Time Factor*: $F [7, 98] = 4.056$; $p\text{-value} = 0.0006$) and 9 (*Time Factor*: $F [7, 98] = 4.502$; $p\text{-value} = 0.0002$; *Interaction Factor*: $F [7, 98] = 3.990$, $p\text{-value} = 0.0007$). On day 5 (*Time Factor*: $F [7, 98] = 2.625$; $p\text{-value} = 0.0158$) and day 11 (*Time Factor*: $F [7, 98] = 5.714$; $p\text{-value} < 0.0001$) the significant increase in latencies is completely absent in both genotypes, whereby $\alpha 5$ -KO mice still seem to show a more prominent elevation of paw withdrawal latency on day 5. Furthermore, consistency in weight throughout the whole testing interval (figures 19 A) was observed for both genotypes.

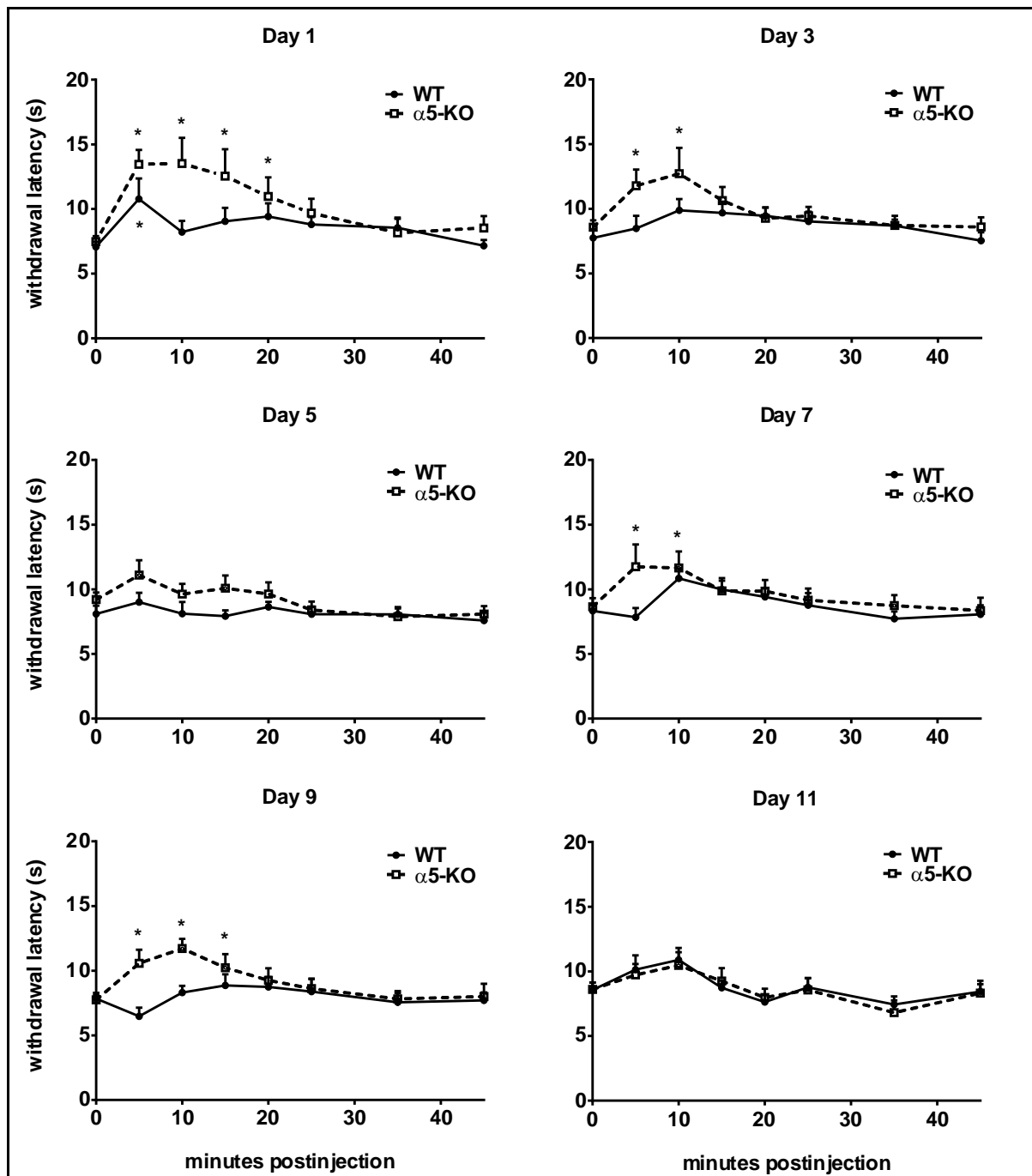


Figure 18: Tolerance development to the thermal analgesic action of nicotine after chronic treatment with 2 mg/kg nicotine. Nicotine was injected 3 times daily for 11 days. Behavioral tests were performed on the Hot Plate test each second day after the first daily injection. WT animals do not show any analgesic sign after nicotine injection on the third day of chronic nicotine treatment. On the following days no more analgesic activity of nicotine could be observed in WT animals. Mice with $\alpha 5$ -KO still show significantly increased withdrawal latencies following nicotine injection on day 9 of chronic nicotine treatment. On treatment day 5 and treatment day 11 no group shows any significant changes from baseline after nicotine injection. Comparisons are shown to the baseline value (x=0) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p-values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: KO; lower stars: WT); n=8 for each group.

