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„Der hetero-pentamere nikotinische Azetylcholinrezeptor
im Rückenmark von Mäusen“

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Abstract

Nicotinic acetylcholine receptor (nAChR) activation in the spinal cord (SC) may cause hyperalgesia as well as analgesia, possibly depending on the concentration of nicotinic agonist being used. RT-PCR experiments have already suggested the expression of a fairly great number of subunits and hence of types of receptors in the rat spinal cord. We confirm that mRNA coding for the subunits $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ and $\beta 4$ is present in the mouse SC. This multiplicity allows, in principle, a great number of different nAChRs. The localization of these receptors, determined by immunohistochemical staining and autoradiography of SC kryosections, showed high densities in dorsal regions of the grey matter, where pain-mediated pathways access the grey matter. In an attempt to assign the analgesic and hyperalgesic properties of nicotinic ligands to a particular type of receptor we have, in a first step, determined the subunit composition of nAChRs by [^3H]-epibatidine binding, combined with single and/or sequential immunoprecipitation (IP). We used self-generated (rabbit) antibodies directed against the cytoplasmic loop. Our affinity-purified antibodies are extensively tested with recombinant receptors expressed in HEK293 cells, with materials taken from wild type (WT) mice, and - as a negative control - with materials from knock-out (KO) mice with deletions of distinct nAChR subunit genes.

IP with the combined use of anti- $\beta 2$ plus anti- $\beta 4$ antibodies revealed a relatively low total number of 43.7 ± 1.16 fmol/mg protein binding sites in the spinal cord of WT C57Bl/6J mice. About 85 % of these receptors contain the subunits $\alpha 4$ and $\beta 2$, whereas $\alpha 3$ co-assembled with $\beta 4$ to make up for about 10% of the receptors. Nonetheless, our data indicate that $\alpha 3$ also co-assembles into receptors that contain $\beta 2$ (but not $\beta 4$). The subunits $\alpha 2$ and $\alpha 5$ were measurable but occurred at low numbers only (2.5% and 2%, respectively), whereas $\alpha 6$ -containing receptors could not be detected.

These observations were confirmed by using mice with targeted deletions of the $\beta 2$ or the $\beta 4$ subunit. In the $\beta 2$ KO, overall receptor levels dropped to 10%, matching the amount of $\beta 4$ – containing receptors in the wild-type. We did not detect any compensation for the dramatic loss of $\beta 2$ -containing receptors by any other subunit. Conversely, receptor frequency in $\beta 4$ KO animals was similar to the wild-type.

The use of mouse KO models also permits an assessment of the kinetics of [3H]-epibatidine binding to defined (yet not recombinant) receptors in a membrane preparation.

Hence, the K_d of $\alpha 3\beta 4^*$ receptors analyzed in the SC of animals lacking $\beta 2$ (160 ± 24 pmol) was three times higher than in WT animals (53.7 ± 6 pmol), where most of the receptors are composed of $\alpha 4$ and $\beta 2$.

Overall, our observations show a fairly heterogeneous entity of nAChRs in the SC for which to find ligands that specifically mediate anti-nociception will be a demanding task.

Zusammenfassung

Aktivierung des nikotinischen Azetylcholinrezeptors (nAChR) im Rückenmark (RM) kann, möglicherweise abhängig von der Konzentration des nikotinischen Agonisten, sowohl Analgesie als auch Hyperalgesie auslösen. RT-PCR Experimente haben bereits die Expression einer relativ großen Anzahl an Untereinheiten, und folglich auch Rezeptor – Subtypen, im Rückenmark von Ratten nahegelegt. Wir konnten im Rückenmark der Maus die Anwesenheit von mRNA's bestätigen, die für die Untereinheiten $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ und $\beta 4$ kodieren. Diese Diversität erlaubt prinzipiell eine große Anzahl an unterschiedlichen nAChR. Die Lokalisierung dieser Rezeptoren mit Hilfe von immunohistochemischer Färbung und Autoradiographie zeigte eine besonders hohe Dichte in dorsalen Bereichen der grauen Substanz, eine Region in der sensible Schmerzfasern synaptische Verbindungen mit Projektionsneuronen des Rückenmarks eingehen. Um die analgetischen und hyperalgetischen Eigenschaften nikotinischer Liganden einem bestimmten Typ von Rezeptor zuordnen zu können wurde in einem ersten Schritt die Komposition der Untereinheiten sowohl mit [^3H]-Epibatidin Bindungsexperimenten als auch mit einfacher und sequentieller Immunpräzipitation (IP) bestimmt. Die in dieser Studie verwendeten selbst hergestellten polyklonalen (Hasen-) Antikörper sind gegen die zytoplasmatische Schleife gerichtet und wurden ausgiebig an in HEK293 Zellen exprimierten, rekombinanten Rezeptoren und Nervengewebe von Wild-Typ (WT) und – als negative Kontrolle - Knock-Out (KO) Mäusen getestet.

IP mit einer Kombination aus anti- $\beta 2$ und anti- $\beta 4$ Antikörpern im Rückenmark von C57Bl/6J Mäusen ergab eine totale Konzentration von 43.7 ± 1.16 fmol/mg an Proteinbindungsstellen. Etwa 85% dieser Rezeptoren beinhalten die Untereinheiten $\alpha 4$ und $\beta 2$, während die Assemblierung von $\alpha 3$ mit $\beta 4$ 10% aller Rezeptoren ausmachte. Des Weiteren konnten wir zeigen, dass $\alpha 3$ auch mit $\beta 2$ (in Abwesenheit von $\beta 4$) Rezeptoren in vivo ausbildet. Die Untereinheiten $\alpha 2$ und $\alpha 5$ konnten nur in sehr kleinen (2.5% und 2%) aber signifikanten Mengen nachgewiesen werden während keine $\alpha 6$ -haltigen Rezeptoren detektiert wurden.

Diese Beobachtungen wurden durch Analyse der Untereinheitskombinationen von Mäusen mit gezielter Gendelektion der $\beta 2$ und $\beta 4$ Untereinheit unterstützt. Die Gesamtmenge der Rezeptoren im Rückenmark von $\beta 2$ KO Mäusen war auf 10% und damit genau auf die Menge an $\beta 4$ – Rezeptoren im WT, reduziert. Trotz dieses dramatischen Verlustes an Rezeptoren konnten wir keinerlei Kompensation durch andere Untereinheiten feststellen. Im Gegensatz dazu war die absolute Anzahl an Rezeptoren in $\beta 4$ KO Mäusen im Vergleich zum WT praktisch unverändert.

Der Einsatz von KO – Mäusen erlaubte uns außerdem eine Berechnung der Bindungskinetik von [^3H] – Epibatidin an definierten (aber nicht rekombinanten!) Rezeptoren mit Hilfe von Membranbindung. Aus diesen Untersuchungen ergab sich für den $\alpha 3\beta 4$ – Rezeptor, analysiert in $\beta 2$ -KO Mäusen, ein K_d - Wert von 160 ± 24 pmol während die Affinität des $\alpha 4\beta 2$ – Rezeptor (vorherrschend in WT-Mäusen) mit einem K_d – Wert von 53.7 ± 6 pmol dreimal so hoch war.

Insgesamt gesehen zeigen unsere Beobachtungen, dass das Rückenmark in Bezug auf den hetero-pentameren nAChR einen sehr heterogenen Bereich darstellt was die Suche nach einem rein analgetisch wirkenden Liganden zu einer anspruchsvollen Aufgabe macht.

1. Introduction

1.1 The nicotinic acetylcholine receptor

The acetylcholine receptor is a pentameric membrane protein that triggers different events when bound by the endogenous neurotransmitter acetylcholine. There are two main classes of acetylcholine receptors: One of them features the muscarinic acetylcholine receptor, a G-protein coupled metabotropic receptor which has been named for its property of being activated by muscarine, a compound that can be found in certain mushrooms. The other class is represented by the nicotinic acetylcholine receptor (nAChR), a ligand-gated ion-channel, which belongs to the superfamily of Cys-loop receptors (as does the GABA-, Serotonin- and Glycinreceptor) and is, among other ligands, opened by the tobacco alkaloid nicotine. As the ionotropic nAChR is a cationic channel which can conduct K^+ , Na^+ and Ca^{2+} , its action at the neuronal synapse is excitatory. Although widely spread throughout the nervous system of mammals, nAChRs can also be found in different non-neuronal cells like endothelial cells (Macklin et al., 1998) or various cells of the immune system, lungs and digestive system (Wessler et al., 2003) where they are acting by regulating signalling mechanisms (Albuquerque et al., 2009). After the discovery of the acetylcholine receptor by Loewi and Dale in 1914, investigation of the nAChR was given further insight after its first isolation and crystallization from the electric organ (which is used to stun prey and which possesses a near crystalline density of nAChR) of *Torpedo* (Kistler and Stroud, 1981). nAChRs are pentamers as they are composed of five subunits which are spanning through the plasma membrane of a cell, closing around a central pore (Fig. 1). With an extracellular N- and C-terminus, each subunit is spanning four times through the plasma-membrane, resulting in four trans-membrane domains (TM1 – TM4) with one extracellular and two intracellular loops (Fowler et al., 2008). By binding of two ligand molecules a conformational change opens the channel pore enabling ion flow through the plasma-membrane.

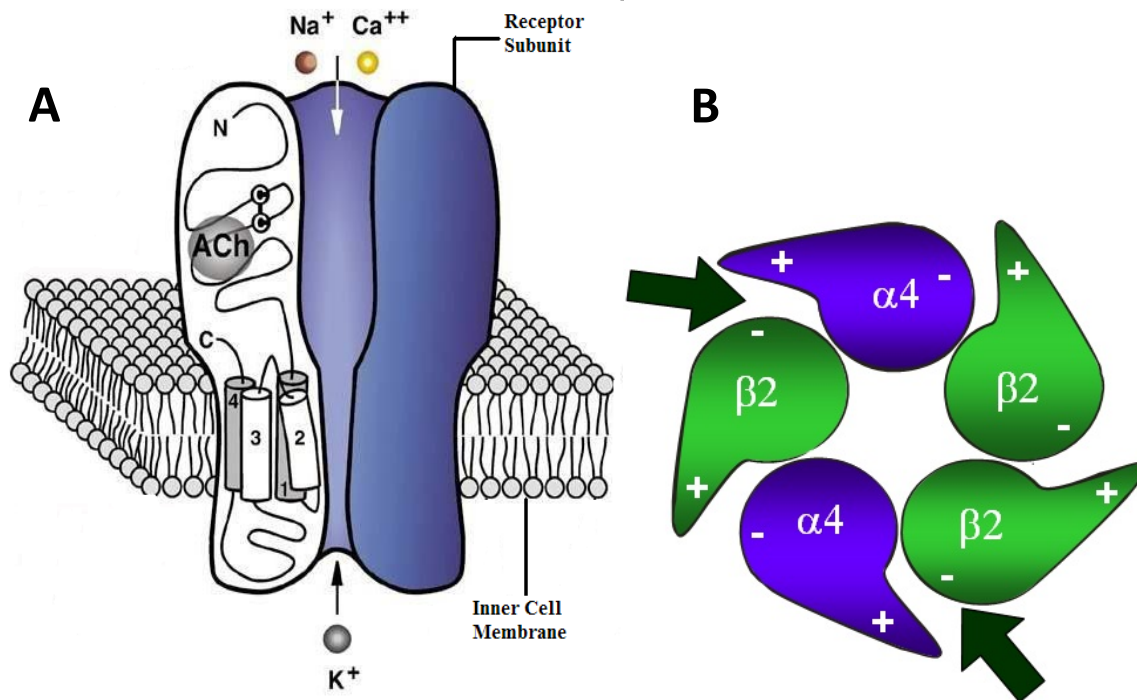


Figure 1: Structure of the hetero-pentameric nicotinic acetylcholine receptor: A shows how a subunit spans through the membrane by crossing it four times resulting in two intracellular and one extracellular loop. A cysteine pair close to the N-terminus of an alpha subunit creates the primary face of the binding pocket for nicotinic agonists. In hetero-pentameric receptors the β -subunits $\beta 2$ and $\beta 4$, which lack this cysteine pair, create the complementary face of the binding site which is therefore located between α - and β -subunits as indicated by the arrows in B. Primary face is marked by +, the complementary face by -.

1.2 Subunit combinations of the hetero-pentameric nAChR

To date, there are 11 subunits identified which contribute to form different variants of the nAChR in neurons of mammals. Eight α -subunits: $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 9$ and $\alpha 10$ and 3 β -subunits: $\beta 2$, $\beta 3$ and $\beta 4$ (McKay et al., 2007). These subunits can either form homo-pentameric receptors by integrating five times the same α -subunit ($\alpha 7$ -homo-pentamers are the most common form in the mammalian nervous system) or hetero-pentameric receptors, which are composed of different α - ($\alpha 2$ - $\alpha 6$) and β -subunits ($\beta 2$ - $\beta 4$) (Fig. 2). The composition of the nAChR greatly influences its biophysical (Sivilotti et al., 1997; Whiteaker et al., 1998; Lewis et al., 1997) and biochemical (Jensen et al., 2005; Kristufek et al., 1999; Xiao et al., 1998; Parker et al., 1998) properties.

As a consequence to the number of subunits, hetero-pentameric nAChRs can form a multitude of different subtypes, which are, however, also limited by certain restrictions:

Every hetero-pentameric nAChRs has to be composed of α - and β - subunits. Given the oddity that the two ligand binding sites of hetero-pentameric nAChRs are located on the interface between an α - and a β -subunit, the stoichiometry is restricted to either $(\alpha)_2(\beta)_3$ (Deneris et al., 1991; Sargent, 1993) or $(\alpha)_3(\beta)_2$. Additionally, the subunits $\alpha 5$ and $\beta 3$ are known to be 'supplementary' as they cannot participate in forming a binding site with a partner subunit and therefore can only be integrated into receptors which contain at least two further subunits (e.g. $\alpha 4\beta 2\alpha 5$, $\alpha 4\beta 2\beta 3$).

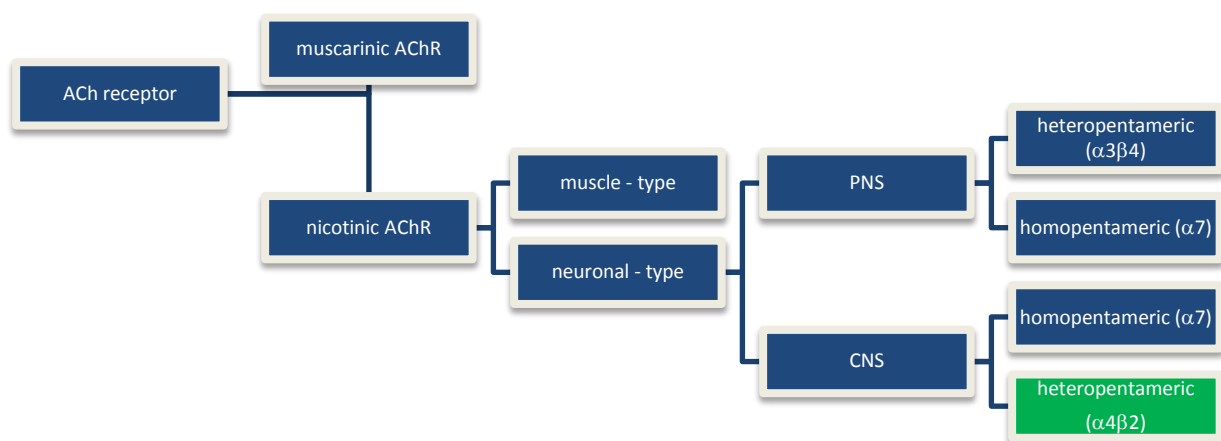


Figure 2: Pedigree of the acetylcholine-receptor: The neuronal type of the nAChR can form homopentameres (predominantly the $\alpha 7$ - homo-pentamere) and hetero-pentameres in regions of the CNS as well as the PNS. Most regions of the CNS, which includes the spinal cord, express the subtype $\alpha 4\beta 2$ (green).

Although, following the restrictions listed above, nearly all variants can be expressed in heterologous expression systems like oocytes and HEK-cells (Parker et al., 1998) it has been demonstrated that *in vivo* less hetero-pentameric nAChR subtypes are expressed than theoretically possible. This is due to the fact that specific subunits have a particular liking, whereas others rather avoid co-integration into a native functional hetero-pentameric nAChR (Gotti et al., 2006). According to this, nearly all regions of the brain are dominated by the $\alpha 4\beta 2$ subtype ('brain type') whereas $\alpha 3\beta 4$ -receptors represent the highest frequent subtype in the PNS ('ganglionic type').

1.3 Ligands for the hetero-pentameric nicotinic acetylcholine receptors

Besides the endogenous neurotransmitter acetylcholine, a vast array of different plant and animal toxins target the hetero-pentameric nAChR in CNS and PNS. Additionally as the perspective of nAChR as potential therapeutic target arose in the last few years, a multitude of different synthetic ligands emerged.

1.4 Agonists and their affinity to different hetero-pentameric nAChR subtypes

Besides nicotine, epibatidine (an alkaloid found in the skin of the Ecuadorian frog *Epipedobates tricolor*) is another well documented nicotinic agonist, which binds the hetero-pentameric nAChR with near covalent strength. Different agonists however differ in their affinity (K_d = dissociation constant) for the binding sites of hetero-pentameric nAChRs. The affinity to nicotinic agonists is an important receptor property and can be used to distinguish different subtypes of the hetero-pentameric nAChR. Accordingly many studies demonstrate that the $\alpha 4\beta 2$ 'brain-type' binds all nicotinic agonists with higher affinity than the $\alpha 3\beta 4$ 'ganglionic type' (Xiao et al., 1998; Perry et al., 2002) (e.g. in table 1). Especially the β -subunit seems to play an important role for the affinity of a binding site as $\beta 2$ -containing hetero-pentameric receptors show similar affinity to agonists as well as $\beta 4$ -containing receptors. The different K_d of $\beta 4$ - and $\beta 2$ - containing receptors to nicotinic agonists can be exploited to differentiate them in binding methods like autoradiography. As the choice of the α -subunit is only sparsely affecting the K_d of nicotinic agonists for a hetero-pentameric nAChR, different α -combinations with the same β -subunit (like $\alpha 4\beta 2$ and $\alpha 6\beta 2$) cannot be differentiated easily with nicotinic ligands.

Agonist	nAChR subtype			
	$\alpha 4\beta 2^*$	$\alpha 3\beta 4^*$	$\alpha 4\beta 2^*$	$\alpha 3\beta 4^*$
	Binding affinity K_i (nM)		Functional potency EC_{50} (μ M)	
Nicotine	0.6 - 10	290 - 476	0.35 - 5	8.1 - 110
Epibatidine	0.042 – 0.15	20 - 240	0.0045 – 0.0085	0.024 – 0.07

Table 1: Binding affinities and functional potencies of selected agonists for the $\alpha 4\beta 2$ and the $\alpha 3\beta 4$ hetero-pentameric nAChR subtypes: The difference of the binding affinities of the two nicotinic agonists nicotine and epibatidine for the subtypes $\alpha 3\beta 4^*$ and $\alpha 4\beta 2^*$ is bigger than the difference of the functional potencies. The * indicates that additional subunits might be part of the receptor. The data shown in the table is a collection of saturation binding experiments from different publications (Xiao and Kellar, 2004; Gerzanich et al., 1995; Rueter et al., 2006; Jensen et al., 2003; Marks et al., 1996).

In assays where not the binding but the opening of the channel due to binding is measured (like in electrophysiological methods), the functional potency (EC_{50} = half maximal effective concentration) of the agonist for the receptor subtype is important and not the K_d . As shown in Table 1, EC_{50} – differences of nicotinic agonist for $\beta 2$ - and $\beta 4$ -containing receptors is always smaller than differences in the binding affinity, wherefore the use of the former to distinguish subtypes of the hetero-pentameric nAChR in functional assays is limited.

As a consequence, there is a certain lack of subtype - specific ligands. The often claimed selectivity of some synthetic ligands for specific subtypes of the hetero-pentameric nAChR has often been proven to be incomplete or conflicting (Gao et al., 2010). Therefore the discrimination of distinct hetero-pentameric nAChR - subtypes in nervous tissues is better ensued by the use of subunit – specific antibodies (AB)

1.5 Antibodies (ABs) against subunits of the hetero-pentameric nAChR

As the specificity of commercially available ABs has been questioned and shown to be insufficient (Moser et al., 2007), the working group of Dr Huck and Dr Scholze decided to self-create polyclonal IgG antibodies against various subunits of the heteromeric nAChR. The characterization of these ABs is described in detail in recent publications (Scholze et al., 2011; David et al., 2010). All of the ABs are specific for an epitope provided on the

intracellular loop between the trans-membrane domains 3 and 4, as this is the most variable protein sequence of different α - and β -subunits.

1.5.1 Difficulties of immunohistochemically staining nAChRs

Although the pharmacological and biophysical profile of different hetero-pentameric nAChR subtypes is a detailed one, data about the morphology in different tissues is rather unsatisfactory. Especially immunohistochemistry (IHC) has been proven to be troublesome and there are several reasons for that. One is the difficult production of highly specific ABs against different subunits of the hetero-pentameric nAChR, which is of course the basis for reliable IHC. In contrast to many other proteins, receptors for neurotransmitters are expressed at a rather low density in the CNS. Therefore it is difficult to separate specific immunoreactivity of antibodies from background signal (Jones and Wonnacott, 2005). As mentioned above, the epitope for subunit-specific antibodies lies intracellular, wherefore samples have to be treated with reagents which open cell membranes, which in turn elevates the danger of destroying cellular structures. Finally, the only absolute reliable control for the specificity of an antibody is to test it on tissue where the source of specific protein is knocked out. As these controls exist for various other types of neurotransmitter-controlled receptors (e.g. cannabinoid, purine and glutamate receptor) (Bridges et al., 2003; Mateos et al., 1998; Sim et al., 2004), reliable equivalents for the nAChR are missing (Jones and Wonnacott, 2005).

1.6 Knock-Out mice for the nAChR

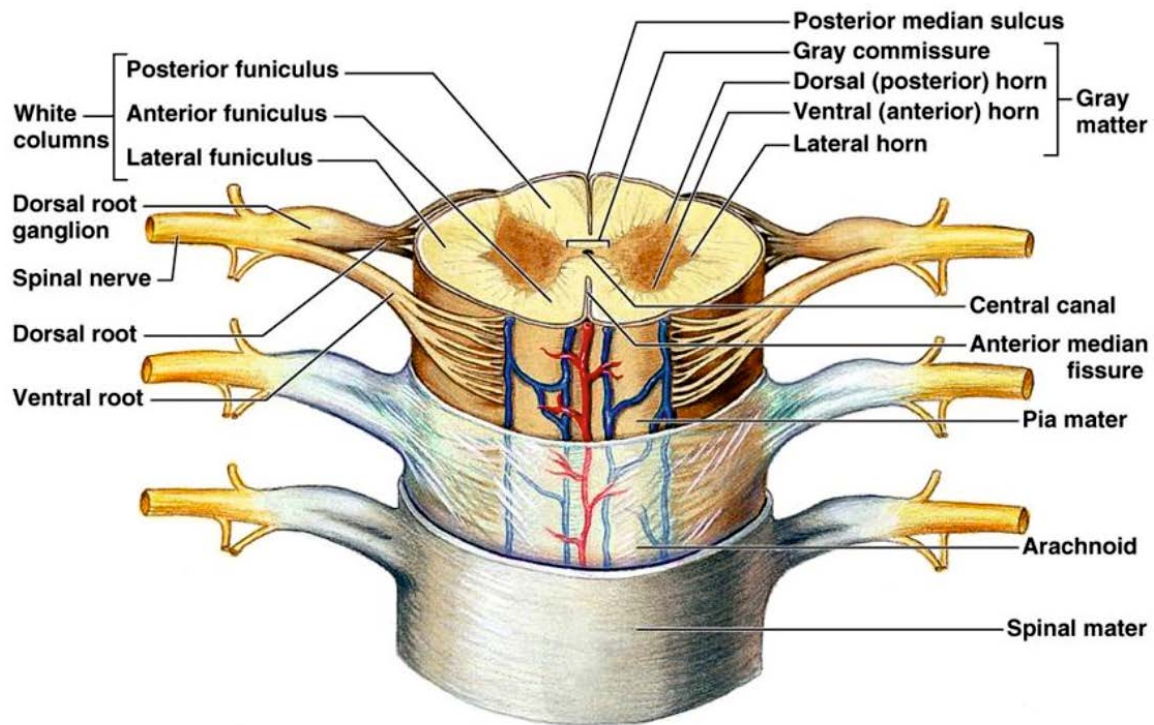
Several constitutive knock-out (KO) mice for one or more subunits of the hetero-pentameric nAChR have been bred and utilized to identify the role of specific subtypes. Apart from the obvious advantage of investigating the consequence of the loss of a specific subunit, KO-mice can also provide access to defined receptor subtypes in certain tissues. Superior cervical ganglia (SCG) of mice for example feature the hetero-pentameric nAChRs $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$ and $\alpha 3\beta 4\beta 2$ (David et al., 2010). In case of the $\alpha 5\beta 2$ double KO-mouse, SCG only contain $\alpha 3\beta 4$ -receptors, which consequently allows convenient investigation of the properties of this subtype in vivo. Surprisingly, the phenotype for mice, which lack a highly frequent subunit like $\beta 4$ or $\beta 2$, is rather mild.

1.7 The spinal cord and pain

The spinal cord is part of the CNS and processes information that is sent from the brain to the periphery (motoric output) and vice versa information recognized by sensory neurons in the periphery that is sent to the brain. The signals are conducted over spinal nerves, which enter/leave the spinal cord in the space between two vertebrae (foramen intervertebralia). The spinal nerve is a fusion of fibres, which run in opposite directions: The dorsal root fibres, which contain axons of sensory neurons, whose soma lie in the dorsal root ganglia and which connect to the dorsal horn of the butterfly shaped grey matter. Soma of motor-neurons, which are located in the ventral horn of the grey matter, send their axons over the ventral root fibres, where they join the spinal nerve (Fig. 3). Nearly all conducted signals get integrated / relayed at the grey matter of the spinal cord, which, apart from various glia cells, also provides a multitude of different neuronal cell-types (listed in table 2).

Neurons in the spinal cord	Function
<i>motor neurons</i>	Their soma is located in the ventral gray matter, axons end directly at muscles, glands or neurons of the PNS.
<i>local interneurons</i>	Their processes are restricted to small areas of the gray matter in the spinal cord. They can modulate conducted sensory input and motoric output by inhibition via GABA.
<i>projection neurons</i>	Possess long axons and span over longer distances in the spinal cord.

Table 2: Rough classification of different neurons, which can be found in the grey matter of the spinal cord.



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Figure 3: Structure of the spinal cord: A shows the principal anatomical organization of the spinal cord. Sensory input is sent via dorsal root to the grey matter and transduced over funiculi to higher procession. Motoric output is sent from the brain to motor neurons which transduct the signal over the ventral root to the periphery. (Illustration of the spinal cord retrieved from: <http://www.alamo.edu/pac/faculty/lgonzales/aplabs/2401/exercises/Ex17SpinalCord,SpinalNerves,andReflexes/Objective2SpinalCordMeninges.htm>)

Pain is the result of the activation of nociceptors, which sit at the periphery and conduct adequate stimuli over slow C-fibres and fast A δ -fibres. The signal is either relayed at the substantia gelatinosa (the most dorsal area of the grey matter) to soma of projection neurons which contra-laterally send the signal to higher structures (supra-spinal) of pain-processing (Fig. 4A), or to motor-neurons leading to direct reflex arcs in response to painful stimuli (spinal pain processing).

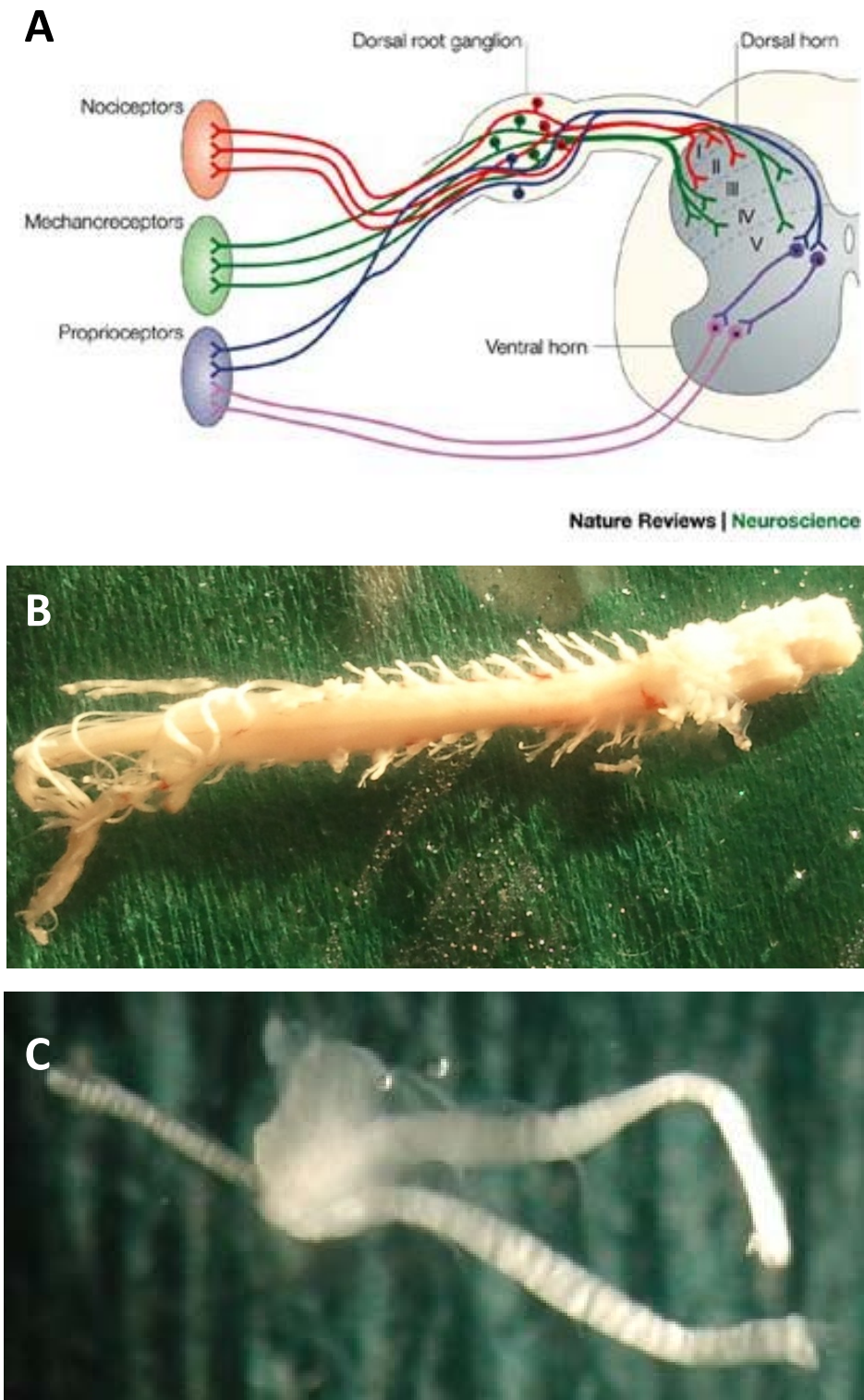


Figure 4: Sensory input from the periphery is caused by activation of nociceptors (pain, touch), mechanoreceptors (touch) and proprioceptors (positioning and movement of the body) as shown in **A**. Temperature and pain are sensed by nociceptive neurons (red) and conveyed to laminae I and II of the dorsal horn grey matter, where they contact soma of projection neurons, which send the signal to higher processions in the brain (Scheme retrieved from (Caspary and Anderson, 2003)). **B** offers a view of a complete mounted murine spinal cord with still attached dorsal root ganglia (DRG). A dissected DRG is shown in higher magnification in **C**. (Photos in B and C were taken by the author)

1.8 How do nAChRs influence pain-mediated pathways?

Nicotinic agonists are known to be effective and potent analgesics. Recent clinical trials which aimed to benefit from this effect have not met primary endpoint, whereas others show efficacy but lack the therapeutic window (Gao et al., 2010). The site of action of this analgesic effect by nicotinic agonists is divided into spinal (Aceto et al., 1986) and supra-spinal (processing of pain mediated pathways at the level of the brain) mechanisms. The former has been shown to be involved in pain processing by the circumstance that injection of nicotinic agonists into the spinal cord liquor (Fig. 5A) of rodents results in analgesia. This oddity has been supported by spinal-specific pain-tests of animals (tail-flick test, Fig. 5B) which have been given nicotine systemically. This effect could be observed as a reaction to various different nicotinic agonists, indicating, that nAChRs are involved in pain-mediated pathways at the spinal cord. The strongest effect, which showed the same efficacy like morphium with 200-fold greater potency, could be elicited with epibatidin, probably due to its high binding affinity for hetero-pentameric nAChR.

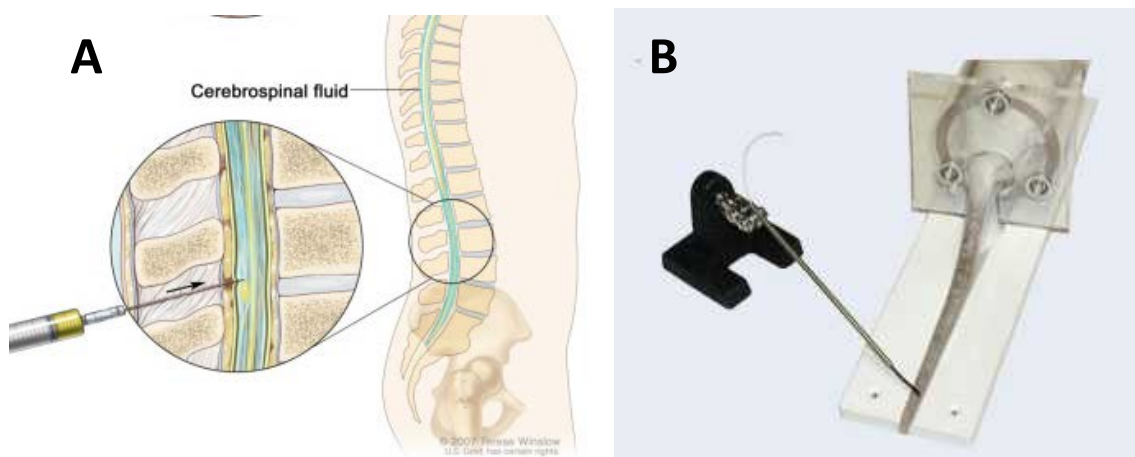


Figure 5: Evidence for the involvement of spinal nAChR in pain mediated pathways: Intrathecal injection of nicotinic agonists into the liquor of the spinal cord (A) provides insight into local effects of nAChRs. The tail-flick test (B) is believed to reflect activation of spinal reflex bows in response to thermal pain applied by a rod. The time and heat needed to elicit this reflex can be influenced by systemic and intrathecal administration of nicotine. (Source for picture A: <http://www.stfranciscare.org/saintfranciscoctors/cancercenter/nci/popUpDefinitions.aspx?id=CDR46681.xml> , for B: <http://www.labsupply.com.hk/Physiology%20&%20Pharmacology%20001.htm>)

As demonstrated with autoradiography in rats (Khan et al., 2003; Perry et al., 2002) nAChRs are distributed over the whole grey matter of the spinal cord, with their highest density in the dorsal horn. With the help of subunit specific knock-out mice, different

publications (Marubio et al., 1999; Matsumoto et al., 2007; Damaj et al., 1998a; Gao et al., 2010) were able to show that not only one specific but different subtypes of the hetero-pentameric nAChR seem to be involved in the analgetic mechanism at the level of the spinal cord.

Although the involvement of spinal nAChRs in pain-mediated pathways is (especially due to its potential clinical benefit) of interest, the complexity of the neuronal network in the spinal cord offers plenty of possibilities for further participation of the nAChR in different other mechanisms. In contrast to nearly all other nervous regions of the CNS, the characterization of the spinal cord according to its occurring hetero-pentameric nAChRs has been surprisingly neglected thus far. The attempt of a characterization in this study is aiming to support the interpretation of existing and future data from functional investigations of spinal nAChRs.

2. Material & methods

2.1 Animals

C57/B6J WT mice and mice which lack the $\beta 2$ (Picciotto et al., 1995) or the $\beta 4$ (Kedmi et al., 2004) subunit were used for experiments in this study. None of the KO animals showed phenotypic differences concerning anatomy or behavior in comparison to the wild-type. Animals were kept in thermally stable environment at 21°C in a light/dark cycle of 10h/14h with free access to food and water.

2.2 Preparation of the spinal cord

For all experiments in this study, mice were in general sacrificed on day 18 (P18, range ± 1 day) by deep anesthesia with CO₂ and subsequent decapitation. Dead animals were fixed dorsally on a polystyrene plate and cut open ventrally. The spine was exposed by removal of skin and organs followed by opening of the vertebral canal. The spinal cord was then completely removed without the dura mater (as dorsal root ganglia, which would have influenced some experiments, stick to the dura mater) under the light microscope and transferred on a plate with Ca²⁺ - free 4°C cold Ca²⁺ - free Tyrode's solution (see reagents) on ice. 5-10 spinal cords from the same genotype were collected and transferred into an eppendorf tube with 1 ml Ca²⁺ - free 4°C cold Tyrode's solution. After 1 minute centrifugation (13 000 rpm) the Tyrode's solution was removed, the tube shock-frozen in liquid nitrogen and stored at -80°C.

2.3 Membrane preparation of spinal cord tissue

In order to homogenize spinal cord tissue, the spinal cord samples (at -80°C) were thawed gently on ice to approximately 4°C. 1 ml ice-cold homogenization puffer (see reagents) was added to 4-6 spinal cords per eppendorf tube. The tissue was dissolved by two ultrasonic pulses (5 sec., 30% power) of the ultrasonic homogenizer (Bandelin Sonopuls UW2200). The samples were cooled on ice between the pulses to avoid heat-damage.

Equally important was keeping the sample volume in eppendorf tubes at 500 μ l and to correctly position the MS73 Sonotrode tip in the tube (as deep as possible in the tube without touching the rim) in order to avoid the formation of foam during pulsing. Afterwards samples were centrifuged in a Sorvall RC 6+ (20 000 g, 4°C, 30 minutes) and the supernatant discarded. 500 μ l washing puffer (see reagents) was added to each tube and homogenized with the ultrasonic device as described before. Afterwards samples were incubated on ice for 30 minutes followed by centrifugation in the Sorvall RC 6+ (20 000 g, 4°C, 30 minutes).

2.4 [³H]-Epibatidin membrane binding of hetero-pentameric nAChRs

Homogenized spinal cord tissue (after membrane preparation) was re-suspended in TRIS Puffer (50 mM TRIS/HCl, pH=7.4). An aliquot was saved on 4°C for subsequent protein determination. 100 μ l of the spinal cord suspension each was transferred into 24 polystyrene tubes (Sarstedt, 5ml, 75x12 mm $\varnothing$). Each experiment was conducted in duplicates. An increasing gradient of Triton-labelled epibatidin ([³H]-epi) was added every two tubes (8 steps: 0.01/0.02/0.05/0.1/0.2/0.5/1/2 nM). Unspecific binding was determined by adding 300 μ M nicotine 5 minutes in advance of the radioligand (although higher affine than nicotine, [³H]-epi is displaced from nAChR binding sites by the much higher concentration of nicotine) to three separate concentration steps of [³H]-epi. Each sample was filled up to 500 μ l. This volume in combination with the receptor concentration per sample was experimentally determined to show no significant depletion of the radioligand [³H]-epi. (As epibatidin binds hetero-pentameric nAChRs with extremely high affinity, there is an increased risk that the desired added total concentration of [³H]-epi for a sample is not approximately the free [³H]-epi concentration as plenty of radioligand is immediately bound by nAChRs, in which case the results for the membrane binding would be incorrect (Swillens, 1995).) All samples were incubated for 2h at room temperature followed by vacuum filtration over Whatman glass microfibre filters. The filters were soaked in 0.5% Polyethlenimine (PEI) for 30 minutes in advance to minimize unspecific binding of the radioligand on the filter. Filters were placed on the vacuum pump and after washing with ice-cold H₂O, samples were filtrated. The aim was to keep pieces of membrane, which contain [³H]-epi bound hetero-pentameric nAChRs in the filter, whereas unbound

radioligand passed through (Fig. 6). Filters were separately transferred into 18 ml Sarstedt tubes, which contain 5 ml scintillation cocktail (Rotszint Eco Plus, ROTH). This cocktail eluted the sample out of the filters and 'translates' radioactivity of [^3H]-epi direct proportionally into flashes of light, which could be detected by the liquid scintillation counter.