3.2.3.2. Chronic administration of 4 mg/kg nicotine

The first day of nicotine treatment with 4 mg/kg and the results for heat sensitivity have already been described in section 3.2.1. However, on the third day of nicotine treatment (*Time Factor: F [7, 91] = 2.424; p-value = 0.0254*) WT animals do not show a paw withdrawal latency that is significantly changed in comparison to the baseline responses. The same is the case for all subsequent experiments on days 5 (*Time Factor: F [7, 91] = 2.692; p-value = 0.0140; Interaction Factor: F [7, 91] = 2.310; p-value = 0.0325; Genotype Factor: F [1, 13] = 8.265, p-value = 0.0130*), 7 (*Time Factor: F [7, 84] = 5.989; p-value < 0.0001; Interaction Factor: F [7, 84] = 6.637; p-value < 0.0001; Genotype Factor: F [1, 12] = 6.846; p-value = 0.0225*), 9 (*Time Factor: F [7, 84] = 4.701; p-value = 0.0002; Interaction Factor: F [7, 84] = 5.261; p-value < 0.0001*) and 11 (*Time Factor: F [7, 77] = 4.531; p-value = 0.0003; Interaction Factor: F [7, 77] = 2.970; p-value = 0.0082; Genotype Factor: F [1, 11] = 8.036; p-value 0.0162*). Mice with $\alpha 5$ -KO show significantly elevated withdrawal latencies from baseline following nicotine injection throughout all experimental days. On the third day of treatment the significant analgesic action of nicotine in $\alpha 5$ -KO mice only takes place 20 minutes after nicotine was injected. For all the other experimental days the paw withdrawal latency is significantly increased from baseline at least 5 and 10 minutes after injection. Furthermore, we could observe an increased risk of occurring seizures in WT compared to $\alpha 5$ -KO animals (18 for WT versus 2 for $\alpha 5$ -KO) as well as an increased risk of nicotine induced deaths (3 for WT), only during the 4 mg/kg injection paradigm, which is consistent with earlier reports (Kedmi et al., 2004). Again consistency in weight throughout the whole testing interval (figures 19 B) was observed for both genotypes.

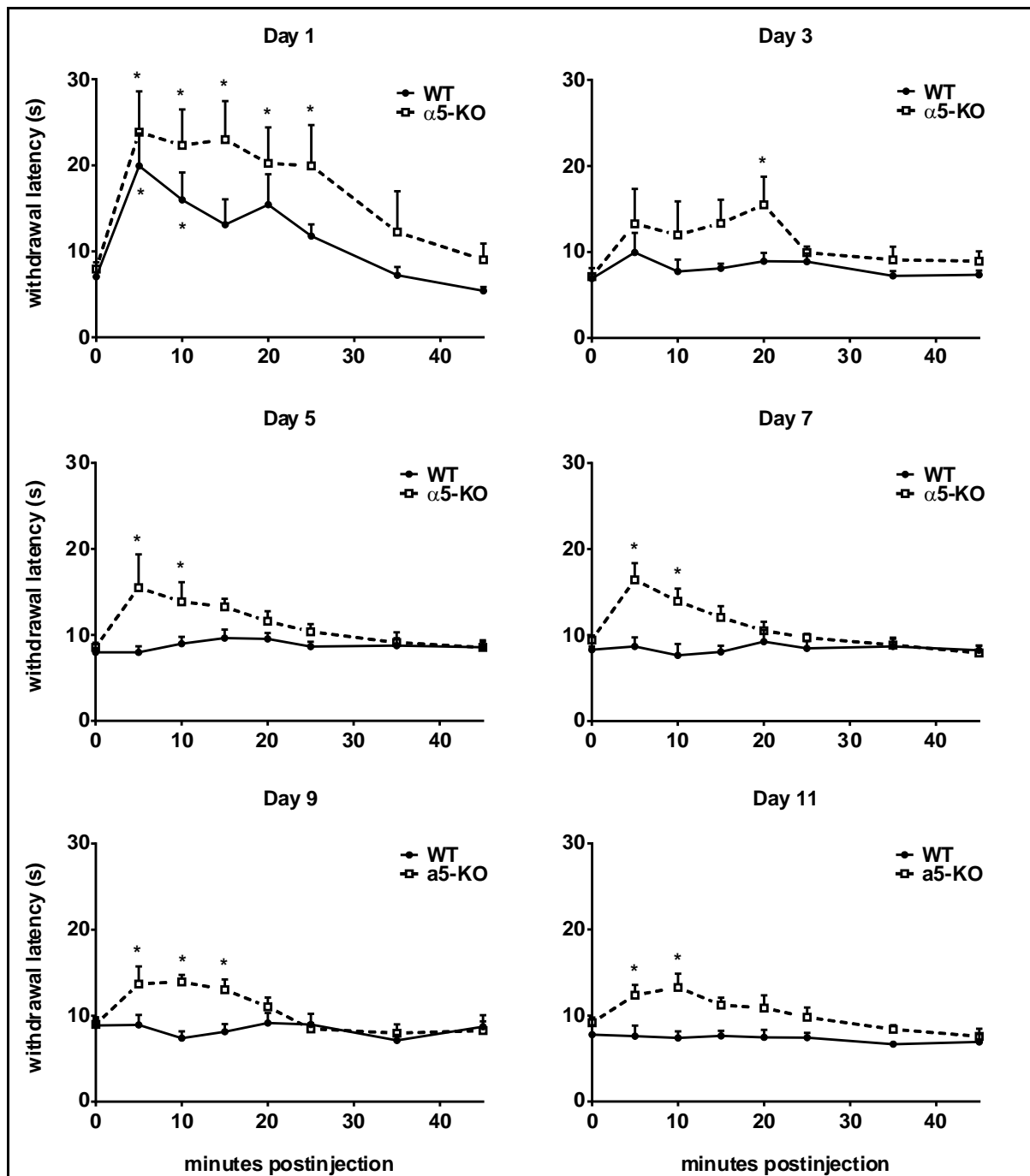


Figure 19: Tolerance development to the thermal analgesic action of nicotine after chronic treatment with 4 mg/kg nicotine. Nicotine was injected 3 times daily for 11 days. Behavioral tests were performed on the Hot Plate test each second day after the first daily injection. WT animals do not show any analgesic sign after nicotine injection on the third day of chronic nicotine treatment. On the following days no more analgesic activity of nicotine could be observed in WT animals. Mice with $\alpha 5$ -KO consistently show a significantly increased paw withdrawal latency after 4 mg/kg nicotine injection for all days tested. Comparisons are shown to the baseline value (x=0) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p-values ≤ 0.05 and error bars show standard error of mean (SEM; upper stars: KO; lower stars: WT); n=6 - 9 for WT and n=7 for $\alpha 5$ -KO.

4. Discussion

The involvement of nAChRs in pain transmission is well known since nAChR agonists can induce potent analgesic efficiency (Nirogi et al., 2013). This effect may be due to a variety of different subunit combinations of nAChRs, which are expressed in peripheral, spinal as well as supraspinal loci that are relevant for pain transmission (Umana et al., 2013). The analgesic efficiency of nAChR agonists is limited due to side effects, especially after administration of higher doses. In mice these can include seizures, hypothermia, and hypolocomotion (Kedmi et al., 2004; Umana et al., 2013). In humans, nausea, increased heart rate and the powerful addictive properties are frequently reported besides other side effects (Henningfield et al., 1985; Flood and Daniel, 2004; Hong et al., 2008). This suggests that the identification of nAChRs subunit compositions can be an important issue for a possible clinical use of nAChRs agonists as analgesics. Receptor subtype specific ligands could then be developed, in order to specifically target receptors which are relevant only for desired effects. In this context, our study increases understanding for the $\alpha 5$ subunit of nAChRs and its role for the nicotine-mediated analgesia as well as its potential role in neuropathic pain.

4.1. Paw withdrawal latency in $\alpha 5$ -KO and WT Mice after CCI

A CCI or a SHAM surgery was performed in $\alpha 5$ -KO and WT mice in order to test their sensitivity for mechanical, heat, and cold stimuli for 29 days after surgery. Although all animals with CCI showed hyperalgesia in all three modalities tested (figures 9 - 11), there were no statistically significant differences between $\alpha 5$ -KO and WT animals at any day after surgery nor for any modality tested. These results had been previously verified in our laboratory with another model for neuropathic pain, the peripheral sciatic nerve ligation (master thesis of Ariane Thrun, unpublished data). From these results we can conclude that $\alpha 5$ -containing nAChRs do not play a major role for the increased sensitivity to mechanical, heat and cold stimuli after peripheral nerve injury. These findings contradict some interpretations of previous studies in the literature for an important role for the $\alpha 5$ subunit

after nerve injury. The increase in mechanical withdrawal latencies after spinal nerve ligation (SNL) which was observed after treatment with $\alpha 5$ antisense (Vincler and Eisenach, 2005) contrasts our results. Besides the rather weak effect of antisense treatment Vincler and Eisenach present, knock down of the $\alpha 5$ subunit mRNA only in lumbar regions L4 to L6 and 10 to 12 days after surgery is one obvious difference to a knock out of the $\alpha 5$ subunit gene. First of all, the localized suppression of protein translation is in contrast to the universal suppression of protein formation in all tissues. Secondly, animals which are born with universal $\alpha 5$ -KO might compensate the subunit loss differently than adult animals that only suffer from temporary and localized deficits in protein formation. Also the discrepancies in the model of neuropathic pain, SNL versus CCI, in used behavioral tests, paw pressure test versus von Frey, Hargreaves and Acetone tests, and the species, rat versus mouse, might play a crucial role for observed differences. Furthermore, we could not confirm reports about an upregulation of the $\alpha 5$ subunit in spinal segments after peripheral nerve injury (Vincler and Eisenach, 2004). We also tested distinct brain regions for subunit expressions with immunoprecipitation, but again we cannot demonstrate an upregulation of any nAChR subunit after CCI (data not shown).