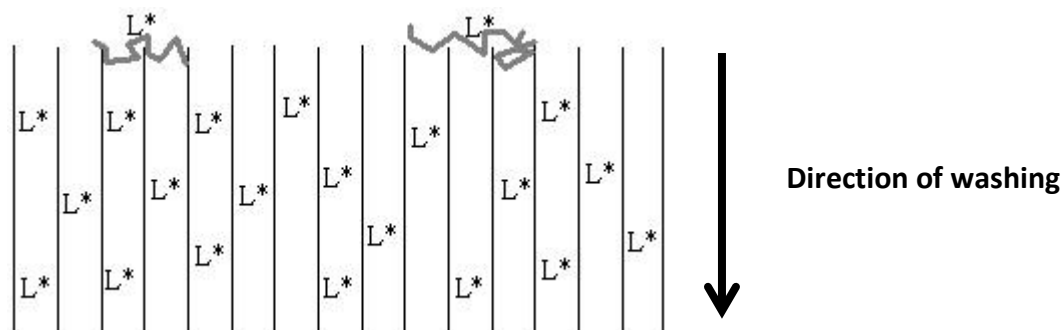


Figure 6: Radioligand, not bound to receptors on membrane pieces was washed through the filter. (Thanks to Michael Berger <http://homepage.univie.ac.at/michael.berger/>)

Values were measured in decays per minute (dpm). Amount (in fmol/mg) of radioligand bound receptors in a sample can be calculated from specific activity of the Triton labeled epibatidine and the previously determined protein concentration of the membrane suspension. All experiments were conducted in duplicates and replicated three times

2.5 Immunoprecipitation (IP) of hetero-pentameric nAChRs with subunit specific antibodies

As dissected spinal cords are free of the dura mater, they can be dissolved directly in 2% - Triton lysis puffer (see solutions). Two spinal cords per tube in 500 μl lysis puffer were pulsed for 5 seconds with the ultrasonic homogenizer (30% power) and put on ice immediately afterwards. Samples were then incubated on ice for 2h and subsequently centrifuged (16 000 g, 15 min, 4°C). The supernatant, which contained the solved nAChRs, was saved and the pellet discarded. An aliquot of 50 μl was taken at this point and stored at 4°C for later protein determination. 150 μl lysate was added per sample in combination with 7 μg subunit specific antibody in 15-20 μl 1x phosphate-buffered saline (see solutions), 1nM [^3H]-epi and incubated on a shaker o/n at 4°C. Unspecific binding was determined in the presence of an excessive (compared to [^3H]-epi) concentration of 300 μM nicotine to half of the samples.

Heat-killed *S. aureus* cells, whose membrane possesses protein A (Pansorbin, Calbiochem) were centrifuged (2300 g, 5 min 4°C) and washed twice with IP-High (see solutions), once in IP-Low (see solutions), and re-suspended in IP-Low. 20 µl of the washed Pansorbin was added to the samples followed by 2h of incubation on the shaker at 4°C, which resulted in the subunit-specific antibody binding to the receptor containing the subunit whereas the Fc part of the antibody was bound by Pansorbin, which increases the mass of the complex for centrifugation as shown in figure 7.

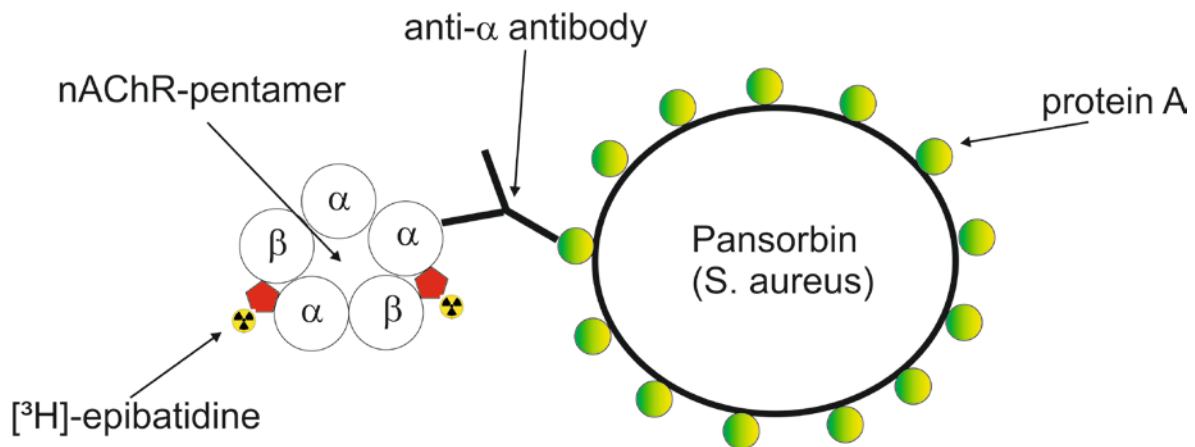


Figure 7: Complex at the end of immunoprecipitation

Following incubation with Pansorbin, samples were centrifuged at 2300 g for 5 min at 4°C, washed twice with IP-High and once with IP-Low (2300g, 1 min, 4°C) and the resulting pellets re-suspended in 200 µl 1 N NaOH. The suspensions were transferred into 5ml Sarstedt tubes containing 5 ml scintillation cocktail (Rotszint Eco Plus, ROTH) and measured in the liquid scintillation counter.

2.6 Sequential immunoprecipitation (seq. IP)

IP was performed as described above (first round of IP) until the first centrifugation step after 2h of incubation with pansorbin at 4°C, where the supernatant of each sample (which contains all of the receptors except those which were bound by the subunit-specific antibody) was not discarded, but transferred separately in new eppendorf tubes and put on ice. After that, the desired subunit-specific antibody was added to this supernatant (as 1nM [3H]-epi and 300µM nicotine, in half of the samples, was already present from the first IP). The procedure of IP (second round of IP) is then repeated as described previously.

The loss of protein due to the process of the first round of IP has been determined by comparing protein concentrations of the lysate before and after IP. Additionally, we determined how the procedure of the first IP influenced nAChRs, which were not precipitated. Protein concentration and levels of hetero-pentameric nAChR equally dropped to 80%. $\frac{1}{5}$ of the result of sequential IP was therefore added to the value to compensate the loss of receptors in the second round (e.g. if the result for $\beta 2$ -containing receptors would result in a theoretical value of 40 fmol/mg MP in sequential IP, the real value would be 50 fmol/mg MP) and be therefore able to compare results from IP to results from seq. IP. An example for sequential IP is given in figure 8.

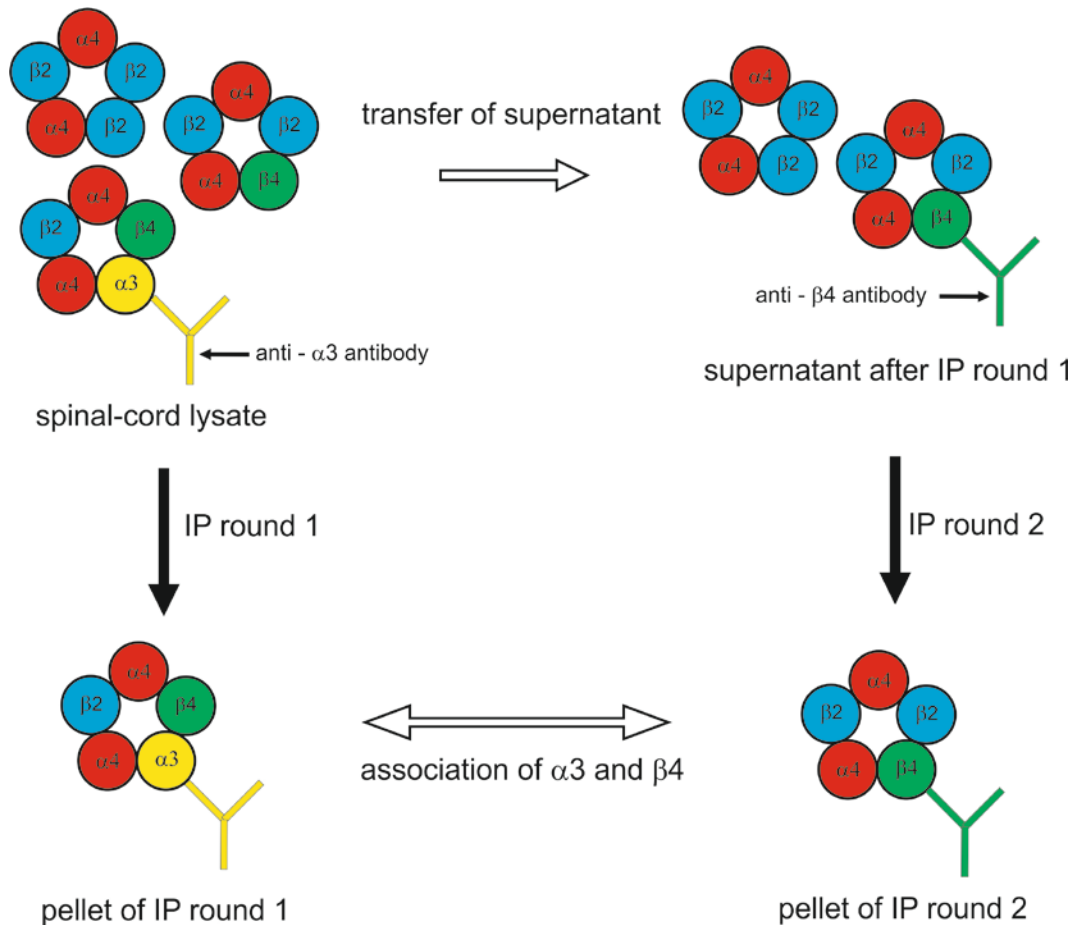


Figure 8: scenario of an experimental setup with seq. IP. This figure illustrates how association between $\alpha 3$ - and $\beta 4$ containing receptors were analyzed with the help of seq. IP. All $\alpha 3$ -containing receptors are precipitated from Spinal cord lysate with the anti – $\alpha 3$ antibody. The supernatant, devoid of all $\alpha 3$ -containing receptors is subjected to another round of IP with the anti $\beta 4$ – antibody. Being aware of the absolute levels of $\beta 4$ in the spinal cord, it is possible to calculate the amount of $\beta 4$ – containing receptors, which do and which do not contain $\alpha 3$ as well.

2.7 Statistics

All data in Immunoprecipitation and sequential immunoprecipitation were means $\pm$ SEM. Data sets were analyzed by 1way ANOVA and Bonferroni's post-test for multiple comparisons. Curves in membrane binding experiments were fitted to a hyperbolic curve based on a one-site binding model as described in (David et al., 2010). Scatchard plot (Rosenthal equation) was performed on binding curves to visualize one-site binding.

2.8 Preparation of spinal cord samples for cryo-sectioning

Spinal cords from P18 (± 1 day) mice were dissected as described above. To analyze different levels of the spinal cord it was cut into three parts (cervical, thoracic and lumbar), while still residing in the spinal canal. Spinal nerve tissue above the most cervical rip was designated as cervical, between this rip and the most caudal rip as thoracic and beyond that as lumbar. Segments were further dissected into approximately 1 cm apiece and then transferred separately to eppendorf tubes with 4% paraformaldehyde (PFA) and fixed for 12h. To avoid crystallization artifacts, samples were first transferred into 1xPBS with 10% Sucrose o/n and then into 1xPBS with 20% Sucrose o/n. This dehydration process was followed by shock-freezing in -50°C isopentan (2-methylbutane) and storage at -80°C .

2.9 Coating of slides

500 ml H_2O were heated to 55°C and 2.5 g gelatin with 0.25 g chromalaun $\text{KCr}(\text{SO}_4)_2$ * 12 H_2O slowly added. After complete dissolution, the still warm chromalaun solution filtered and stored at -4°C . To coat, clean slides were dipped into the chromalaun solution and dried o/n. Coated slides were stored at room temperature.

2.10 Cryo-sectioning for immunohistochemistry and autoradiography

Samples were tempered from -80°C to -20°C and transported to the cryomicrotome on dry-ice. The spinal cord tissue, chromalaun covered slides, the cryomicrotome knife and

the cooling cylinder (specimen holder) were cooled to -15°C in the cryomicrotome chamber. Single segments of the spinal cord were mounted vertically on a cooling cylinder with the help of Tissue- TEK and cut into 16 µm thick slices, which were, directly from the blade, thaw mounted on pre-cooled slides (3-4 slices per slide). The cryo-sections were either used immediately or stored at -20°C.

2.11 Autoradiography

Spinal cord sections from cervical and lumbar regions of wild-type and $\beta 2$ KO mice on slides, freshly cut on the cryomicrotome, were dried and tempered to room temperature for 5 minutes. The slices on the slides were encircled with a hydrophobic fat barrier using a Dako Pen to build an incubation room and subsequently subjected to ice-cold Tris puffer (see reagents) for 20 minutes at room temperature. This was followed by incubation of ice-cold Tris puffer containing 1 nM [3 H]-Epi for 2h at room temperature. To determine unspecific binding, 300 µM nicotine was added half of the samples. All samples were then washed twice with ice-cold Tris puffer for 5 seconds and once in aqua dest. for 2-3 seconds. Slides were then thoroughly dried with a cold fan and exposed to film in a cassette for 2 month at room temperature in complete darkness. Standard stripes for 3 H-radioactivity were co-incubated to get a measurement for the level of bound [3 H]-epi afterwards. The film was developed after the incubation and dried for several hours at room temperature. Finally the film was digitalized by scanning with an Epson 1600 at maximal high resolution.

2.12 Immunohistochemistry

Spinal cord sections were thawed and dried for 5 minutes at RT and transferred to a water-filled moist chamber. Sections were encircled with a hydrophobic fat pen (Dako pen) to create an incubation space and washed three times with 1xPBS, incubated with 0.2% Triton in 1xPBS (20 min, RT) to permeable cell membranes (as the epitope for antibodies against hetero-pentameric nAChR subunits is located intracellular) and blocked with 0.2% Triton and 3% normal donkey serum in 1xPBS. Subsequently, the primary antibody was added and incubated o/n at 4°C. On the next day, samples were washed three times with 1xPBS, followed by incubation with the secondary antibody for 1h at RT in darkness. From

this point, samples were kept as dark as possible to avoid bleaching of the fluorochromes. Slices were washed three times with 1xPBS and, if required, DAPI was added and incubated for 10 minutes. This was followed by another washing-step with aqua dest. and mounting with ProLong gold anti-fade mounting medium (Invitrogen). Samples were dried in darkness for 10h before regarding under the confocal fluorescence microscope (Leica TCS SP5 II).

2.13 Antibodies

2.12.1 Primary antibodies

manufacturer	antigen	host	dilution	type
Millipore	Map 2 (microtubuli associated Protein 2)	chicken	1:1000	polyclonal
SySy	SYN (synaptophysin, presynaptic)	mouse	1:200	monoclonal (Cy5 – conjugated)
Covance	SMi 31 (neurofilaments)	mouse	1:1000	monoclonal
SySy	vAChT (vesicular acetylcholine transporter)	rabbit	1:200	polyclonal
Huck Lab	α3 (S2)	rabbit	9.5 µg/ml	polyclonal
Huck Lab	α4 (S26)	rabbit	2.2 µg/ml	polyclonal
Huck Lab	β2 (S19)	rabbit	40 µg/ml	polyclonal

2.12.2 Secondary antibodies

manufacturer	antigen	host	dilution	fluorochrome
Invitrogen	anti-mouse/chicken/rabbit	goat	1:200	Alexa 568
Invitrogen	anti-mouse/chicken/rabbit	goat	1:200	Alexa 488

2.14 Solutions

Ca²⁺-free Tyrode's solution pH 7.4 (adjusted with 1N NaOH)

reagent	molarity
NaCl	150 mM
KCl	4 mM
MgCl ₂	2 mM
glucose	10 mM
HEPES	10 mM

10x phosphate buffered saline (PBS): pH 6.8 (adjusted with 1N NaOH)

reagent	molarity
NaCl	140 mM
NaH ₂ PO ₄ *2H ₂ O	30 mM
Na ₂ HPO ₄	120 mM

Tris/HCl buffer: pH 7.4 (adjusted with 1M HCL)

reagent	molarity
Tris	50 mM

Homogenisation buffer: pH 7.5 (adjusted with 1N NaOH)

reagent	molarity
HEPES	10 mM
EDTA	1 mM
sucrose	300 mM
protease inhibitors (freshly added before use, stock at -20°C)	concentration
aprotinin	1 µg/ml
leupeptin	0.5 µg/ml
PMSF	1 mM
pepstatin	0.5 µg/ml

Washing buffer: pH 7.5 (adjusted with 1N NaOH)

reagent	molarity
HEPES	10 mM
EDTA	1 mM
protease inhibitors (freshly added before use, stock at -20°C)	concentration
aprotinin	1 µg/ml
leupeptin	0.5 µg/ml
PMSF	1 mM
pepstatin	0.5 µg/ml

2% - Triton lysis puffer: pH 7.5 (adjusted with 1N NaOH)

reagent	molarity
Tris	50 mM
NaCl	150 mM
Triton X-100 (Fluka)	2 %
protease inhibitors (freshly added before use)	
1 tablet Roche mini complete per 10 ml	

IP – High: pH 8.3 (adjusted with 1N NaOH)

reagent	molarity
Tris	50 mM
NaCl	600 mM
EDTA	1 mM
Triton X-100 (Fluka)	0.5 %

IP – Low: pH 8.0 (adjusted with 1N NaOH)

reagent	molarity
Tris	50 mM
NaCl	150 mM
EDTA	1 mM
Triton X-100 (Fluka)	0.2 %

3. Results

3.1 Subunit mRNA levels in the spinal cord

nAChR are not expressed homogeneously in the CNS and the receptor frequency and composition of specific subunits can vary considerably within different regions. However, the dominant backbone subunits of the CNS are considered to be $\alpha 4$ and $\beta 2$ ('brain type') in contrast to PNS ($\alpha 3$ and $\beta 4$, 'ganglionic type'). In order to determine all possible occurring subunits the mRNA levels of spinal cord tissue were screened by Gabi Koth and Petra Scholze via semi-quantitative RT-PCR (Fig. 9). They could detect transcripts coding for the subunits $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ and $\beta 4$, the strength of the signal for $\alpha 5$ and $\beta 4$ was particularly weak.

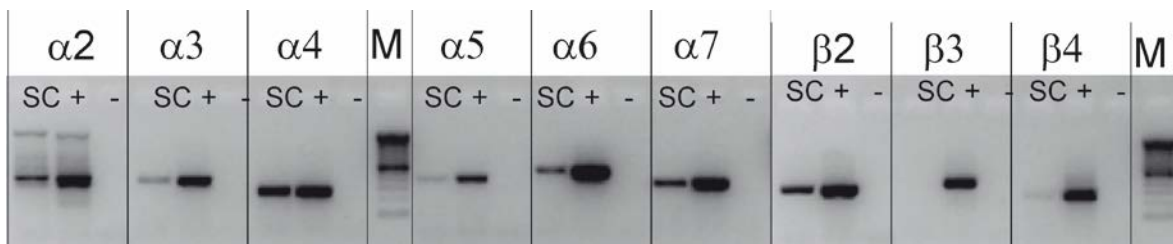


Figure 9: Identification of occurring subunit mRNA's in the spinal cord indicates subunit existence on protein level. mRNA of different nAChR subunits was reverse transcribed into cDNA with Invitrogen's ThermoScript reverse transcriptase. Transcripts for nAChR subunits were amplified in a PCR reaction using subunit-specific primers as described (Putz et al., 2008). SC: RT-PCR product of spinal cord. +: positive control (cDNA from brain or dorsal root ganglia). -: negative control in the absence of template. M: molecular weight marker (100 bp ladder, Invitrogen).

3.2 Immunoprecipitation

nAChR subtypes are defined by their variable subunit composition. Although existence of mRNA is not a guarantee for translation into functional subunits, it indicates the possibly occurring protein products. Immunoprecipitation (IP) was carried out on [3 H]-epi labeled, solubilized spinal cord nAChR with the according selective antibodies against those subunits, whose mRNA could be detected in RT-PCR. The subunit specific antibodies we used in this study are self-generated and extensively tested on heterologous systems (HEK293 cells) as well as on native material from wild-type (positive control) and KO mice (negative control) (David et al., 2010) [3 H]-epi binds to all heteropentameric nAChR, which integrate

alpha- and beta-subunits regardless of their exact composition as the binding pocket for nicotinic agonists is created between an α - and a β -subunit (Xiao et al., 1998; Xiao and Kellar, 2004). As $\beta 3$ is an accessory subunit only (and RT-PCR revealed that $\beta 3$ is not occurring in the spinal cord), every receptor contains at least a $\beta 2$, a $\beta 4$ or both subunits. As a consequence, nAChR precipitated with a conjunction of antibodies against $\beta 2$ and $\beta 4$ resemble the absolute number of receptors that can be found in the spinal cord. We exploited this rational by using the result of IP with a mix of anti- $\beta 4$ and anti- $\beta 2$ ABs together, as the 100% reference (Fig 2A). Consequently, the use of either anti – $\beta 4$ or anti – $\beta 2$ will suffice to precipitate all heteromeric nAChR in $\beta 2$ and $\beta 4$ KO animals.

3.2.1 Wild – type nAChR in the spinal cord are fairly heterogenous / What we learn from the frequency of subunits as determined by IP.

IP with a combination of anti- $\beta 2$ and anti- $\beta 4$ AB on the same sample determined the absolute number of hetero-pentameric nAChR in the spinal cord at 43.7 ± 1.16 fmol / mg membrane-protein (MP). It has been demonstrated in previous studies (Piciotto 2002; Gotti et al 2005; Turner and Kellar 2005) that $\alpha 4$ and $\beta 2$ are the dominant CNS nAChR subunits according to frequency, forming the CNS backbone receptor $\alpha 4\beta 2$ ('brain type'). I could confirm this by IP on spinal cord tissue, where the antibody against $\beta 2$ precipitated 85%, whereas $\alpha 4$ -containing receptors could be found in 87% of all heteromeric nAChR (Fig. 10A). $\alpha 3$ (18%) and $\beta 4$ (10%), the backbone subunits of the PNS, showed significantly lower levels in the wild-type spinal cord. $\alpha 2$ (2.5%) and $\alpha 5$ (2%) gave only very little contribution to the number of hetero-pentameric nAChR, but still significantly more than zero, whereas $\alpha 6$ could not be detected. The results of IP show that neither $\alpha 4$ nor $\beta 2$ reached the level of 100%. Consequently, neither $\alpha 4$ nor $\beta 2$ can be found in every nAChR in the spinal cord. However, this fact raised the question whether in any case one of the two subunits is present in every spinal cord receptor. To resolve this I set up an IP to compare the absolute number of nAChR ($\beta 2 + \beta 4$) with the amount of receptors, which could be precipitated with a combination of our antibodies against $\alpha 4$ and $\beta 2$ ($\alpha 4 + \beta 2$) (Fig. 10B). This experimental setup showed that 91% of receptors, significantly less than 100%, contain either $\alpha 4$, $\beta 2$ or both subunits in one nAChR. The fact that these two antibodies could precipitate 85 and 87% on their own demonstrates that a majority of $\alpha 4$ and $\beta 2$ are always assembled in one pentamer, whereas only a minority of nAChR can be found, where these two subunits are

separated (~5%). Furthermore, the results give evidence, that the remaining ~10% of overall receptors which neither contain an $\alpha 4$ nor a $\beta 2$ - subunit have to be composed of $\alpha 3$ and $\beta 4$.

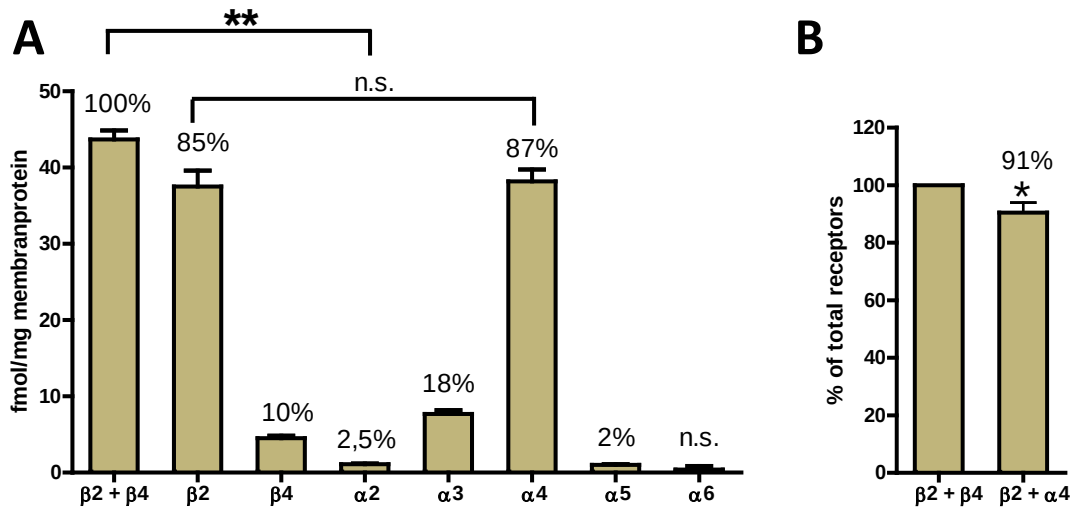


Figure 10: $\alpha 4$ and $\beta 2$ dominate nAChR-numbers, but they are not ubiquitous: Absolute numbers of nAChR, which contain specific subunits in fmol/mg membrane protein from wild-type spinal cord as determined by immunoprecipitation. Spinal cord was removed from P18 wild-type mice and nAChR from the tissue solubilized in Triton-Lysisbuffer. All nAChR were labeled with [3 H]-epi (1nM) followed by addition of a specific antibody, indicated on the abscissa. Non-specific Binding was measured by adding excessive nicotine (300 μ M) and subtracted from the total value to receive specific binding of [3 H]-epi. The number of precipitated receptors was measured in fmol and related to protein levels of the samples in mg. **A:** Comparison of different amounts of receptors precipitated with specific subunits. As $\beta 2$ and $\beta 4$ are the only β subunits in the spinal cord, the $\beta 2 + \beta 4$ column represents the amount of all hetero-pentameric (consisting of α and β - subunits) nAChR in the spinal cord at 43.7 ± 1.16 fmol / mg MP. **B:** Evidence for the existence of hetero-pentameric nAChR in the spinal cord devoid of $\alpha 4$ and $\beta 2$ (=10% $\alpha 3\beta 4^*$). Data are the mean $\pm$ S. E. M. of 6 experiments (**A**) respectively 3 experiments (**B**) *, $p < 0.05$; **, $p < 0.01$ statistical significance between columns was analyzed with 1way ANOVA and Bonferroni's post-test

Although IP revealed the absolute contribution of single subunits occurring in the spinal cord of mice, the degree to which certain subunits are associated with each other cannot be resolved with this technique.

3.2.2 Association of subunits in the wild-type spinal cord

To further resolve the hetero-pentameric nAChR of the spinal cord I additionally performed sequential IP. This technique allows analysis of nAChR that are left behind after all receptors which contain a specific subunit are removed. It therefore gives insight into whether two subunits are co-expressed in a receptor or not (Flores et al., 1992; Hernandez et al., 2004). The rational of this particular experimental setup is that you perform an IP (first

round) with a specific antibody (clearing AB). After the removal of all receptors which could be precipitated in this first round you perform a second IP (second round) with a different AB (capturing AB) on the nAChR remaining in the sample after the first round. If the capturing AB, specific for subunit 1, is not detecting significant levels of subunit 1-containing receptors, the clearing AB, specific for subunit 2, was able to remove all nAChR's which are associated with this hypothetical subunit 1. The consequence is that subunit 1 and 2 can only be found together in one receptor and never separated. Vice versa in a case where the subunit 1 specific capturing AB detects 100% of receptors (which is the same amount that you would detect in conventional IP with this AB), removal of subunit 2 – containing nAChR in the first round by the subunit 2 – specific clearing AB, didn't remove subunit 1 – containing receptors. Therefore subunit 1 and subunit 2 are never associated in the same hetero-pentameric nAChR. However, in most cases I didn't retrieve all - or - nothing results, but merely situations where two subunit were only associated to a certain percentage. To proof the reliability of the test system I performed negative control, where the clearing and capturing AB were one and the same. In this case the clearing AB was able to remove all nAChR which contained the corresponding subunit, whereas the capturing AB showed no signal. As a positive control the antibody against $\alpha 6$ (which is not expressed in the spinal cord) has been used as clearing AB. With $\beta 2 + \beta 4$ as capturing AB, I was then able to detect similar levels of signal as with $\beta 2 + \beta 4$ in conventional immunoprecipitation (data not shown). In my first test row I tried to prove the existence of non $\alpha 4$ - and $\beta 2$ – containing receptors (~10 % were predicted from the results of IP as described earlier). This could be fulfilled by sequentially precipitating receptors with $\alpha 3$ as capturing AB, which could not be precipitated with $\alpha 4$ and $\beta 2$ as clearing AB's (Fig. 11A). When I removed those nAChR that contain $\alpha 4$ and $\beta 2$ in the first round, the antibody against $\alpha 3$ could still identify 5% of total heteromeric nAChR as $\alpha 3$ -containing receptors in the second round, which are not associated with either $\alpha 4$ or $\beta 2$. As a negative control sequential immunoprecipitation with $\alpha 3$ as capturing AB was performed on samples which were cleared of all nAChR by using a combination of our antibodies against $\beta 2$ and $\beta 4$ as clearing ABs. As the latter two are precipitating all possible hetero-pentameric nAChR in the spinal cord, none should be left for further precipitation with other antibodies as turned out to be the case. As previously discussed every hetero-pentameric nAChR is comprised of α - and β subunits, therefore the receptors precipitated with $\alpha 3$ in this experiment that do not contain $\alpha 4$ and $\beta 2$ have to

contain $\beta 4$, which is the only β - subunit of the mouse spinal cord except $\beta 2$. Therefore 5 % of all nAChR in the spinal cord are $\alpha 3\beta 4$ containing receptors, which are not associated to $\alpha 4$ and $\beta 2$.

In order to resolve all associations of nAChR I did extensive analysis of $\alpha 3$ and $\beta 4$ with the help of further sequential IP and screened to which amount the $\alpha 3\beta 4$ subtype is associated with the dominant subunits $\alpha 4$ and $\beta 2$, with the minor frequent $\alpha 5$ and $\alpha 2$ and with each other. The rationale behind choosing $\alpha 3$ and $\beta 4$ as capturing ABs in this experimental setup is that the receptor number of these two subunits is in a range ($\alpha 3$ 18%, $\beta 4$ 10%) where results of sequential IP can be interpreted more clearly and provide more information as it would be the case with the huge number of $\alpha 4$ - and $\beta 2$ -, or the small number of $\alpha 5$ - and $\alpha 2$ - subunits containing receptors.

3.2.3 $\beta 4$ - associations of nAChR

In the first series of sequential IP I used $\beta 4$ as the capturing AB (Fig. 11B) on samples which were pre-cleared separately with different clearing antibodies against the subunits $\beta 2$, $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\alpha 5$. The combination of anti- $\beta 2$ and anti- $\beta 4$ and the anti- $\beta 4$ alone as clearing ABs were done as negative control because no $\beta 4$ -containing receptor should be left in those samples after the first round of sequential IP. Consequently the capturing AB against $\beta 4$ did not detect any receptors in those cases. Setting up seq. IP with anti- $\alpha 6$ as clearing AB would not remove any nAChR's from samples in the first round ($\alpha 6$ is not expressed in the spinal cord, Fig. 10A) and could be used as positive control. The number of receptors, which could be found in those samples with our AB against $\beta 4$, therefore represents the number of all $\beta 4$ - containing receptors of the spinal cord, the 100% value of this particular test series. 100% of nAChR which contain a $\beta 4$ subunit are 10 % of all heteromeric nAChR (Fig. 10A). Concerning associations of $\beta 4$ with $\alpha 3$, we could show that pre-clearing samples with $\alpha 3$ also removed all $\beta 4$ - containing receptors in the first round of sequential IP, as $\beta 4$ does not capture any nAChR in those samples in the second round. This result demonstrates that every $\beta 4$ -containing receptor in the spinal cord (10% of total receptors) is an $\alpha 3\beta 4^*$ receptor (The asterisk indicates that this composition can contain additional subunits). When I used $\alpha 2$ and $\alpha 5$ as clearing ABs, the amount of nAChR, which could be precipitated with $\beta 4$ as capturing AB was as high as in samples pre-cleared with the AB against $\alpha 6$, which does not occur in the spinal cord. This would mean that $\alpha 5$ and $\alpha 2$ are never associated with $\beta 4$. However, it may also be possible that the number of nAChR,

where $\beta 4$ are associated with $\alpha 5$ or $\alpha 2$ is below the level of the detection system, as these two subunits can only be found to a marginal degree in the spinal cord (Fig. 10A)

Concerning the issue of how the highly frequent $\alpha 4$ and $\beta 2$ are associated to the $\alpha 3\beta 4$ receptor, removal of $\alpha 4$ -containing nAChR decreased the amount of all $\beta 4$ -receptors (100% $\beta 4$ receptors = 10% of all receptors, Fig 10A) to 70% (Fig 11B) which means that the 30% of $\alpha 3\beta 4$ receptors, which are lost in the first round of sequential IP are associated with $\alpha 4$ ($\alpha 3\beta 4\alpha 4$). Similarly, removing $\beta 2$ -containing receptors in the first round of sequential immunoprecipitation left 60% of $\beta 4$ -receptors in the supernatant. Therefore the other 40% of $\alpha 3\beta 4$ receptors is associated with $\beta 2$ ($\alpha 3\beta 4\beta 2$). Of course it is also possible that $\alpha 4$ and $\beta 2$ are associated with $\alpha 3\beta 4$ to a certain degree forming the complex $\alpha 3\beta 4\alpha 4\beta 2$ subtype. As this cannot be completely resolved I deduce 5% pure $\alpha 3\beta 4$ and 5% $\alpha 3\beta 4^*$ with possible additional $\alpha 4$ or/and $\beta 2$ – subunits (= $\alpha 3\beta 4\alpha 4/\alpha 3\beta 4\beta 2/\alpha 3\beta 4\alpha 4\beta 2$).

When pinpointing at percentages of receptor combinations by bringing together results of different experimental setups, it appears more convenient to rather stay in a resolution of 5% steps than trying to assume the exact percentage. Additionally it might also be possible that the receptor composition is exposed to slight variation depending on the individual mouse. However, in agreement with experimental results, the possible receptor distribution thus far concerning $\alpha 4$, $\beta 2$, $\alpha 3$ and $\beta 4$ would be: 5% $\alpha 3\beta 4$; 5% $\alpha 3\beta 4\alpha 4/\alpha 3\beta 4\beta 2/\alpha 3\beta 4\alpha 4\beta 2$; 10% $\alpha 4\beta 2\alpha 3^*/\alpha 3\beta 2^*$; 80% $\alpha 4\beta 2^*$. The asterisks indicate a possible additional $\alpha 5$ and/or $\alpha 2$ –subunit assuming that these two subunits prefer to assemble with the $\alpha 4\beta 2^*$ combination.

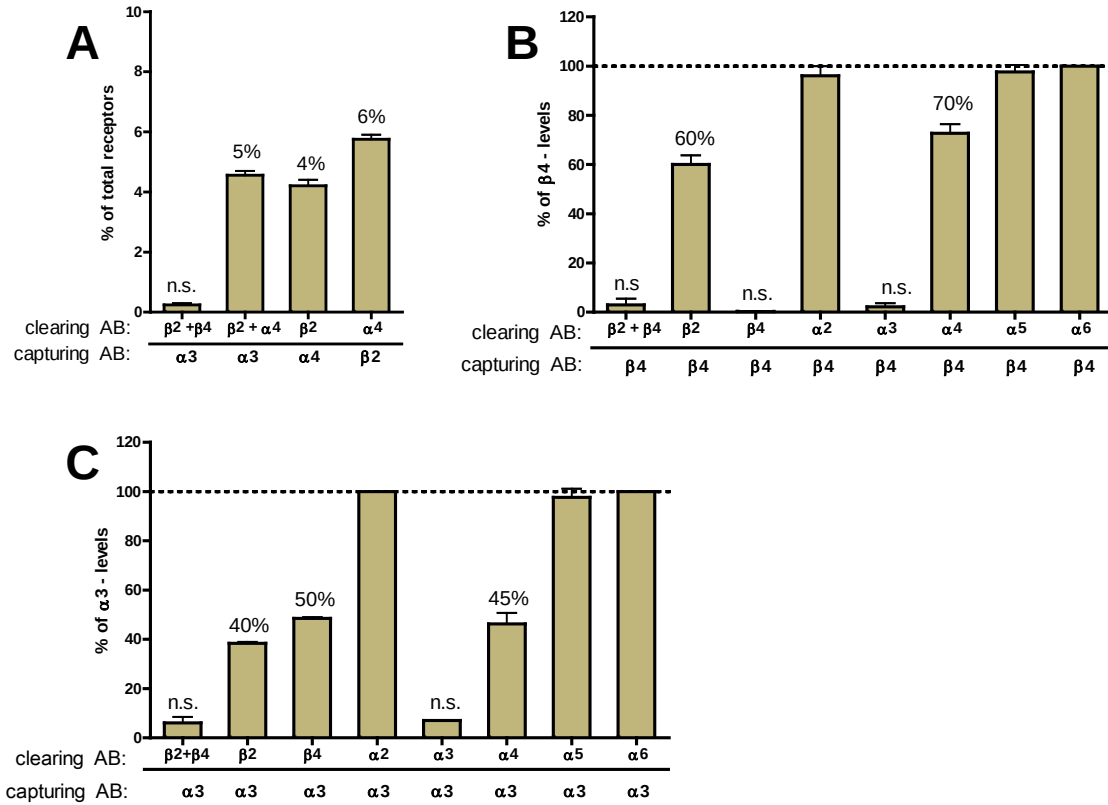


Figure 11: Sequential Immunoprecipitation reveals possible nAChR – subtypes in the wild-type spinal cord. [^3H] – epi labeled spinal cord extracts of P18 wild-type mice were immunoprecipitated with a clearing AB. Receptors, which could be precipitated in this first round were removed by centrifugation. nAChR remaining in the supernatants were precipitated in a second round with a different (capturing) AB. The bars demonstrate the amount of [^3H] – epi labeled receptors caught with the capturing AB. The subunits on the x-axis indicate the specificity of clearing and capturing AB's. $\beta 2 + \beta 4$ represents a combination of AB's against these two subunits, used together on one sample. As these two represent all possible β -subunits in the spinal cord, a combination of clearing AB against them always captures all hetero-pentameric receptors in the first round of sequential IP, serving as negative control. **A:** Evidence for existence of the $\alpha 3\beta 4$ nAChR subtype, devoid of $\alpha 4$ and $\beta 2$. Samples were either pre-cleared from all nAChR ($\beta 2 + \beta 4$) or from receptors containing $\beta 2 + \alpha 4$, $\beta 2$, or $\alpha 4$. Additionally the evidence for receptors where $\alpha 4$ and $\beta 2$ are separated is demonstrated, as clearing with one of these two left ~5% of the other one and vice versa. Bars represent either $\alpha 3$, $\alpha 4$ or $\beta 2$ containing receptors, remaining after the first round of sequential IP. The percentage of receptors is relative to the overall number of all hetero-pentameric nAChR from single IP. **B:** Associations of $\beta 4$ to all other subunits, which occur in the wild-type spinal cord. Bars represent the percentage of nAChR immunoprecipitated with our AB against $\beta 4$, relative to all $\beta 4$ – containing receptors. The amount of percentage denotes the number of receptors that are not associated to a specific clearing AB, represented by a corresponding bar. $\alpha 6$ doesn't occur in the spinal cord and designates the 100% value of all $\beta 4$ receptors in this experiment. Note that $\alpha 3$ removes all $\beta 4$ -containing receptors in the first round of sequential IP **C:** Associations of $\alpha 3$. Experimental setup and table are identical to sequential IP described in 3B. AB's against $\alpha 4$ and $\beta 2$ reduce $\alpha 3$ levels to 45% and 40%, indicating that ~half of all $\alpha 3$ containing receptors assemble to form $\alpha 4\beta 2\alpha 3$. Data are the

mean $\pm$ SEM of three independent experiments. Statistical differences between bars were indicated by 1way ANOVA and Bonferroni's post-test.

3.2.4 $\alpha 3$ - associations of nAChR

After revealing the associations of $\beta 4$ with other subunits of nAChR I set up sequential immunoprecipitation with the AB against the subunit $\alpha 3$ as the capturing AB and different clearing ABs. As a negative control, $\alpha 3$ could not detect significant levels of $\alpha 3$ -containing nAChRs in samples, which were pre-cleared with either a combination of $\beta 2$ plus $\beta 4$ or $\alpha 3$ itself. By using the AB against $\alpha 6$, which doesn't occur in the spinal cord, as the clearing AB, all nAChR were left in the sample after the first round. The amount of receptors precipitated with $\alpha 3$ as capturing AB in the second round was therefore referred to as our 100% value in this test row. 100% of receptors, which contain $\alpha 3$ are equivalent to ~20% of all nAChR (Fig. 10A).

Pre-clearing samples with antibodies against $\alpha 2$ and $\alpha 5$ in the first round of sequential IP did not affect the amount of $\alpha 3$ – containing nAChR, which could be precipitated in the second round. As we already observed in combination with the $\beta 4$ subunit, $\alpha 2$ and $\alpha 5$ show no association to $\alpha 3$ as well. That means that in spinal cord nAChR $\alpha 2$ only forms either $\alpha 2\beta 2$ or $\alpha 2\alpha 4\beta 2$, whereas $\alpha 5$, which is only an accessory subunit (introduction), can only be found in $\alpha 4\beta 2$ receptors.