4.2. Peak Effect of the Acute, Analgesic Action of Nicotine in $\alpha 5$ -KO and WT Mice

In order to test the analgesic effect of 2 and 4 mg/kg subcutaneously applied nicotine, we tested $\alpha 5$ -KO and WT mice for 45 minutes on the Hot Plate after injection. The peak effect of nicotine at 5 minutes after injection did not show any statistically significant differences between the two genotypes for both doses tested (figures 12 and 13). These results are in contrast to the results previously described by Jackson et al., (2010). In their dose-response curve for the analgesic action of nicotine on the Hot Plate, a significant difference between $\alpha 5$ -KO and WT mice was described for all doses tested (figure 7). For this discrepancy with published results, it is difficult to offer a single satisfactory explanation. We also tested $\alpha 5$ -KO mice and their WT littermates on the Hot Plate with the same temperature (55°C) and cut-off latency (40s) as Jackson et al., (2010) did. Speculations can be made about observed discrepancies include housing conditions and care of animals just as the separate evolution

of our $\alpha 5$ -KO animals. With breeding over several generations, it cannot be excluded that the lack of the $\alpha 5$ subunit was compensated in a different manner than it was in Jackson's laboratories. However, for the possibility of an error a random population of our animals was rechecked concerning their $\alpha 5$ subunit expression. All animals tested showed the desired $\alpha 5$ -KO (data not shown). We further added an additional study with independent lines of $\alpha 5$ -KO* and WT* animals born from homozygous parents (supplementary material: figures 20 and 21). It is important to note that the comparison between these lines is limited, as they evolved separately from each other and thus might have generated independent genetic adaptation which in turn might influence the response to nicotine. This is not uncommon, as the exact physiological effect of nicotine strongly depends on the genetic background (Aschhoff et al., 1999; Stolerman et al., 1999). However, again in contrast to Jackson et al., 2010, in these experiments, the response of $\alpha 5$ -KO* mice 5 minutes after nicotine injection is significantly increased for 2 mg/kg (p-value: <0.0001) and 4 mg/kg (p-value: 0.003), when compared to WT* animals. Furthermore, in our results from $\alpha 5$ -KO and WT littermates with SHAM or CCI surgery which were tested on the Hargreaves test with 2 mg/kg of nicotine (figures 14, 15 and 16), again we could not observe any statistically significant difference between $\alpha 5$ -KO and WT mice 5 minutes after nicotine injection at any point after surgery. This is true for the ipsilateral paw of mice with SHAM surgery or CCI as well as for the contralateral paw which might somehow reflect the response of a healthy individual. However, it should be noted that the cut-off level in our Hargreaves experiments might restrict otherwise detectable differences, even though 20s is a reasonable value for repeated stimulation with higher temperatures, in order to avoid tissue injury.

4.3. Timecourse of the Acute, Analgesic Action of Nicotine in $\alpha 5$ -KO and WT Mice

Beyond the peak effect of nicotine, we also measured the duration of the nicotine analgesic effect in $\alpha 5$ -KO and WT mice. The response in the Hot Plate (figures 12 and 13) was tested for 45 minutes, whereas in the Hargreaves test (figures 14, 15 and 16) this was tested for 60 minutes after injection. In the Hot Plate test, the increased duration of analgesic efficacy in $\alpha 5$ -KO mice is obvious for both the 2 mg/kg and for the 4 mg/kg doses. Mechanisms

underlying this effect may be speculated to be due to altered nicotine receptor properties due to lack of the $\alpha 5$ subunit. The $\alpha 5$ subunit has been indeed described to potentially influence Ca^{2+} permeability, channel opening times, burst durations, and desensitization properties depending on the receptor it is integrated in (Ramirez-Latorre et al., 1996; Gerzanich et al., 1998; Ciuraszkiewicz et al., 2013). All of these effects can potentially play a prominent role for the observed results. Desensitization properties of $\alpha 4\beta 2$ receptors play an important role for the analgesic action of nAChR ligands (Nirogi et al., 2013) and especially the desensitization property of $\alpha 4\beta 2\alpha 5$ receptors is reported to influence pain transmission (Zhang et al., 2012). A missing $\alpha 5$ subunit could result in disturbed receptor desensitization which may result in increased receptor activation times and longer lasting analgesic effects of nicotine.

Furthermore, changes in receptor composition during prolonged receptor activation are reported for nAChRs (Fenster et al., 1997; Lester et al., 2009; Govind et al., 2012) and can additionally affect incorporation of the $\alpha 5$ subunit into $\alpha 4\beta 2$ receptors (Mao et al., 2008; Zhang et al., 2012). Since nicotine injection leads to prolonged receptor activation compared to activation by endogenous acetylcholine, these mechanisms could additionally be absent or partially disturbed in $\alpha 5$ -KO animals.

Further explanations for the longer lasting effect of nicotine could be due to the nicotine metabolism or sympathetic nervous system responses. A large proportion of nAChRs in autonomic ganglia integrate the $\alpha 5$ subunit (David et al., 2010) and it is likely that they modulate the receptor properties and autonomic organ functions there (De Biasi, 2002; Wang et al., 2002). Most autonomic functions have been previously reported to be unaltered in $\alpha 5$ -KO mice in the absence of nicotine (Salas et al., 2003), but this could be speculated to change in the presence of nicotine.

Besides the possibility that the half-life of nicotine differs for both genotypes, the interaction of cotinine with nAChRs could differ as well. Since cotinine is reported to have a very increased half-life as compared to nicotine (Siu and Tyndale, 2007) as well as the possibility to influence receptor desensitization (Buccafusco et al., 2007). The half-life of nicotine and the exact effect of cotinine injection concerning analgesia and antinociception could be compared in $\alpha 5$ -KO and WT mice.