As a consequence to my earlier statements, that every $\beta 4$ -containing receptor contains $\alpha 3$ and that the amount of $\alpha 3$ (~20% of total receptors) doubles the amount of $\beta 4$ (~10% of total receptors), half of all $\alpha 3$ -containing receptors are $\alpha 3\beta 4^*$. This is reflected in the result that pre-clearance with $\beta 4$ left 50% $\alpha 3$ -containing receptors in the supernatant (Fig. 11C). Furthermore, if one half of all $\alpha 3$ assembles with $\beta 4$ to form heteromeric nAChR, the other half has to be associated with $\beta 2$, the only remaining β -subunit in the spinal cord next to $\beta 4$. Subsequently, samples treated with $\beta 2$ as capturing AB left 40% $\alpha 3$ -containing receptors. We could find similar results when pre-clearing samples with our antibody against $\alpha 4$ in the first round, leaving behind 45% of receptors, which could be precipitated with $\alpha 3$ as capturing AB. However, the fact that $\alpha 4$ and $\beta 2$ are almost always co-assemble in one receptor (Fig 10B), doesn't necessarily mean that those $\alpha 3$ subunits, which are not associated with $\beta 4$ are in any case $\alpha 3\alpha 4\beta 2$ receptors. In the rare population of receptors, where $\beta 2$ is separated from $\alpha 4$ (Fig 10B), $\alpha 3$ is the only reasonable α -subunit (by means of

frequency) which can assemble with $\beta 2$ ($\alpha 2$ might be possible, but its frequency is very low, $\alpha 5$ is only an accessory subunit) forming $\alpha 3\beta 2^*$ receptors.

In consensus with our results from investigation of the wild-type nAChR with the help of IP and sequential IP I propose the following receptor distribution in the spinal cord: 5% $\alpha 3\beta 4$; 5% $\alpha 3\beta 4\alpha 4/\alpha 3\beta 4\beta 2/\alpha 3\beta 4\alpha 4\beta 2$; 10% $\alpha 4\beta 2\alpha 3/\alpha 3\beta 2$; 75% $\alpha 4\beta 2$; 2,5% $\alpha 2\beta 2/\alpha 2\alpha 4\beta 2$ and 2% $\alpha 5\alpha 4\beta 2$. Note the absence of any asterisks.

3.3 Lessons from KO-mice

Originally we utilized the $\beta 2$ - and $\beta 4$ KO mice to adequately test the specificity of our self-generated ABs, however, there are also some interesting aspects about how the distribution of nAChR-subtypes changes when a particular subunit is missing. The idea of analyzing nAChR in KO-mice with IP is originating from two general questions: 1) Is there any compensation for the loss of one subunit by others? 2) Can I observe any preferences of certain subunits to co-assemble together in one receptor?

3.3.1 $\beta 4$ KO

According to the results from wild-type animals, knocking out the $\beta 4$ subunit was expected to show less impact than knocking out $\beta 2$, as $\beta 4$ only occurs in 10% of heteropentameric receptors. Due to the lack of $\beta 4$, every heteropentameric nAChR in $\beta 4$ KO mice contains the only remaining β -subunit $\beta 2$. Therefore, Immunoprecipitation with anti- $\beta 2$ revealed the total number of receptors (42 ± 2.7 fmol / mg MP, Fig. 12A), which was not significantly different from the overall amount of nAChR in the wild-type (represented by IP against $\beta 2$ and $\beta 4$: 43.7 ± 1.16 fmol / mg MP, Fig 10A). Hence, the overall frequency of receptors is equal in these two genotypes. However, $\beta 2$ -containing receptors of animals lacking $\beta 4$ turned out to be slightly increased in comparison to wild-type animals (37.5 ± 5.14). It might therefore be possible that knocking out $\beta 4$ resulted in a slight up-regulation of $\beta 2$ for means of compensation. However, this result should be considered with care as the difference might be just below the range of where our testing system is reliable.

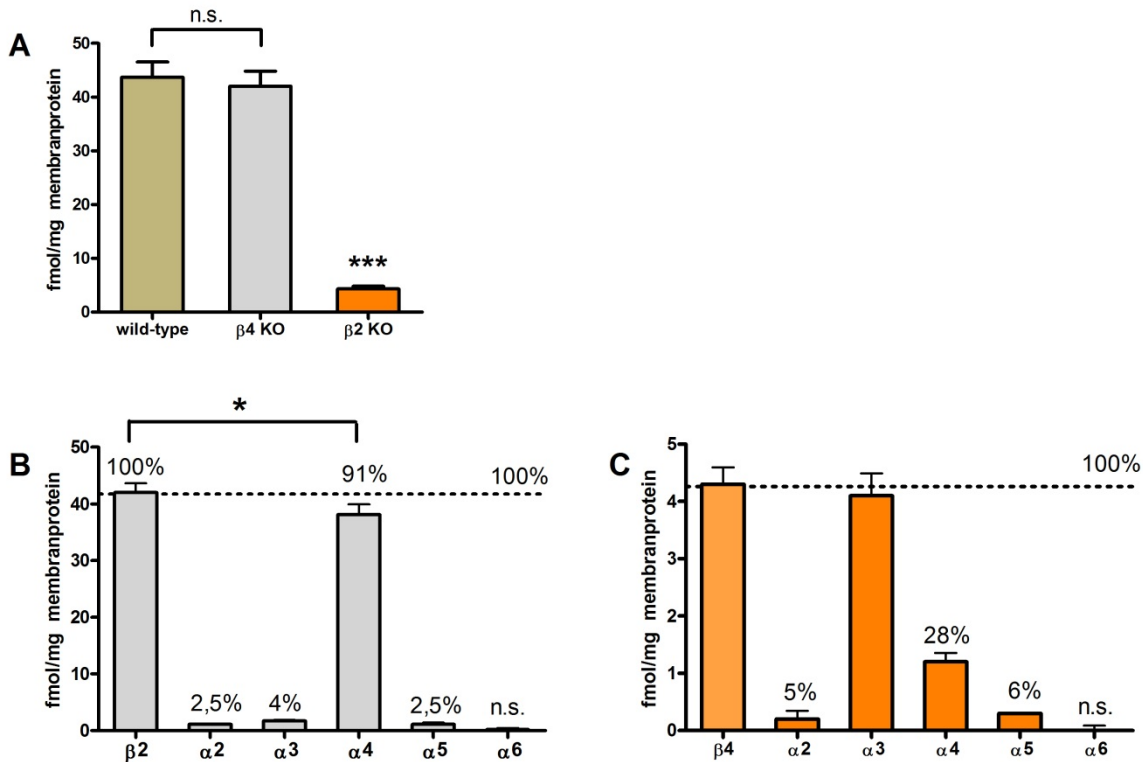


Figure 12: Number of nAChR in β4–KO is equal to wild-type receptors, but drops to 10% in β2-KO mice. Figures show the absolute numbers of nAChR, which contain specific subunits in fmol/mg membrane protein from β2 KO and β4 KO spinal cord tissue as determined by immunoprecipitation. Spinal cord was removed from mice of the two genotypes and nAChR from the tissue solubilized in Triton-Lysisuffer. All nAChR were labeled with [³H]-epi (1nM) followed by addition of a specific antibody, indicated on the abscissa. Non-specific Binding was measured by adding excessive nicotine (300μM) and subtracted from the total value to receive specific binding of [³H]-epi. The number of precipitated receptors is demonstrated in fmol and related to protein levels of the samples in mg. Data are the mean ± SEM of three independent experiments. A: Comparison of the absolute numbers of hetero-pentameric nAChRs in wild-type, β4 KO and β2 KO. Bars represent the 100% value of receptors which could be precipitated with anti-β2 + anti-β4 in wild-type, anti-β2 in β4 KO and anti-β4 in β2 KO. B: IP on spinal cord tissue from mice with distinct deletion of β4. Bars represent the amount of receptors, which contain the specific subunit marked on the x-axis. 100% value refers to receptors precipitated with β2, the only remaining subunit in this genotype. Level of α4 is significantly lower than β2, concluding that β2 also assembles to form α2β2 and α3β2 next to the major α4β2 C: IP on spinal cord tissue from β2 – KO mice. Bars represent the amount of receptors, which contain the specific subunit marked on the x-axis. 100% value refers to receptors precipitated with β4, the only remaining subunit in this genotype. 70% of receptors form the α3β4 – subtype, whereas 30% contain an additionally α4 to compose α3β4α4. Levels of α2 and α5 could only be found in traces (0.2 fmol/mg MP), which are below the resolution of our detection system in terms of significance. Statistical differences between columns were indicated by 1way ANOVA and Bonferroni's post-test. *, p < 0.05; ***, p < 0.0005

The anti- $\alpha 4$ antibody precipitated ~90% of all receptors, leaving 10% of $\beta 2$ – receptors which lack $\alpha 4$. These 10% most likely form 4% of the $\alpha 3\beta 2$ - and 2.5% of the $\alpha 2\beta 2$ - subtype. The fact that the sum of α -subunits does not quite reach the level of $\beta 2$ indicates that the detection range of IP is limited to a certain degree. However, the strong reduction of $\alpha 3$ from 18% in the wild-type to 4% in the $\beta 4$ KO underlines the preference of this subunit to assemble with $\beta 4$ rather than with $\beta 2$. The frequency of $\alpha 5$ - subunit in $\beta 4$ KO mice was unaffected by the lack of $\beta 4$ which underlines the statement from initial experiments that $\alpha 5$ has a liking to integrate with $\alpha 4\beta 2$ to form $\alpha 4\beta 2\alpha 5$ receptors. This is in keeping with several studies which also report the existence of this particular subtype in various other CNS-regions (Conroy and Berg 1998; Gerzanich et al. 1998; Mao et al. 2008). Similarly to $\alpha 5$, the level of $\alpha 2$ -containing receptors was not affected in the $\beta 4$ KO, resulting in 2.5% of total receptors, whereas $\alpha 6$ could not be detected.

Summarized, my findings in the $\beta 4$ KO indicate 90% $\alpha 4\beta 2^*$, 4% $\alpha 3\beta 2^*$ and 2.5% $\alpha 2\beta 2^*$. The asterisks indicate that all these compositions may contain an additional $\alpha 5$ subunit, although by sheer numbers, the probability for $\alpha 5$ integration into the $\alpha 4\beta 2\alpha 5$ subtype is of course high.

3.3.2 $\beta 2$ KO

IP on spinal cord of $\beta 2$ KO mice yielded striking differences of general nAChR expression as the overall number of receptors, determined by IP with AB against $\beta 4$, resulted in 4.5 ± 0.51 fmol/mg MP (Fig. 12B), revealing a tenfold reduction of heter-opentameric receptors compared to the wild-type (43.7 ± 1.16 fmol / mg MP). At a closer look it becomes obvious that the receptors are leveled to match the amount of $\beta 4$ (the only remaining β subunit of hetero-pentameric nAChR in this genotype) at ~4 fmol/mg MP. This is the same amount of $\beta 4$ as observed in the wild-type (Fig 10A) demonstrating that the expression of $\beta 4$ is not affected at all by the lack of $\beta 2$.

The amount of $\alpha 3$, the preferred α -subunit partner of $\beta 4$, adjusts to the available β -containing receptors resulting in the fact that all nAChR of $\beta 2$ KO are $\alpha 3\beta 4^*$ -receptors. The number of $\alpha 4$ containing receptors drops dramatically to ~1 fmol/mg MP (from nearly 40 fmol/mg MP in the wild-type), assembling with $\alpha 3\beta 4$ to form the $\alpha 3\beta 4\alpha 4$ subtype. This experiment proves the ability of neurons to express this composition in vivo, about which we already speculated from results of initial experiments in WT IP and sequential IP (Fig. 11B and 11C). $\alpha 5$ and $\alpha 2$ - containing receptors seem to be increased in the $\beta 2$ –KO (6% and 5%)

in comparison to 2 and 2.5% in the wild-type. However, as the percentage is relative to the absolute number of receptors in each genotype, 2% and 2.5% of these two subunits make up to ~1 fmol/mg MP in the wild-type, whereas 5 and 6% resemble ~0.2 fmol/mg MP in the $\beta 2$ -KO, which is by high chance below the range of our detection system. Therefore these results rather back up the hypothesis, that $\alpha 5$ and $\alpha 2$ have a preference for $\alpha 4\beta 2$ - than $\alpha 3\beta 4$ -containing receptors in the spinal cord. As observed in the wild-type and the $\beta 4$ KO also $\beta 2$ KO provided no detectable amounts of $\alpha 6$.

Results of IP suggest only two subtypes of hetero-pentameric nAChRs in the spinal cord of $\beta 2$ KO mice: 70% $\alpha 3\beta 4$ and 30% $\alpha 3\beta 4\alpha 4$. Keep in mind that the overall number of heteromeric nAChRs is reduced to 10% (compared to wild-type and $\beta 4$ KO) in this genotype.

3.4 Membrane Binding of [3 H]-epibatidine reveals three times higher affinity of $\alpha 3\beta 4^*$ compared to $\alpha 4\beta 2^*$ receptors

Kinetics of nAChR have been investigated in defined compositions of heterologous systems (Parker et al., 1998; Perry et al., 2002) as well as in native tissues from various regions of the nervous system (Champtiaux et al, David et al.) In our study we use the advantage of defined receptor compositions in the native spinal cord tissue of wild-type (dominant $\alpha 4\beta 2$) and $\beta 2$ KO (dominant $\alpha 3\beta 4$) mice to distinguish binding site pharmacology.

At first we determined kinetics of [3 H]-epi -binding to spinal cord membrane homogenates of wild-type animals. The binding was saturable with a dissociation constant (K_d) of ~54 pmol and a maximum binding capacity (B_{max}) of ~70 fmol/mg MP. As we showed in this study in initial experiments, ~80% of receptors in the wild-type spinal cord are composed of $\alpha 4\beta 2$. Therefore the affinity of wild-type nAChR to [3 H]-epi is represented by this subtype. The contribution of other receptor subtypes in the wild-type, if different in their kinetic properties, could not be shown as their contribution is relatively low. This was supported by analysis of the membrane binding data as the fitting of our data points by non-linear regression was better suited for one than multiple binding sites determined with Graph Pad Prism version 4.0. To visualize this we transformed the curve to Scatchard blot.

Kinetics of [3 H]epi binding to spinal cord tissue of $\beta 2$ KO mice yielded a three times higher K_d of ~160 pmol in comparison to wild-type tissue. As we could show by IP, this genotype produces $\alpha 3\beta 4^*$ only, therefore binding analysis in mice lacking $\beta 2$ represents the

affinity of this composition for [^3H]epi. B_{max} value of ~ 7.7 fmol/mg MP showed an approximately tenfold reduction of overall nAChR in the $\beta 2$ KO in contrast to wild-type, which we could also detect by IP. The difference of absolute numbers of receptors between the two techniques is a result of the different spinal cord tissue pre-processing. Membranes are prepared for membrane binding experiments, a procedure where cytosolic proteins are lost, which is not the case in IP experiments.

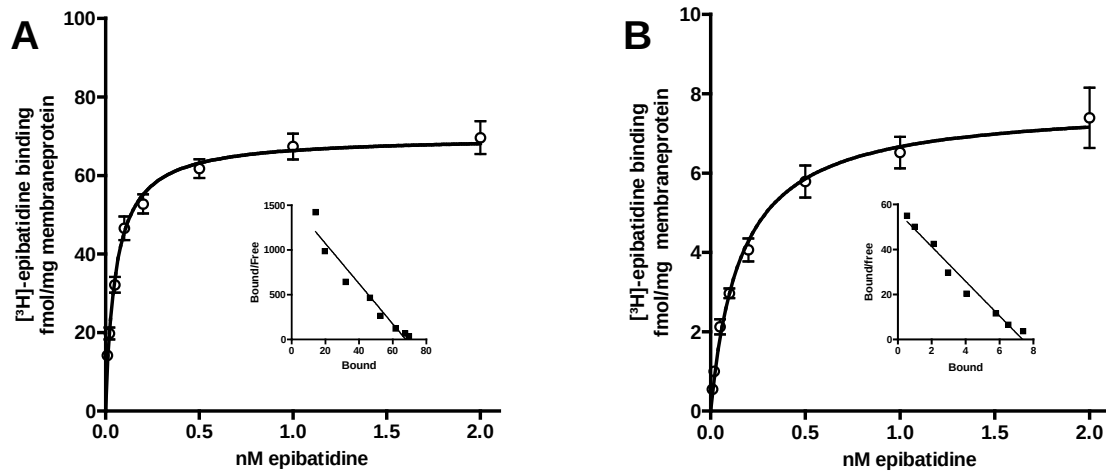


Figure 13: $\alpha 4\beta 2^*$ have a three times higher affinity to [^3H]epi than $\alpha 3\beta 4^*$ in native tissue. Membrane binding of (A) wild type ($B_{\text{max}} = 69.92 \pm 1.75$ fmol/mg MP, $K_d = 53.67 \pm 5.9$ pmol) and (B) $\beta 2$ KO ($B_{\text{max}} = 7.73 \pm 0.33$ fmol/mg MP, $K_d = 160.02 \pm 24.3$ pmol) spinal cord tissue with according Scatchard plot transformation. Wild-type tissue was homogenized by membrane preparation and incubated with 12 different [^3H]epi concentrations (0.01 – 2 nM) in sample volumes of 0.5 ml for 2h at RT and separated by centrifugation. Note the different slope between the two curves indicating the different affinity and the tenfold reduction of receptors in the $\beta 2\text{KO}$. Data are the mean $\pm$ SEM of three independent experiments.

3.5 Localization of specific nAChR subunits in the spinal cord

Besides frequency and properties of specific nAChR subtypes in the spinal cord, their distinct function and involvement in specific processes is undoubtedly of major interest. Therefore my next task was to determine localization of different receptor compositions within the wild-type spinal cord to finally be able to assign them to spinal mechanisms as there is detailed information about regional anatomical structures and their function

3.6 Autoradiography

In situ localization of nicotinic agonist binding sites in spinal cord regions was done by incubating spinal cord slices with 1nM [3 H]epi (As we learned from membrane binding experiments in this study that this concentration is sufficient to saturate all heteropentameric nAChR subtypes) and exposing tissue with bound radioligand to film. We chose the $\alpha 4\beta 2^*$ subtype dominated wild-type and $\beta 2$ KO, where all nAChR contain $\alpha 3\beta 4^*$ to distinguish localization between these two compositions. Additionally we tested for differences between lumbar and thoracic areas as distribution of receptors subtypes might vary depending on the axial position in spinal cord. Initial IP experiments on spinal cord tissue of separated lumbar and thoracic regions did not yield different results according to receptor frequency (data not shown). However, as we could find [3 H]epi binding concentrated in regions of the dorsal horn and dorsal to the central canal (Fig. 14), this was true for thoracic as well as lumbar sections. The lesser binding of nicotinic agonist to $\beta 2$ KO slices was expected given the fact that this genotype contains only a tenth of nAChR (in contrast to the wild-type). However, although definitely [3 H]epi bound receptors were visible in this genotype in comparison to negative control, the weakness of the signal prevented assignment to a specific area.

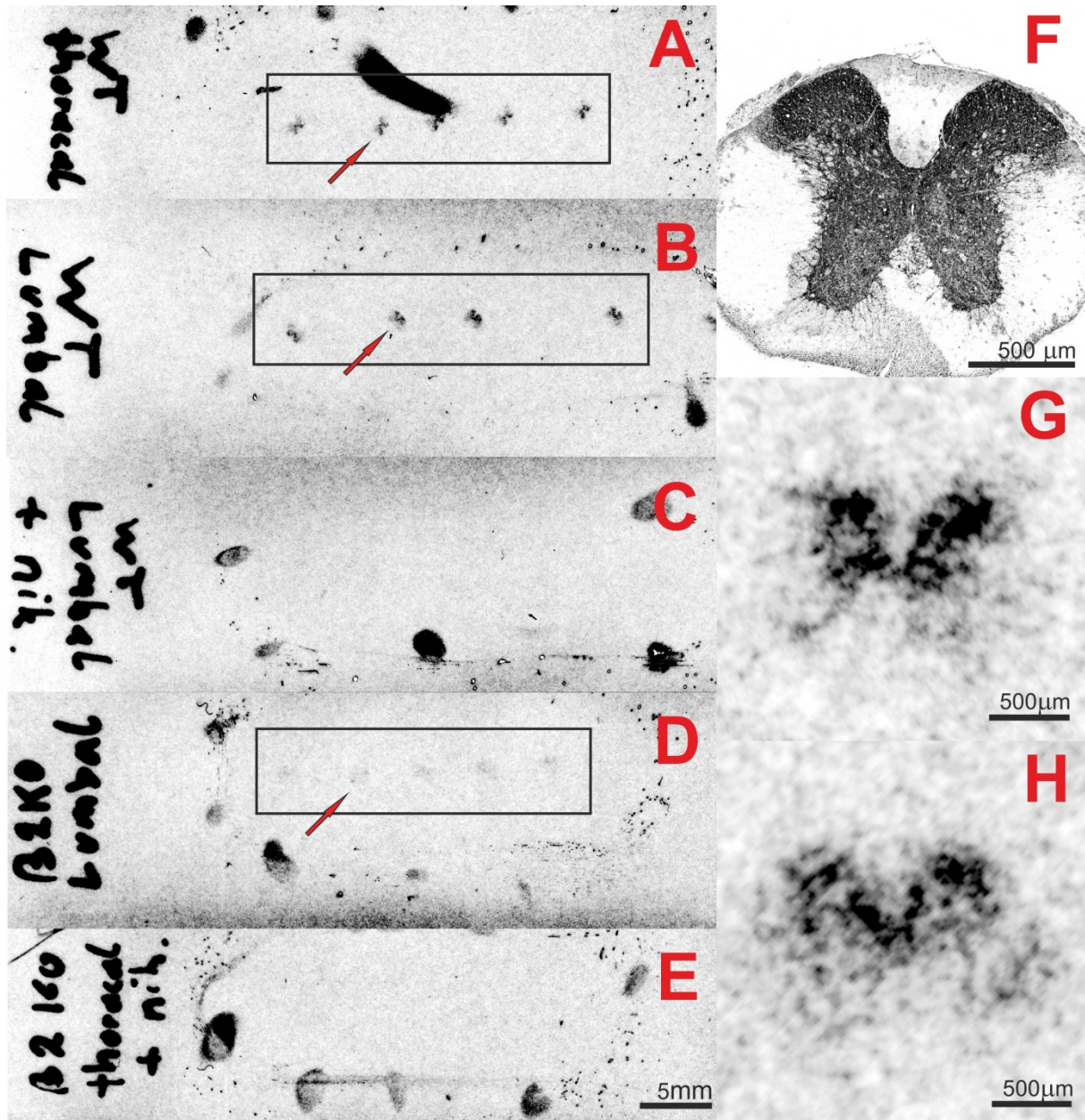


Figure 14: High density of nAChR in the dorsal horn region of the spinal cord Autoradiograms display [^3H]epi binding to wild-type thoracic (A), wild-type lumbar (B) and $\beta 2$ KO lumbar (D) spinal cord sections. 16 μm thick slices were cut on a kryotome, mounted on glass slides 4-5 apiece and incubated with 1nM [^3H]epi. Detection for unspecific binding was done by parallel addition of 300 μM nicotine and neither showed any signal in wild-type lumbar (C) nor $\beta 2$ KO thoracic (E) slices. Slices were air – dried and exposed to film for 2 month. Black rectangular squares narrow the area where single slices (tagged with red arrows) showed impact on the film. Additional slices were dyed immunocytochemically with an AB against MAP2 to visualize the entire grey matter in more detail (F). Note that magnified slices of wild-type thoracic region (G) and wild-type lumbar region (H) show stronger [^3H]epi binding in the dorsal and central area of the spinal cord. As [^3H]epi binding to $\beta 2$ KO slices was barely visible, magnifying autoradiograms of this genotype revealed no details.

3.7 Immunohistochemistry

Staining of nAChR subunits has been performed on different tissues in rat and mouse (Gahring and Rogers, 2008; Khan et al., 2003). Still, there is not much known about exact receptor targeting and assembly of distinct nAChR subunits on the various types of neurons in the spinal cord.

3.7.1 Various markers against spinal cord neurons

In order to restrict nAChR to cellular morphological structures we immunolabelled spinal cord cryosections with different antibody markers additionally to our subunit specific polyclonal AB's. The grey matter of the spinal cord could be visualized with an AB (Millipore, polyclonal) against MAP2 (Microtubule Associated Protein 2), a cross-linker of microtubule (MT) in dendrites and cell bodies of neurons. This AB revealed the classic H-shaped or butterfly outline of the grey matter (Fig. 15). Confirming this, staining with an AB (Covance, monoclonal) against pan-neuronal neurofilaments provided similar results. However, as the grey matter of the spinal cord is packed with cell bodies of various types of neurons, these two markers give no insight into detailed cellular morphology. This high density of neurons logically produces an enormous amount of synaptic connections between somas and dendrites. Co-staining with AB's against the vesicular acetylcholine transporter (vAChT, SYSY, polyclonal) and synaptophysin (SYN, SYSY, monoclonal) indicated presynaptic sites of potentially cholinergic nerve terminals. vAChT showed great distribution all over the spinal cord and could not be restricted to a defined area or lamina. However, especially the outline of motoneurons, by far the biggest sized cells in the spinal cord, in the ventral horn could be identified to contain cholinergic varicosities which showed nice co-localization with synaptophysin.

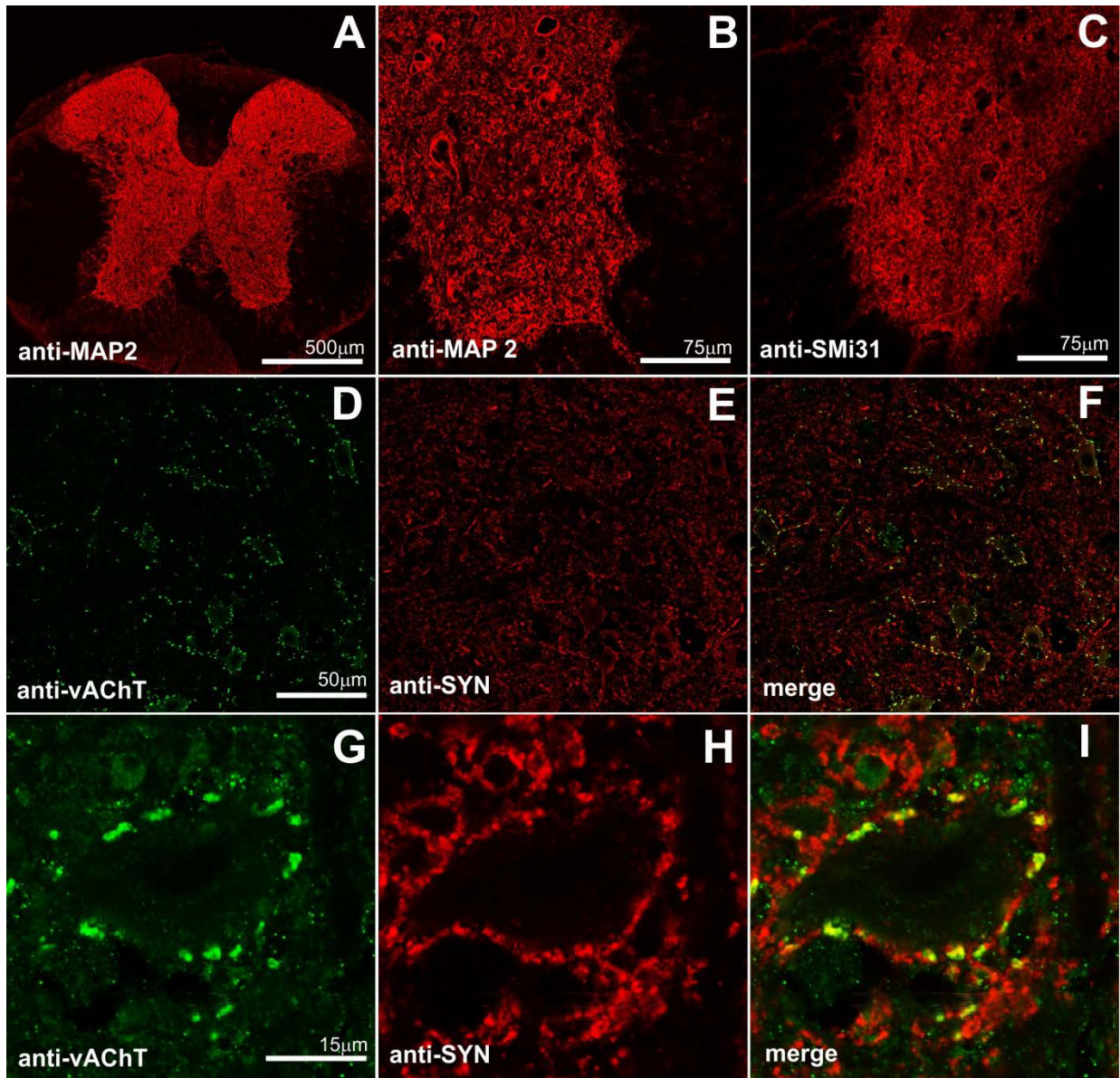


Figure 15: Different markers reveal morphology of the spinal cord and its synaptic varicosities. Photos depict immunofluorescence of 4% PFA post-fixed 16 µm thick thoracic spinal cord cryosections from P18 wild-type mice incubated with primary and secondary AB **A:** Visualization of the characteristic H-shaped grey matter of the spinal cord with anti-microtubule associated protein 2 (MAP2) AB coupled with red fluorescence secondary AB (Alexa 568). **B:** Higher magnification of MAP 2 signal from left ventral horn shows the neuropil of the grey matter. **C:** Similar signal in the ventral horn was retrieved by staining with AB against pan-neuronal neurofilaments (SMi31). **D, E, F** show co-staining of ventral region with AB against vesicular acetylcholine transporter (vAChT, green) and synaptophysin (SYN, red). Note that the characteristic outlines of motorneurons are visualized by vAChT. **G, H, I** depict a motorneuron in higher magnification co-stained with vAChT (green) and SYN (red). Note that vAChT could only be found on varicosities around the motorneuron, whereas SYN additionally marked presynaptic terminals of smaller, most likely interneurons around the motorneuron. Yellow areas in the merged picture show clear co-localization of the two markers. Photomicrographs on this Figure and in Figures 8-10 depict one representative of a minimum of three separate experiments.

3.7.2 Immunohistochemical labeling of nAChR subunits

Immunohistochemical stainings of wild-type spinal cord cryosections with our self-produced AB's against the $\alpha 3$, $\alpha 4$ and $\beta 2$ nAChR-subunits showed enhanced nAChR density in the dorsal horn, confirming our results from autoradiography. Since anti- $\alpha 4$ provided constantly strong signal in IHC, we utilized this AB as our reference for further stainings with other anti-nAChR subunit AB's. Fig. 16 shows that the frequency of $\alpha 4$ positive neurons faded from dorsal, central to ventral, whereas the density of overall cells, as marked by DAPI, was constant. In agreement with studies where IHC was performed on nAChR in various neuronal tissues (Dehkordi et al., 2007; Duncan et al., 2008; Picciotto et al., 2000) we observed that especially cytoplasm of nAChR positive neurons could be stained with our anti-nAChR AB's. Higher magnification of $\alpha 4$ positive cells in dorsal regions of the spinal cord reveals this strong intracellular staining of some small diameter interneurons (Fig. 17). Co-staining of $\alpha 4$ and synaptophysin showed only very little co-localization on the extracellular surface of these interneurons. Analysis was particularly difficult as due to their small size details of those cells were limited by the highest possible resolution of our confocal fluorescence microscope. However, we could identify interneurons with synaptophysin, which lack signal of $\alpha 4$, indicating that nAChR are not ubiquitously expressed in all interneurons. Higher magnification of ventral regions provided additionally signal from characteristically bigger sized motoneurons. Although we could show vAChT in varicosities around motoneurons in initial stainings, the $\alpha 4$ signal was interestingly restricted to cellular compartments around the nucleus. Unfortunately, DAPI was not prone to infiltrate motoneurons and mark nuclei of these big cells, although the staining of nuclei from smaller cells was flawless.

Fig. 18 shows immunolabeling for antibodies against $\alpha 4$, $\alpha 3$ and $\beta 2$ in wild-type and different KO mice. As for the remaining subunits which occur in the spinal cord according to our results from IP, anti - $\beta 4$ showed unspecific signal (data not shown), whereas no signal could be detected with AB's against the minor frequent $\alpha 2$ and $\alpha 5$ subunit. The observed strong $\alpha 4$ signal in wild-type and $\beta 4$ KO mice, goes in agreement with results from IP, which provided high frequency of $\alpha 4$ -containing nAChR in these two genotypes (85% in wild-type, 91% in $\beta 4$ KO). In mice lacking $\beta 2$, the signal was generally diminished, fitting into the scheme that $\alpha 4$ is drastically reduced in this genotype when compared to wild-type and

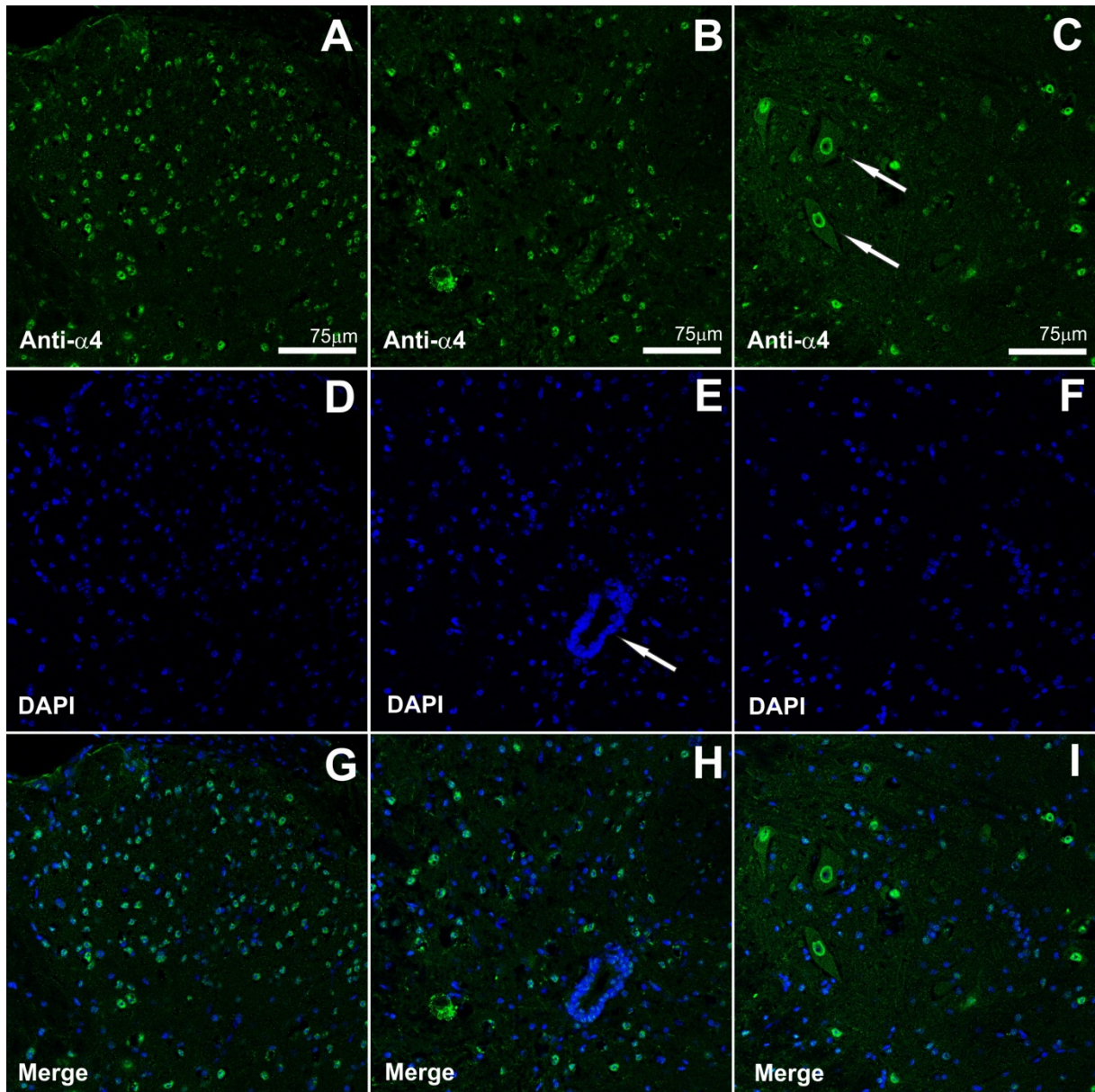


Figure 16: nAChR's density gradually decreases from dorsal to ventral. Photos depict immunofluorescence of 4% PFA post-fixed 16 μm thick thoracic spinal cord cryosections from P18 wild-type mice incubated with anti- $\alpha 4$ AB and DAPI. The figure demonstrates the higher density of $\alpha 4$ containing neurons dorsally A, D, G: Co-staining of $\alpha 4$ -containing receptors (green) and nuclei of spinal cord cells (blue) by DAPI in the dorsal horn. B, E, H: Identical staining procedure on central area of the wild-type spinal cord. The arrow on picture E depicts nuclei of epithelial cells surrounding the central canal of the spinal cord. C, F, I: anti- $\alpha 4$ / DAPI co-staining of the ventral horn. The two arrows mark two motoneurons, which can easily be distinguished from other types of spinal cord neurons. Note differences of the density of $\alpha 4$ positive neurons between pictures A, B and C, whereas frequency of DAPI stained nuclei stays constant unimpaired of whether being localized dorsally (D), centrally (E) or ventrally (F)

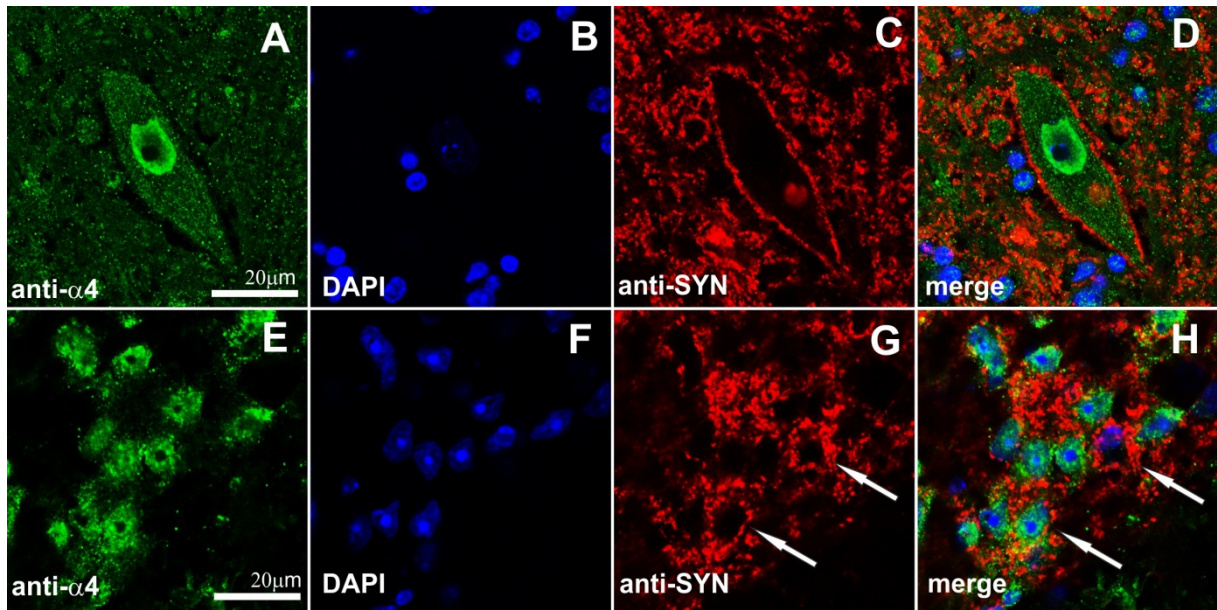


Figure 17: $\alpha 4$ – containing nAChR are found in ventral horn motorneurons and dorsal horn interneurons. Photos depict immunofluorescence of 4% PFA post-fixed 16 μ m thick thoracic spinal cord cryosections from P18 wild-type mice incubated with anti- $\alpha 4$ AB (green), synaptophysin (SYN, red) and DAPI (blue). A, B, C, D: Co-staining of a motorneuron in the ventral horn in high magnification. $\alpha 4$ signal could only be detected inside the cell perinuclearly. SYN outlines the cell, but there is no sign of $\alpha 4$ -containing nAChR co-localizing with SYN on the surface of motorneurons. E, F, G, H: Co-staining of dorsal horn interneurons. Few co-localisation of SYN and $\alpha 4$ – containing nAChR could be observed, yielding yellow fluorescence. White arrows indicate a $\alpha 4$ -positive and $\alpha 4$ -negative neuron.

$\beta 4$ KO (We tried to pick representative pictures to denote differences between genotypes). Staining with our anti- $\alpha 3$ antibody showed signal pattern similar to $\alpha 4$ with less intensity. Weak, but still specific signal could be identified in dorsal horn interneurons in the wild-type, whereas we detected no $\alpha 3$ positivity in motorneurons of ventral areas. In agreement with low levels of $\alpha 3$ subunit, as observed with IP measurements in $\beta 4$ KO and $\beta 2$ KO mice, staining showed no specific signal in those two genotypes. Unfortunately, IHC with our anti $\beta 2$ antibody gave only very sparse signal in wild-type and $\beta 4$ KO spinal cord slices.