In the Hargreaves test, differences are not as obvious and rather point towards an increased nicotine antinociceptive effect in WT as compared to KO animals at early timepoints at least for 4 days after surgery (figure 14 and 17). Early timepoints of CCI are characterized by strong inflammatory responses at the site of injury. It can be speculated that the $\alpha 5$ subunit is involved in these mechanisms, which might be absent in KO animals. In a later timepoint, 11 days after surgery, the effect for CCI WT animals starts earlier and lasts 35 minutes longer if compared to SHAM WT animals (figure 15). Hence, the nerve injury might have an effect on the duration of nicotine-induced analgesia in WT but not in KO animals at early timepoints of CCI. However, the calculation of AUC for the 60 minute timecourse did not unveil any significant differences for $\alpha 5$ -KO animals. Also no clear pattern or obvious difference is detectable for the duration of antinociceptive activity in $\alpha 5$ -KO or WT animals 4, 11 and 29 days after CCI. These results and the possibility of artifacts could best be confirmed with an increase in the number of animals tested although the impact of the results needs to always be balanced with an ethical use of animals. However, with our results at hand we assume no major involvement of the $\alpha 5$ subunit in nicotine-induced antinociception tested on the Hargreaves test.

The Hargreaves test was used in order to discriminate the hind paws that are affected or not affected by the nerve injury. Compared to the Hargreaves test, the Hot Plate activates more supraspinal circuits related to pain, as the whole animal is moved directly from its home cage environment to the Hot Plate. Furthermore, both paws are stimulated and the animal is freely moving on the plate which is already heated to the set temperature. In contrast to that, the stimulation with the Hargreaves test rather involves spinal reflexes and may not even be recognized by the animal before reaching pain relevant temperatures and subsequent withdrawal reflexes. Thus, an activation of different nociception or pain related circuits involving the $\alpha 5$ subunit might be an explanation for the discrepancies we observed in $\alpha 5$ -KO mice between Hot Plate and Hargreaves tests after injection of 2 mg/kg nicotine.

4.4. Tolerance to the analgesic activity of nicotine in $\alpha 5$ -KO and WT mice

Besides the acute effect of nicotine on $\alpha 5$ -KO animals, we were also interested in the effect of chronic, intermittent nicotine injections for the development of tolerance to the analgesic

effects (figures 17 and 18). This research might be of special interest for smokers, since their analgesic action of nAChRs agonists differs compared to the non-smoking population (Habib et al., 2008; Yagoubian et al., 2011) and since the $\alpha 5$ subunit gene was related to nicotine intake and dependence (Wang et al., 2009).

WT animals became completely tolerant to the effect of nicotine already within a few days after injections started. In contrast, $\alpha 5$ -KO mice still showed remaining responses at least 11 days after their first injection of 4 mg/kg. Also for the 2 mg/kg injection paradigm, we found indications for disrupted tolerance development in $\alpha 5$ -KO animals. To our knowledge, this was the first time an involvement of the $\alpha 5$ subunit is described for the tolerance development to the analgesic actions of nicotine in behavioral experiments. It has been described that an incorporation of the $\alpha 5$ subunit can prevent upregulation of $\alpha 4\beta 2^*$ receptors (Mao et al., 2008). The mechanisms for physiological tolerance of nicotine are not fully understood but strong evidence points towards a general upregulation of nAChRs after repeated or chronic nicotine exposure (Nguyen et al., 2003). With immunoprecipitation studies for nAChR subunits in distinct spinal and supraspinal structures it could be investigated if $\alpha 5$ -KO mice show any discrepancy in receptor upregulation after repeated nicotine treatment *in vivo*.

During the intermittent nicotine injection paradigm, we could not observe major shifts in basal nociceptive responses or in the weight (supplementary material figure 20) of treated animals on the Hot Plate. It is consistent with earlier reports that nicotine-induced seizures and deaths occur less frequently in $\alpha 5$ -KO animals (Kedmi et al., 2004). These seizures lasted only for a few seconds and if seizures were observed in animals before behavioral testing, these animals were not considered for stimulation on the Hot Plate. In this way possible interference of seizures with our results was limited.

4.5. Tolerance to the analgesic activity of nicotine in an independent set of nAChR-KO mice

An enduring significant analgesic effect of nicotine 11 days after initiation of chronic, intermittent injections was seen for $\alpha 5$ -KO* mice for the 2 mg/kg as well as for the 4 mg/kg

paradigm (supplementary material figures 20 and 21). For WT* animals, the nicotine analgesic effect was lost on the third day in both cases. This is further consistent with our results in WT and $\alpha 5$ -KO littermates. Interestingly also $\beta 2$ -KO mice still show significantly increased withdrawal latencies from baseline on treatment day 11 after either 2 mg/kg or 4 mg/kg injections. Animals with $\beta 4$ -KO develop a delayed tolerance with onset on day 5 after the first injection day. It can be speculated if the initial effect of nicotine on day 1 and/or the different weight of animals (supplementary material figure 20) might play a role for the subsequent development of physiological tolerance.

4.6. Conclusion

Loss of the $\alpha 5$ subunit of nicotinic receptor is suggested not to affect the development of peripheral neuropathic pain after CCI. Furthermore, the basal nociceptive responses as well as the peak analgesic responses to nicotine do not seem to be affected by the deletion, indicating a minor role of the $\alpha 5$ subunit for this effect. In contrast, the full duration of the analgesic action of nicotine is elongated in $\alpha 5$ -KO animals, indicating an involvement of the subunit for receptor properties relevant for the duration of the analgesic action. Interestingly, tolerance for intermittent nicotine administration appears to require $\alpha 5$ -containing receptors.