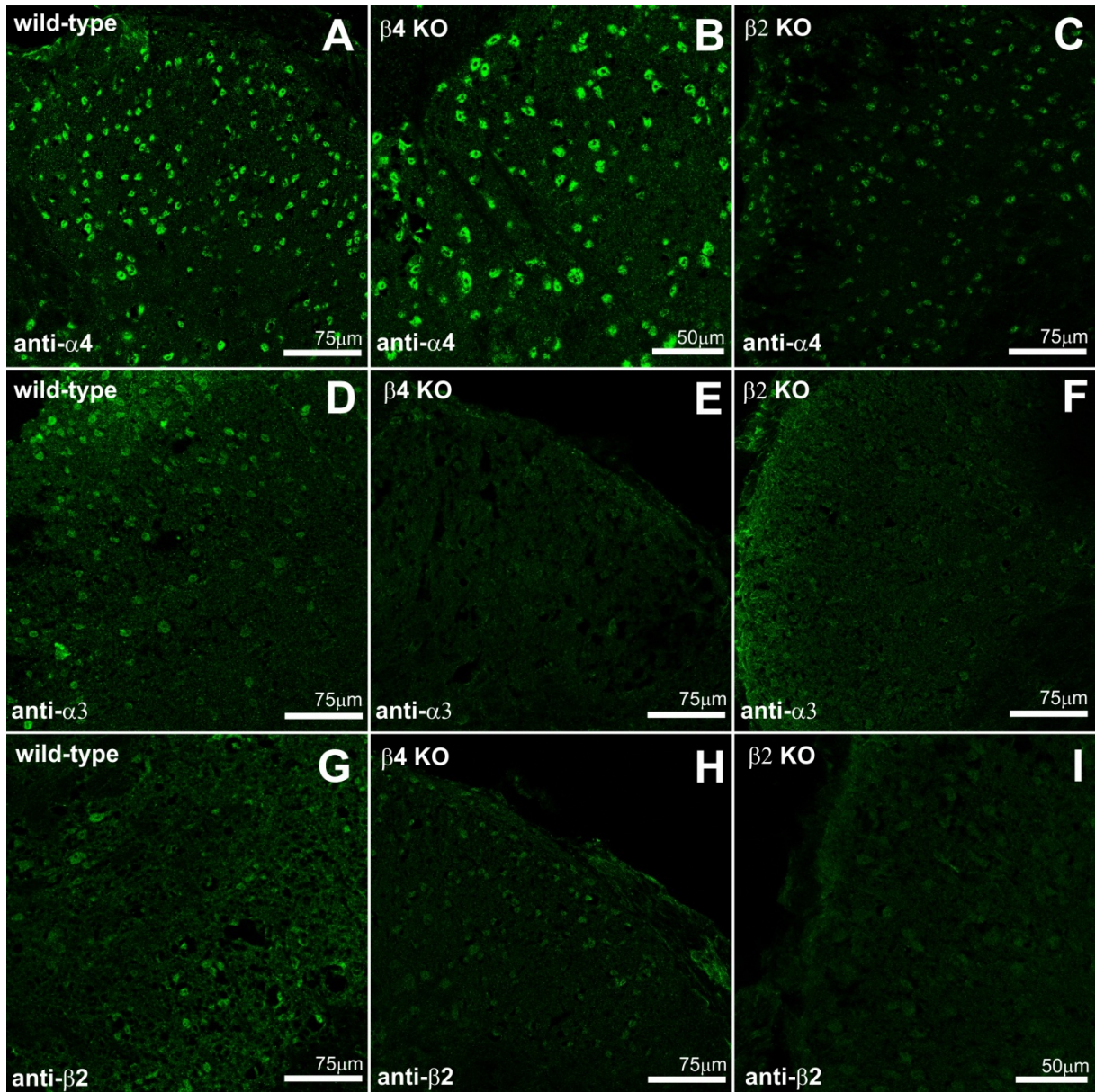


Figure 18: AB's against different nAChR subunits show similar signal pattern. Photos depict immunofluorescence of 4% PFA post-fixed 16 μ m thick thoracic spinal cord cryosections from P18 wild-type, $\beta 4$ -KO and $\beta 2$ KO mice incubated with anti-nAChR AB's (green). Photomicrographs depict Immunofluorescence labeling of anti-α4 (A), anti-α3 (D), anti-β2 (G) in wild-type dorsal horn regions. Specificity of anti-α4 AB was better than anti-β2, since anti-α4 signal was much stronger, although these two subunits showed similar frequency in wild-type IP. Weaker signal of α3 compared to α4 in the wild-type goes in agreement with IP results. α4 signal was similar to wild-type in $\beta 4$ KO mice (B) but diminished in $\beta 2$ KO (C). Anti-α3 yielded no specific fluorescence in $\beta 4$ KO (E) and $\beta 2$ KO (F), whereas specific β2 signal could be detected in $\beta 4$ KO (H), but not in $\beta 2$ KO (I).

Additionally the control of the anti-β2 AB in $\beta 2$ KO animals demonstrates that there seems to be very slight unspecific background staining of neurons, which has to be subtracted from the already weak signal in wild-type and $\beta 4$ KO.

4. Discussion

Scarce data about the occurrence of a few subtypes in the spinal cord has been provided with the help of different selective agonists and antagonists (Khan et al., 2001; Young et al., 2008) but we are the first to give a detailed overview of the exact distribution and frequency of subunits and their association to form distinct subtypes. Our studies demonstrate, that nAChR's show a great heterogeneity of subtypes in the mouse spinal cord, including combinations which contain only two subunits, as well as compositions containing three or four subunits. Fig. 19 is listing the proposed nAChR subtypes and their frequency in wild-type, $\beta 4$ KO and $\beta 2$ KO, which can be deduced from the experiments of this study. Consequently one of the main goals in the field of hetero-pentameric nAChR's is to connect specific compositions to specific functions. With this study we tried to provide data for further investigations, which thus may be able to link a specific subtype of the spinal cord to a certain task.

4.1 The choice of the β -subunit determines nAChR - pharmacology

Previous investigations demonstrated that the pharmacological profile of heteromeric nAChR is reflected primarily by the presence of either the $\beta 2$ or $\beta 4$ subunit (Turner and Kellar, 2005). Within the range of hetero-pentameric nAChR most studies differentiate $\alpha 4\beta 2^*$ and $\alpha 3\beta 4^*$ containing receptors by their functional potency to different nicotinic agonists, as $\beta 2$ has a certain liking to co integrate with $\alpha 4$, whereas $\beta 4$ prefers $\alpha 3$. In contrast to the β -subunits, good assessment for the influence of different α subunits on pharmacological properties of nAChR is not possible at the moment. In general $\beta 2$ containing nAChR's show higher affinity for nicotinic agonists compared to nAChR's which integrate $\beta 4$. The K_d for acetylcholine for example is four times higher in the $\alpha 3\beta 4^*$ subtype (Jensen et al., 2003; Xiao and Kellar, 2004) compared to $\alpha 4\beta 2^*$, meaning that in case you have both receptor subtypes in a synapse, only the $\alpha 4\beta 2$ -containing population is activated at a low concentration of neurotransmitter which reflects a possible regulatory mechanism for hetero-pentameric nAChR. Our results from the membrane binding experiments could confirm this difference of affinity between nAChR subtypes for the nicotinic agonist

epibatidine. The main difference of our experimental setup to a majority of similar investigations was the usage of native tissue. This was possible due the finding that the spinal cord of the wild-type consists of ~80% $\alpha 4\beta 2^*$, whereas mice lacking $\beta 2$ showed 100% of the $\alpha 3\beta 4^*$ subtype. Although 10% of $\alpha 3\beta 4^*$ receptors can be found in the wild-type as well, their contribution is very likely negligible due to the much greater number of $\alpha 4\beta 2$ – containing receptors. In comparison to our setup, most of the pharmacological data from nAChR subtypes has been observed in different cell lines and oocytes which were transfected with combinations of subunit mRNAs. Caveat of this technique is that there is probably a difference of nAChR's expressed in vivo in comparison to nAChR's expressed heterologously in oocytes and various cell lines due to potential different post-translational modifications and a different subcellular environment of the nAChRs. There are quite a lot of these modifications on nAChR as described in a review of Albuquerque (Albuquerque et al., 2009). However, deduced from our experiments, the binding affinity for wild-type spinal cord, representing $\alpha 4\beta 2^*$ was three times higher (~50 pM) than in $\beta 2$ KO mice, representing $\alpha 3\beta 4^*$ (~150 pM) and therefore did not much differ from findings in heterologous systems. In a previous study from our working group, consequently a K_d of 150 pM in the superior cervical ganglion (SCG) of wild-type mice, which contain a 100% $\alpha 3\beta 4^*$ nAChR could be observed (David et al., 2010), supporting the veracity of our recent findings in the spinal cord.

4.2 $\alpha 4\beta 2^*$ predominates the spinal cord

As demonstrated in results from our IP experiments, the dominance of $\alpha 4\beta 2$ and $\alpha 4\beta 2^*$ throughout various CNS regions (Flores et al., 1992; Whiting and Lindstrom, 1987; Perry et al., 2002) is consequently pertained in the spinal cord. Moreover we could show that 75 % of all nAChR of the wild-type spinal cord comprise only of these two subunits (Fig. 19A). The rest of $\alpha 4\beta 2$ containing receptors combined in traces with either an additional $\alpha 2$ ($\alpha 4\beta 2\alpha 2$ 2%), $\alpha 3$ ($\alpha 4\beta 2\alpha 3$ ~5%), $\alpha 5$ ($\alpha 4\beta 2\alpha 5$ 2%) or even possibly with $\alpha 3\beta 4$ forming $\alpha 4\beta 2\alpha 3\beta 4$. In cases where $\alpha 4\beta 2$ combinations contain just one more additional subunit (e.g. $\alpha 2$, $\alpha 3$, $\alpha 5$) one might speculate that the difference is possibly rather small as the two binding sites of e.g. the two combinations $\alpha 4\beta 2\alpha 3$ and $\alpha 4\beta 2\alpha 5$ might be the same (always

between $\alpha 4$ and $\beta 2$). A hypothetical $\alpha 4\beta 2\alpha 3\beta 4$ nAChR in contrast would definitely provide two different binding sites per receptor molecule between different $\alpha \beta$ interfaces.

Although the predominant role of $\alpha 4\beta 2$ suggests a particular liking of these two subunits to assemble together in one receptor, we also found them in separation by detecting ~5% of receptors containing $\alpha 4$ but no $\beta 2$ and another ~5% vice versa. This finding enhances the possible variation in which hetero-pentameric nAChRs can be composed of. The most prominent subtype where we could find $\beta 2$ in isolation of $\alpha 4$ in the wild-type spinal cord is $\alpha 3\beta 2$ (~5%), which has been described previously (Gotti et al., 2005) in the CNS and which we could additionally observe in $\beta 4$ KO animals. However, we showed association of $\alpha 3$ and $\beta 4$ with $\alpha 4$ and $\beta 2$ in our sequential IP experiments and therefore conclude (although at low frequency) that $\alpha 3\beta 4$ containing receptors can be associated with either $\alpha 4$ or $\beta 2$ to form $\alpha 3\beta 4\beta 2$ and $\alpha 3\beta 4\alpha 4$. We could observe the latter subtype in $\beta 2$ KO mice, corroborating that this composition can be formed in native tissue of mice.

4.3 $\alpha 3\beta 4^*$ is rare in the CNS and rare in the spinal cord

$\alpha 3\beta 4$ - containing receptors, although the dominant composition of the PNS in autonomic ganglia and the adrenal medulla, play a minor frequent role in the CNS. In the case of rodents, pure $\alpha 3\beta 4$ and $\alpha 3\beta 4^*$ (including various additional subunits) nAChR's have been discovered in rat cerebellum (Turner and Kellar, 2005), rat pineal gland (Hernandez et al., 2004), rat retina (Marriott et al., 2005) and the mouse habenulo-interpeduncular pathway (Grady et al., 2009). Tissue of the latter structure (medial habenula and interpeduncular nucleus) is providing the main contribution of $\alpha 3\beta 4^*$ receptors in the CNS of rodents (Scholze et al., submitted; (Marks et al., 2010). In this study we added the mouse spinal cord to the list of $\alpha 3\beta 4^*$ containing CNS-areas as we could detect 10% $\alpha 3\beta 4^*$ nAChR's of which 5% contain solely $\alpha 3$ and $\beta 4$ subunits ($\alpha 3\beta 4$), whereas the remaining 5% contain either an additional $\alpha 4$ ($\alpha 3\beta 4\alpha 4$), $\beta 2$ ($\alpha 3\beta 4\beta 2$) or both ($\alpha 3\beta 4\alpha 4\beta 2$). The strong preference of $\beta 4$ to assemble with $\alpha 3$ together in one receptor is shown in this study with sequential IP, where precipitating $\alpha 3$ – containing receptors with our specific anti- $\alpha 3$ AB left no $\beta 4$ – containing receptors behind.

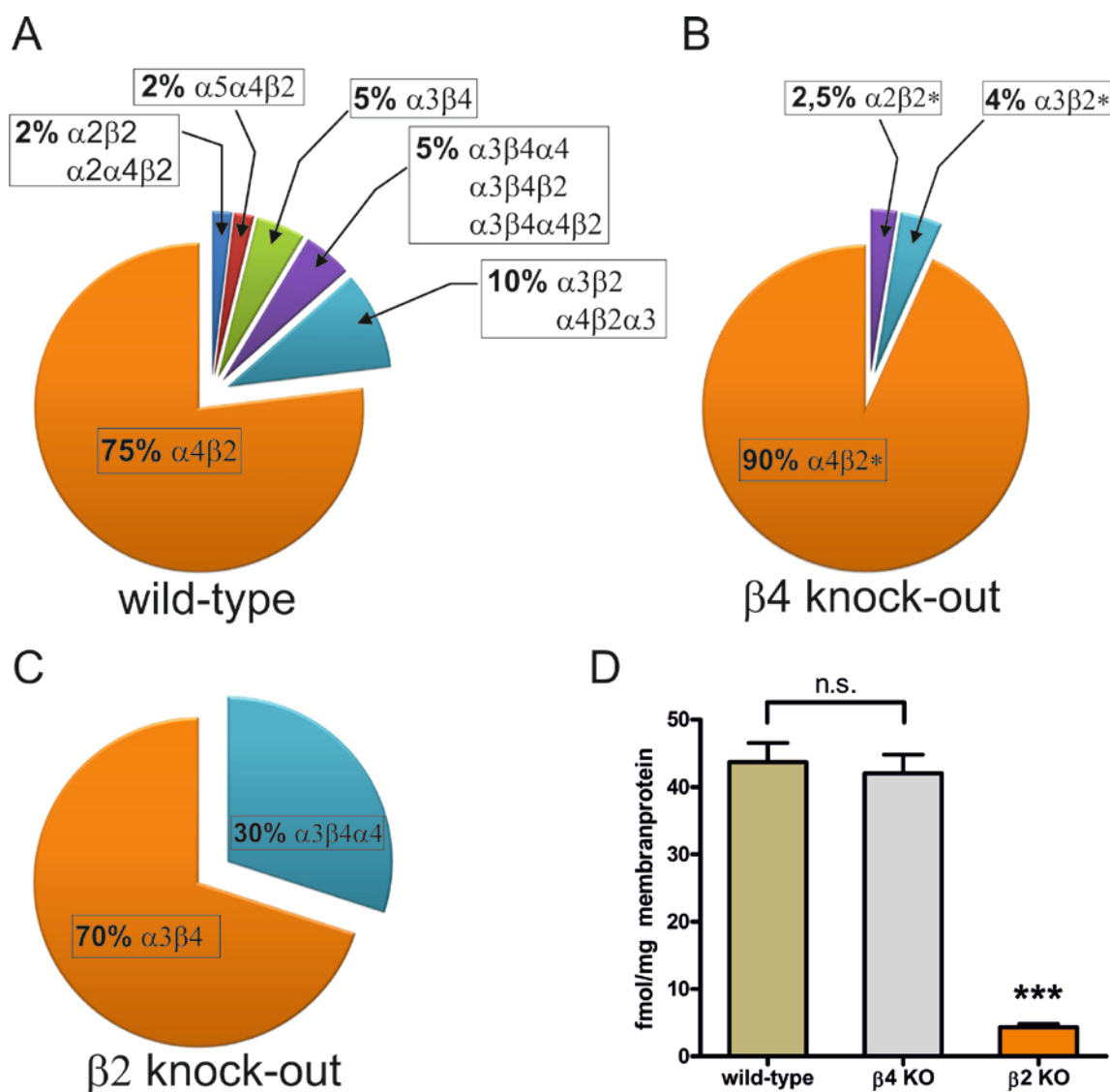


Figure 19: Knock-out of β -subunits drastically reduces the variety of subtypes. A, B and C summarize the hetero-pentameric nAChR subtype variety deduced from results of IP and sequential IP in the spinal cord of wild-type, $\beta 4$ KO and $\beta 2$ KO. The asterisks next to the receptor compositions of the $\beta 4$ KO in B indicate an additional possible $\alpha 5$ subunit in each occurring subtype of this genotype. In the case of wild-type (A) due to the contribution of many different subunits, some receptor subtypes could not be resolved completely. If more than one subtype is listed next to a percentual share it means that this share can possibly contain all of the listed compositions, but doesn't necessarily have to. D offers a comparison between the different genotypes regarding overall frequency of heteromeric nAChRs as determined by IP, which is not shown in the pie-charts. Statistical differences between columns were indicated by student's t-test. ***, $p < 0.0005$

4.4 How does the subunit composition change in response to complete lack of a β subunit?

In order to take full advantage from the accessibility of $\beta 2$ - and $\beta 4$ - KO mice we analyzed consequent rearrangement of nAChR compositions as well as absolute numbers of nAChRs to supplement results from experiments with wild-type spinal cords. The distribution of receptor subtypes in the spinal cord of the two knock-out animals is shown in figure 19.

4.4.1 Loss of β -subunits is not compensated at all

Although $\beta 2$ (in combination with $\alpha 4$) is accounting for the major part of nAChR subunits in the spinal cord and nearly all regions of the brain, we could not detect any means of compensation among the remaining nAChR subunits. Regarding receptor composition, the level of expressed $\beta 4$ subunit in the $\beta 2$ -KO is as frequent as in the wild-type, so the transcription and expression of $\beta 4$ is not influenced by the loss of $\beta 2$ at all. A possible explanation of how $\beta 2$ KO neurons keep up a seemingly functioning cholinergic system in spinal cord with only 10% of the original number of hetero-pentameric nAChRs might be the fact that in wild-type, 65-85% of receptors are kept in intracellular pools (Fenster et al., 1999; Pakkanen et al., 2006; Whiteaker et al., 1998). This circumstance might enhance the ability of hetero-pentameric nAChRs for turn-over and up-regulation under certain conditions. Possibly, the 10% of $\beta 4$ receptors are enough to provide functional cholinergic synapses in the spinal cord of $\beta 2$ -KO mice under most conditions.

However, interestingly the non-compensation for $\beta 2$ -KO has been also observed in regions within the CNS where $\beta 2$ is the only β -subunit of hetero-pentameric nAChRs (Hippocampus, mouse P18, Scholze et al. unpublished). In case of $\beta 2$ KO, hetero-pentameric nAChRs seem to be lost entirely in this region and not compensated by $\beta 4$. Compensation for hetero-pentameric nAChRs by $\alpha 7$ homo-pentameric nAChRs is not likely as binding of α -Bungarotoxin ($\alpha 7$ selective antagonist) in brain of wild-type and $\beta 2$ KO mice is unchanged (Marks et al., 2010)

In contrast to the dramatic uncompensated loss of $\beta 2$ containing receptors in the spinal cord we could not observe any obvious phenotype (without animal testing) of $\beta 2$ KO mice, demonstrating that the consequences of $\beta 2$ deficiency are more subtle as shown in different publications (Picciotto et al., 1995; Picciotto et al., 1998; Marubio et al., 1999) To

understand how the CNS is coping with the loss of certain receptor subtypes or the complete loss of all hetero-pentameric nAChRs in certain tissues and the consequences of these changes will be a challenging task for future investigations.