With our study we address at least two possible side effects of nicotine treatment. Thus the $\alpha 5$ subunit could be relevant for the limited duration of acute analgesic action and the rapid development of tolerance. Also the occurrence of seizures after nicotine injection is reduced in $\alpha 5$ -KO mice.

The exact molecular mechanisms and the role of the $\alpha 5$ subunit in these mechanisms, the affected receptor subtypes that potentially integrate the $\alpha 5$ subunit, as well as the anatomical sites of action, all need to be addressed in further studies. Further experiments could also address a chronic, continuous or intermittent, nicotine injection paradigm in nerve injured animals, because this would best mimic a possible clinical intervention to treat chronic pain disorders with nAChR agonists. In this context, the best suitable dose to treat neuropathic pain might become an interesting aspect. A dose that manages to elevate

withdrawal latencies only back to basal levels in nerve injured animals might also have limited negative side effects. This may then translate to the human clinical condition.

5. Supplementary Material

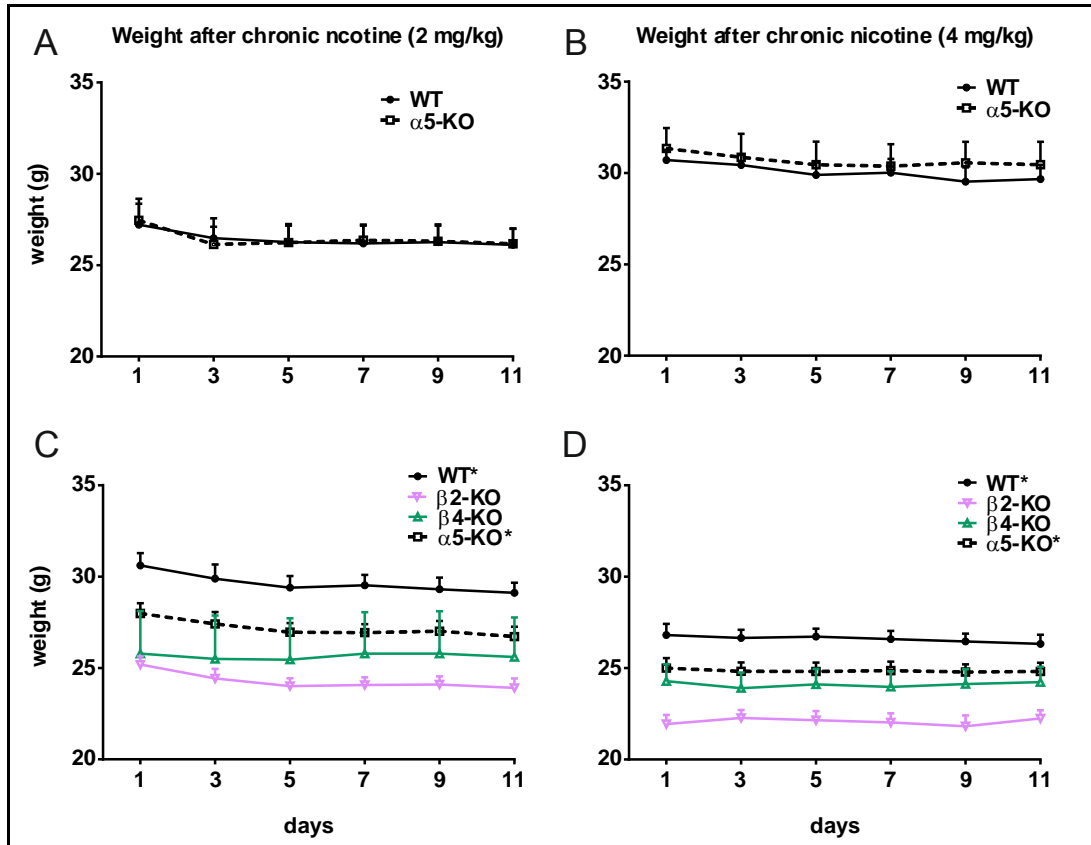


Figure 20: The weight of mice with genetic deletions of distinct nAChR subunits before and after chronic treatment with 2 or 4 mg/kg of nicotine. Animals were treated with 3 daily s.c. injections of nicotine. The weight was measured every second day before the first daily injection was performed. WT and $\alpha 5$ -KO littermates with heterozygous parents did not differ in weight at any time after treatment with 2 mg/kg (A) or 4 mg/kg (B) of nicotine. In contrast to that, animals with homozygous parents from independent nAChR KO-lines show slight deviations in their initial weight. These differences are consistently maintained throughout the time of chronic treatment with 2 mg/kg (C) and 4 mg/kg (D). Thereby WT animals have the highest weight before $\beta 4$ -KO and $\alpha 5$ -KO* mice. $\beta 2$ -KO mice always had the lowest weight of all groups tested.