4.5 Preferences of subunits in choosing partners

Although there seems to be no compensation for knocked out subunits, we did observe decrease of integration of certain subunits into hetero-pentameric nAChRs in $\beta 2$ - and $\beta 4$ -KO animals. However, at this point, it should be considered that our IP shows the amount of [^3H]-epi binding to dimers, trimers and heteromers which contain a specific subunit. If a certain subunit is less integrated into such a complex in $\beta 2$ - or $\beta 4$ -KO animals it can be due to two reasons. A: Due to the KO of a β subunit, there might be down-regulation in transcription or translation of other subunits. B: The expression or translation of a certain subunit is the same in wild-type and KO, but the subunit less likely integrates together with subunits that are left after β -subunit KO.

4.5.1 $\alpha 3$ and $\beta 4$ like each other

One example for this phenomenon was the drop of the $\alpha 3$ subunit from 18% in the wild-type to 4% in the $\beta 4$ KO. As discussed above, $\beta 4$ has a liking to co-integrate into a receptor together with $\alpha 3$ as all $\beta 4$ containing receptors in the wild-type possess $\alpha 3$. From the ~20% (compared to all nAChR) of $\alpha 3$ -containing receptors in the wild-type, ~10% go together with $\beta 4$ to form $\alpha 3\beta 4^*$, whereas ~10% form either the subtype $\alpha 3\beta 2$ or $\alpha 3\alpha 4\beta 2$. In the $\beta 4$ KO spinal cord there are not only all $\alpha 3\beta 4$ -receptors lost, also the $\alpha 3\beta 2$ and $\alpha 3\alpha 4\beta 2$ containing receptors (which make up for 10% of heteromeric nAChRs in the wild-type) are decreased to 4%. (As the 100% value in WT and $\beta 4$ KO is similar at around 43 fmol/mg MP it is accurate to compare percentages between these two genotypes which refer to the 100% value of the respective genotype). As the composition $\alpha 3\beta 2$ and $\alpha 3\alpha 4\beta 2$ as mentioned above is possible, not finding a functional partner to form hetero-pentameric nAChR's in the $\beta 4$ KO neurons cannot be the reason for the $\alpha 3$ subunit levels to drop this dramatically. Therefore our experiments in the $\beta 4$ KO demonstrate that the expression of $\alpha 3$ in hetero-pentameric nAChRs is negatively influenced by the lack of $\beta 4$. This is not completely surprising given the fact that $\alpha 3$ (CHRNA 3), $\alpha 5$ (CHRNA5) and $\beta 4$ (CHRNA4) are genetically

associated in a cluster on chromosome 15 (Berrettini, 2008) whereby the KO of $\beta 4$ may influence gene transcription of the whole cluster.

In the case of $\beta 2$ KO animals, $\alpha 3$ is leveled to exactly match the frequency of $\beta 4$, resulting in a decrease from 8 fmol/mg MP in the wild-type to 4 fmol/mg MP in the $\beta 2$ KO. As we measure whole receptors (and not subunits) with IP it is not necessarily the transcription and translation of $\alpha 3$ -subunit, which is affected by the KO of $\beta 2$. It is more likely that $\alpha 3$ subunits are translated in similar amounts as in the wild-type whereas the amount of $\beta 4$ -subunits (the only β -subunit partner for $\alpha 3$ in the $\beta 2$ KO) is the limiting factor. In the wild type, the amount of $\alpha 3$ -containing receptors is two times the number of $\beta 4$ -containing receptors, in which case $\alpha 3\beta 2^*$ receptors are formed besides $\alpha 3\beta 4$.

4.5.2 $\alpha 4$ has no particular interest in $\beta 4$

In contrast to the liking of $\alpha 3$ and $\beta 4$ to form receptors, in the $\beta 2$ KO, the level of $\alpha 4$ shows reduction below the level of $\beta 4$ containing receptors to only ~1fmol/mg MP, which is conform to ~30% of all receptors which are expressed in the $\beta 2$ KO. We cannot determine if the expression of the $\alpha 4$ subunit is influenced by the KO of $\beta 2$, but if we suggest similar levels of translated $\alpha 4$ subunits in this genotype as in the wild-type (where it is expressed in much higher frequency: 43 fmol/mg MP), the fact that $\alpha 4$ doesn't even level the 4 fmol/mg MP in the $\beta 2$ KO (it only shows 1 fmol/mg MP) shows that its ability to form the $\alpha 3\beta 4\alpha 4$ – subtype with $\alpha 3$ and $\beta 4$ is limited.

In this study, the subtype mentioned above ($\alpha 3\beta 4\alpha 4$) was the only one where $\alpha 4$ and $\beta 4$ could be found together in one receptor. This is in contradiction to findings of the $\alpha 4\beta 4$ subtype in the IPN of $\beta 2$ KO mice (Klink et al., 2001) and cerebellum and retina of the rat (Turner and Kellar, 2005; Marritt et al., 2005).

4.5.3 $\alpha 2$ and $\alpha 5$ rather integrate with $\alpha 4\beta 2$ than $\alpha 3\beta 4$

The minor frequent subunits $\alpha 2$ and $\alpha 5$ (~2fmol/mg MP in wild-type each) are not affected by the KO of $\beta 4$, leading to the suggestion that these two subunits co-integrate preferentially together with $\beta 2$. As $\alpha 5$ is accessory this would result in the subtype $\alpha 5\alpha 4\beta 2$, but not $\alpha 5\beta 2$. Concerning $\alpha 2$, the $\alpha 2\alpha 4\beta 2$ subtype is possible, but also the assembly of $\alpha 2\beta 2$ receptors has been reported in the rat retina (Moretti et al., 2004) and the rat IPN (Gotti et al., 2005). The fact, that $\beta 2$ showed significantly higher levels than $\alpha 4$ in IP of $\beta 4$ KO mice might be an indication that $\alpha 2\beta 2$ is preferred over $\alpha 2\alpha 4\beta 2$ as there is not enough $\alpha 3$ and $\alpha 4$

to cope with the $\beta 2$ – levels in this genotype. The levels of $\alpha 2$ and $\alpha 5$ in $\beta 2$ KO animals are below 0.3 fmol/mg MP, a protein concentration which is very likely beyond the level of our detection system. However, it is a fact that $\alpha 2$ and $\alpha 5$ levels drop dramatically in the $\beta 2$ KO (In terms of fmol/mg MP, the percentage of $\alpha 2$ and $\alpha 5$ is higher in $\beta 2$ KO because the 100% value is much lower), but stay the same in the $\beta 4$ KO. This strengthens the suspicion that $\alpha 5$ and $\alpha 2$ form receptors with $\beta 2$ in the wild-type spinal cord.

Investigations of the $\alpha 5$ subunit revealed different results. As $\alpha 5$ has been shown to co-integrate preferentially with $\beta 4$ to form $\alpha 3\beta 4\alpha 5$ in autonomic and sensory ganglia (David et al., 2010; Mao et al., 2006; Conroy and Berg, 1995), it only forms receptors with $\alpha 4$ and $\beta 2$ ($\alpha 4\beta 2\alpha 5$) in various CNS regions of the brain (Mao et al., 2008; Gerzanich et al., 1998; Gotti et al., 2005; Lomazzo et al., 2010; Zoli et al., 2002). Therefore the decision of $\alpha 5$ to form receptors with either $\beta 2$ or $\beta 4$ is probably made by the dominance of either $\alpha 3\beta 4$ or $\alpha 4\beta 2$ in the according region.

A hypothetical $\alpha 2\alpha 5\beta 2$ receptor would also be possible in the spinal cord but this subtype has not been shown yet in mammals and due to the low levels of $\alpha 2$ and $\alpha 5$, the claim of its existence would be highly speculative.

4.6 Can localization of nAChRs in the spinal cord be aligned to the analgetic effect of intrathecally injected nicotinic agonists?

Although the pharmacological and biophysical profile of the various subtypes of hetero-pentameric nAChR's is, thanks to numerous studies, a detailed one, exact morphological data is rather rare. The major reasons for this circumstance is on one hand the problematic of producing specific AB against the different existing subunits (Moser et al., 2007) and on the other hand the lack of adequate KO-controls (see introduction).

Our findings in immunohistochemistry (IHC) and autoradiography confirmed the findings that nAChR's in the spinal cord of wild-type mice tend to show much heavier labeling in dorsal layers than ventral areas of the grey matter (Khan et al., 2003). In more detail, the observed signal of our antibodies against the specific subunits $\alpha 3$, $\alpha 4$ and $\beta 2$ showed strong labeling of cell soma from interneurons or projection neurons in the dorsal horn and much lighter labeling of motoneurons in the ventral horn. This dorsal polarity is in agreement with the assumption that spinal nAChR's play a role in the transfer of nociceptive

stimuli over C-Fibres, which are relaying to soma of projection neurons and interneurons at dorsal layers of the spinal cord.

At least two reasons might contribute to our observed strong intracellular signal. Firstly, as mentioned above, cytoplasmatic pools account for the majority of total pentameric receptors in a nAChR-expressing cell. Secondly AB's bind specific subunits not receptors and therefore consequently label single subunits, dimers and trimers as well as functional pentamers. These factors impede the task of pinpointing active nAChR at synaptic varicosities on neuronal cell bodies as it is hard to tell where the Cytoplasm ends and a potential synapse starts although we could observe some co-localization of nAChR from interneurons and/or projection neurons in the dorsal horn with synaptophysin. As synaptophysin is a presynaptic marker, this would impede that hetero-pentameric nAChR sit pre-synaptically on axons of DRG neurons which reach into dorsal areas of the grey matter to connect to projection and/or interneurons. However due to the strong intracellular staining in these interneurons and/or projection neurons we cannot exclude postsynaptic occurrence of hetero-pentameric nAChR either.

Clearly no synaptic signal of immunoreactivity with our anti-nAChR ABs could be observed in motoneurons in ventral areas, although we could detect some sort of a perinuclear staining, especially with the anti- $\alpha 4$ AB. As nAChR are folded and oligomerized in the endoplasmatic reticulum (Green and Claudio, 1993; Gaimarri et al., 2007; Ren et al., 2005) before they are transported to synaptic varicosities at the cell membrane our observations in motoneurons suggest the assumption that nAChR subunits are translated, but not expressed as functional pentamers which leave the ER. The observed strong vAChT immunoreactivity around the motoneurons arises from C-Boutons, which are known to contain muscarinic AChRs (Miles et al., 2007) but according to our stainings no heteropentameric nAChRs. These results lead us to believe that heteropentameric nAChRs are transcribed and translated in neurons in dorsal and ventral areas, wherefore it is possible to obtain positive signal in IHC and autoradiography in dorsal and ventral areas, but their participation in synaptic transmission seems to be restricted to dorsal areas of the grey matter.

Regarding immunoreactivity of different subunits, we could not detect any differences concerning the location of staining from AB against $\alpha 3$, $\alpha 4$ and $\beta 2$ and therefore it is not likely that different subunits are restricted to different specific areas in the spinal

cord. Still, as we were not able to obtain signal from $\beta 4$ and the minor frequent subunits of the spinal cord, $\alpha 5$ and $\alpha 2$, this possibility remains.

4.7 Contribution of nAChR – subtypes to transmission and modulation of pain-mediated pathways

An important point about investigating the pain alleviation by nicotinic agonists in rodents would be to assign the effect to certain nAChR – subtypes. According this especially the KO of specific subunits and the intrathecal injection of selective nicotinic agonists have been the methods of choice. Analysis of KO animals have demonstrated that analgesia due to injection of nicotine is less effective in $\alpha 4$ KO and $\beta 2$ KO animals tested in pain tests specific for spinal mechanisms (Marubio et al., 1999). This notion is supported by numerous reports about pain relieving effects of potent agonists which are specific for the $\alpha 4\beta 2^*$ receptor (Khan et al., 1998), which is in accordance to our findings that the $\alpha 4\beta 2^*$ subtype occurs in high frequency in the spinal cord and are therefore likely involved in pain-mediated pathways. However, on the other hand, it has been demonstrated recently that some agonists (e.g. varenicline and ABT-594), which were believed to activate $\alpha 4\beta 2$ only, also activate $\alpha 3\beta 4$ receptors (Gao et al., 2010). Furthermore this study showed that the anti-nociceptive effects of thoroughly tested $\alpha 4\beta 2$ - selective agonists never showed the same strength like full agonists indicating that the $\alpha 4\beta 2^*$ subtype is necessary, but maybe not sufficient to elicit analgesia by itself. Consequently, it is possible that our proposed 10% of the $\alpha 3\beta 4^*$ subtype also plays an important role in spinal pain transmission. Interestingly, although we only detected marginal amounts of $\alpha 5$ -containing receptors in the spinal cord, it has been shown that nicotine induced analgesia is greatly diminished in $\alpha 5$ -KO mice (Jackson et al., 2010). Combined with our results the conclusion might be that $\alpha 4\beta 2\alpha 5$ receptors in the grey matter are located restrictive to areas of pain transmission. Also $\alpha 7$ homo-pentameric receptors have been reported to be involved in spinal pain processing as $\alpha 7$ specific antagonists can neutralize the anti-nociceptive effect of nicotine (Damaj et al., 1998b; Damaj et al., 2000; Khan et al., 2001).

As a consequence to the apparent involvement of different nAChR subtypes it is very likely that there is more than one mechanism involved in modulating and transmitting pain signals at the spinal cord level. In order to get clinical benefit from the analgetic potential of

nicotinic agonists on spinal cord level, more information about the exact site of action and the mechanism itself is needed.

4.8 Occurrence of receptors identifies the site of nAChR action, mRNA levels the site of nAChR synthesis

From the subtypes, which might be involved in mediating pain relief at spinal cord level, we could detect $\alpha 3\beta 4^*$ and $\alpha 4\beta 2^*$ (among them $\alpha 4\beta 2\alpha 5$) with IP. Surprisingly, although finding decent levels of $\alpha 3$ and $\beta 4$ – containing receptors in the wild-type spinal cord with the help of IP (20% and 10% respectively), mRNA levels of $\alpha 3$ and especially $\beta 4$ were barely detectable in RT-PCR analysis of wild-type spinal cord tissue. When screening literature we found several different studies backing up the notion that there are no mRNA levels of $\beta 4$ transcripts in rodent spinal cord tissue (Zoli et al., 1995; Dineley-Miller and Patrick, 1992). A plausible explanation for the expression of subunits in heteromeric nAChRs in the spinal cord with no mRNAs could be that instead of being transcribed in neurons whose soma sit in the spinal cord, these two subunits are rather produced by dorsal root ganglion (DRG) neurons, whose axons express $\alpha 3\beta 4^*$ receptors inside the spinal cord (which would explain results from IP) presynaptically (Fig. 20). Further evidence for this scenario are provided by Boyd and Genzen (Boyd et al., 1991; Genzen et al., 2001), which showed strong levels of $\alpha 3$ and $\beta 4$ mRNA levels in DRGs of rodents. A very similar situation has been described in the habenulo-Interpeduncular pathway, where $\alpha 3\beta 4^*$ receptors in the interpeduncular nucleus sit on presynaptic axonal terminals, which origin in the medial habenula (Gotti et al., 2009). Therefore $\alpha 3\beta 4^*$ receptors could be detected in the IPN by IP, but no $\alpha 3$ or $\beta 4$ mRNA levels by RT-PCR.

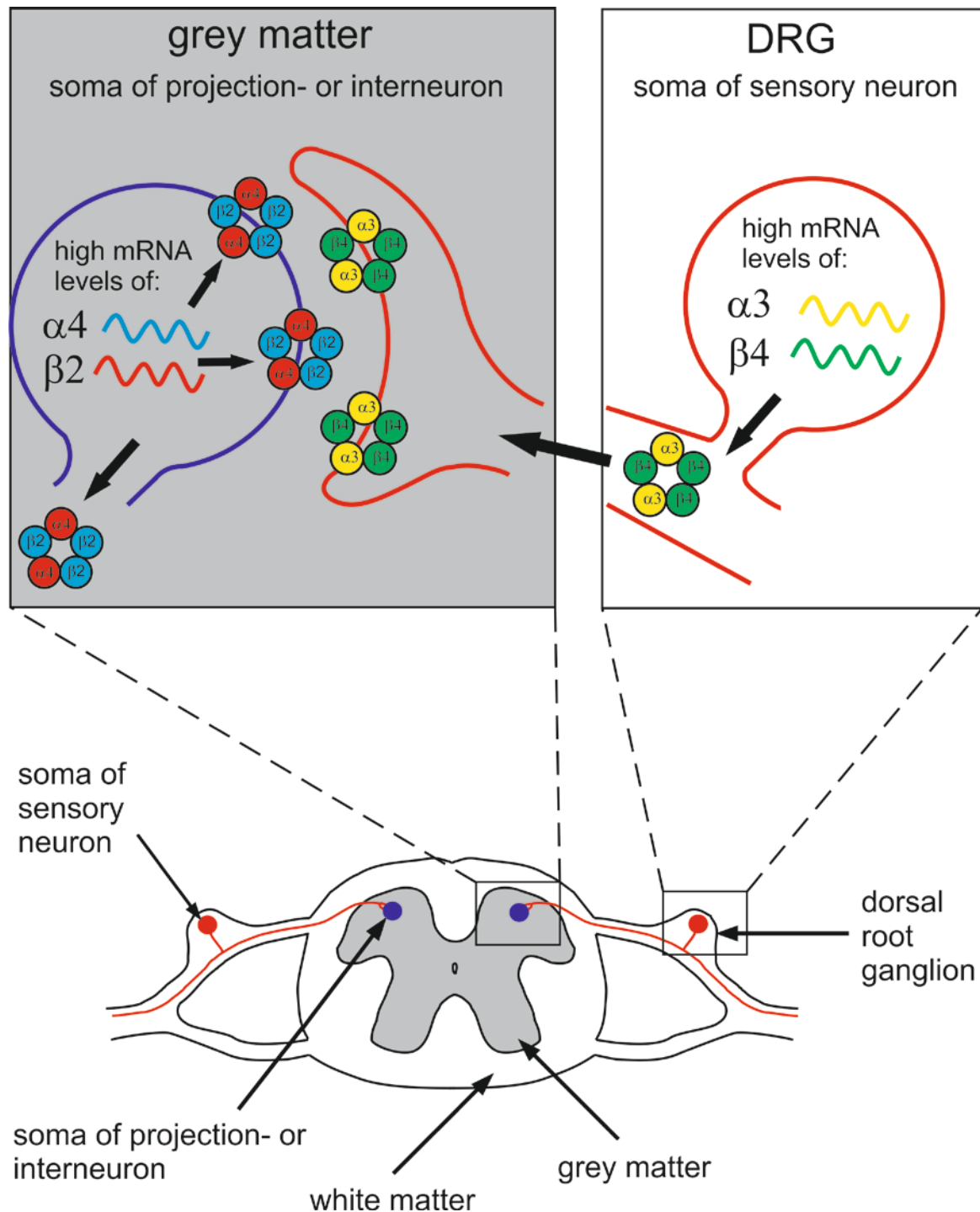


Figure 20: $\alpha 3\beta 4$ – containing receptors might origin in the soma of dorsal root ganglia The lower panel displays a transverse section of the spinal cord including dorsal root ganglia (DRG). DRGs contain soma of afferent sensory neurons, including pain mediating $A\beta$ - and $A\delta$ - fibres (red). Axons of these fibres enter the dorsal horn of the spinal cord grey matter and connect to soma of interneurons- and/or projection neurons (blue). The left upper panel shows a simplified scheme of axonal afferences connecting to soma of projection-or interneurons in the grey matter. As mRNA levels of $\alpha 3$ and $\beta 4$ are very low to non-existent in the grey matter (whereas $\alpha 3\beta 4$ receptors can be found) it is possible that this subtype is transcribed by DRG neurons and expressed presynaptic on their axonal terminals in the grey matter. Of the various subtypes which can be transcribed and expressed by neurons, whose soma sit in the grey matter, only the most abundant heteropentameric nAChR $\alpha 4\beta 2$ is depicted in this simplified overview.

Although our data from IHC provide some insight into possible localization of different nAChR- subunits many questions about the cholinergic role in pain transmission over the spinal cord remain unanswered. One major problem is the complexity of the spinal network, which is affecting the transmission of nociceptive stimuli on the spinal cord level. Recent investigations on the physiological role of afferent cholinergic innervation have shown that nicotinic agonists evoke inhibitory post-synaptic potentials (IPSPs) via GABA evoked currents (Matsumoto et al., 2007; Gao et al., 2010), which would mean that hetero-pentameric nAChRs sit presynaptic and release GABA via binding of acetylcholine. However, there are still plenty of possibilities of how these interneurons can be relayed to either the afferent neuron or the projection neuron, which transmits the signal to supra-spinal levels of pain procession or in reflex arcs to motor neurons.

There seems to be consensus that presynaptic hetero-pentameric nAChRs tend to play a bigger role than postsynaptic receptors (Albuquerque et al., 2009). Therefore, for understanding mechanisms of cholinergic signaling, a crucial task will be investigating which types of neurotransmitters (next to GABA) can be released by activation of different heteromeric nAChRs.

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6. Curriculum vitae

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