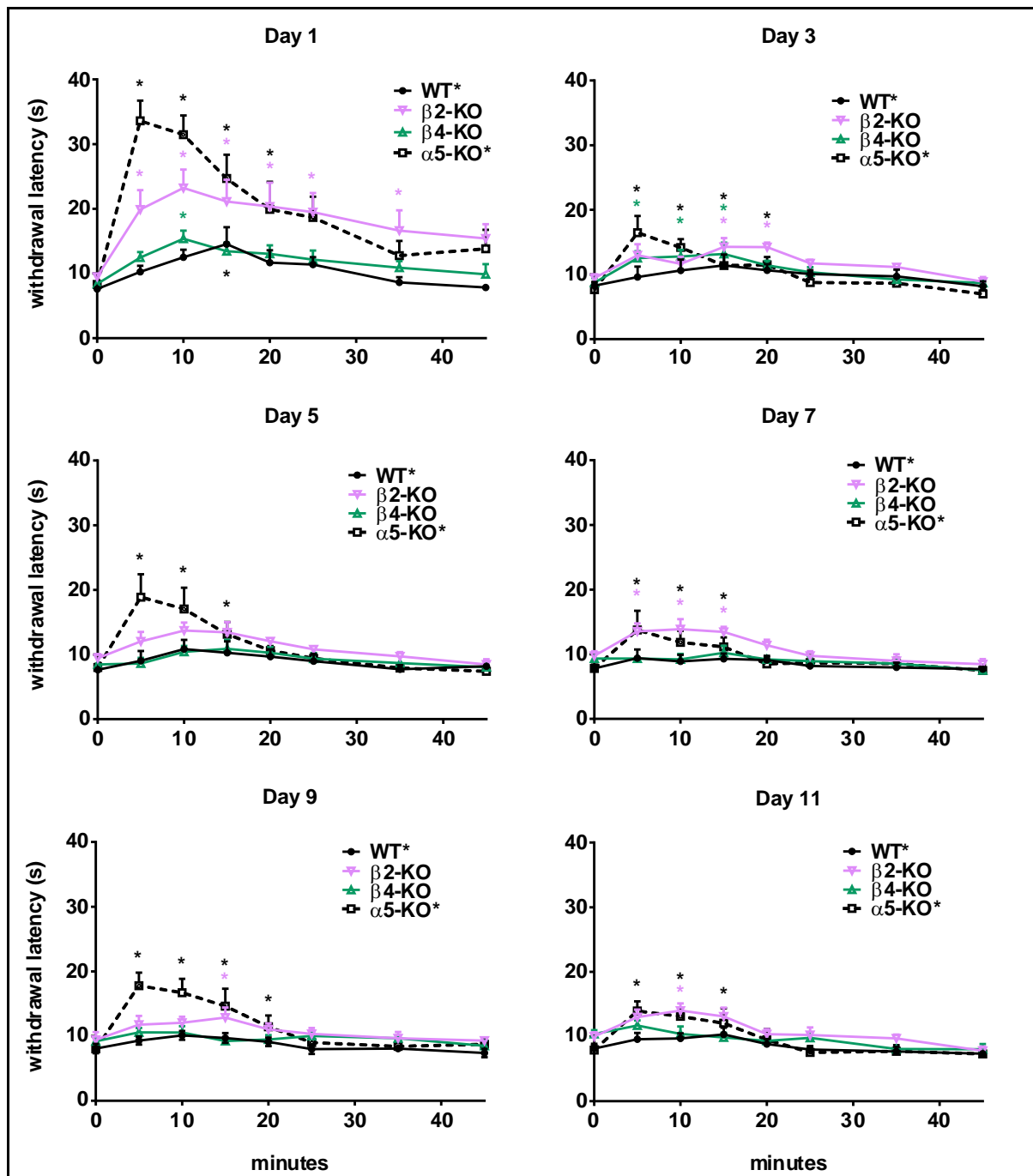


Figure 21: Tolerance development to the thermal analgesic action of nicotine after repeated treatment with 2 mg/kg nicotine. Nicotine was injected 3 times daily for 11 days. Behavioral tests were performed on the Hot Plate test each second day after the first daily injection. For the experiment animals with homozygous parents for a distinct nAChR subunit deletion (α5-KO*, β2-KO, β4-KO) were tested together with an independent line of WT* animals (they were not littermates). Only α5-KO* and β2-KO animals show an analgesic effect of nicotine injection on experimental day 11. WT* animals do only show significantly increased withdrawal latencies after the first injection and β4-KO mice lost the analgesic effect on the fifth day. Analyses refer to the baseline value (x=0) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p-values ≤ 0.05 and error bars show standard error of mean (SEM; upper black stars: KO; lower black stars: WT); n=7 - 8

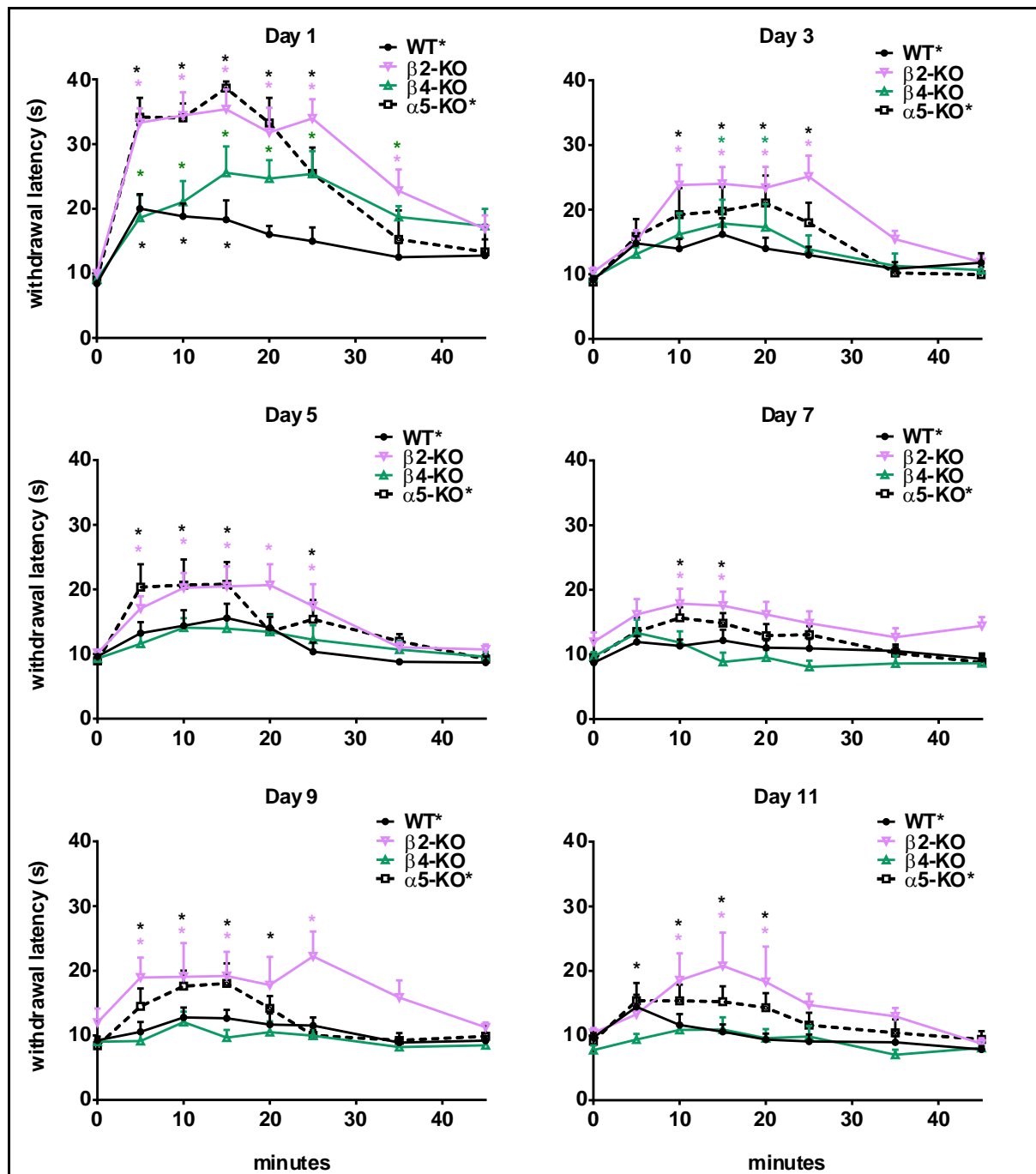


Figure 22: Tolerance development to the thermal analgesic action of nicotine after repeated treatment with 4 mg/kg nicotine. Nicotine was injected 3 times daily for 11 days. Behavioral tests were performed on the Hot Plate test each second day after the first daily injection. For this experiment only, animals with homozygous parents for a distinct nAChR subunit deletion (α5-KO*, β2-KO, β4-KO) were tested together with an independent line of WT* animals (they were not littermates). Only α5-KO* and β2-KO animals show an analgesic effect of nicotine injection on experimental day 11. WT* animals only show significantly increased withdrawal latencies after the first injection and β4-KO mice lost the analgesic effect on the fifth day. Analyses refer to the baseline value (x=0) and are performed with a Two-Way ANOVA followed by Bonferroni multiple comparisons; significance (*) was assumed for p-values ≤ 0.05 and error bars show standard error of mean (SEM; upper black stars: KO; lower black stars: WT); n=5 - 8

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CURRICULUM VITAE

Walter Johannes Beiersdorf

School education

July 1993 – June 1997	Elementary school, Marienschule, Dieburg
July 1997 – June 2003	Gymnasium/ high school, Goetheschule, Dieburg
July 2003 – June 2006	Senior high school, Alfred-Delp-Schule, Dieburg
Emphasis	Biology and history

**Basic social/ military service**

July 2006 – June 2007	Voluntary year of social service at Johanniter-Unfallhilfe, Dieburg
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Studies

October 2007 – August 2010	Bachelor: Biosciences , J.W. Goethe University, Frankfurt am Main
Practical emphasis	Animal physiology, neurobiology, cell biology, molecular biology
January – July 2010	Goethe University Frankfurt, Institute of Anatomy II – Experimental Neurobiology, Prof. Dr. Nürnberger
Bachelor thesis	“Intrinsic and extrinsic pathways of the suprachiasmatic arginin vasopressin and vasoactive intestinal polypeptide systems: A combination of immunocytochemistry and Dil-tracing technique“
Since March 2011	Master: Neurobiology, Behaviour and Cognition , University of Vienna, Vienna
Since January 2013	Medical University of Vienna, Center for Brain Research, Department of Pathobiology of the Nervous System in collaboration with the Department of Neurophysiology
Master thesis	“Alpha 5 containing nicotinic acetylcholine receptors in animal models of neuropathic pain and their contribution to nicotine induced analgesia”

Publications & other merits

September 2013	Attendance at the meeting of the Austrian Neuroscience Association (ANA) with a poster presentation of the master project
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Computational skills

GraphPad - Prism	Advanced level
Wolfram - Mathematica	Basic level

Member of Organizations

Since August 2013	Austrian Neuroscience Association
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