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Role of α5-containing nicotinic receptors in neuropathic pain and response to nicotine

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ABSTRACT

Nicotinic receptors in the central nervous system (nAChRs) are known to play important roles in pain processing and modulate behavioral responses to analgesic drugs, including nicotine. The presence of the α5-neuronal nicotinic accessory subunit in the nicotinic receptor complex is increasingly understood to modulate reward and aversive states, addiction, and possibly pathological pain. In the current study, using α5-knockout (KO) mice and subunit-specific antibodies, we assess the role of α5-containing neuronal nicotinic receptors in neuropathic pain and in the analgesic response to nicotine. After chronic constriction injury (CCI) or partial sciatic nerve ligation (PSNL), no differences in mechanical, heat, or cold hyperalgesia were found in wild-type (WT) *versus* α5-KO littermate mice. The number of α5-containing nAChRs was decreased (rather than increased) after CCI in the spinal cord and in the thalamus. Nevertheless, thermal analgesic response to nicotine was marginally reduced in CCI α5-KO mice at 4 days after CCI, but not at later timepoints or after PSNL. Interestingly, upon daily intermittent nicotine injections in unoperated mice, WT animals developed tolerance to nicotine-induced analgesia to a larger extent than α5-KO mice. Our results suggest that α5-containing nAChRs mediate analgesic tolerance to nicotine but do not play a major role in neuropathic pain.

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated channels formed from multiple α (α2–α10) and β subunits (β2–β4) in various combinations that are widely but not uniformly distributed in the peripheral and central nervous system. Heteropentameric nAChRs with an α3β4 backbone prevail in the PNS, whereas α4β2 receptors are more numerous in most parts of the CNS. Both the pharmacological and biochemical properties of nAChRs are critically determined by their subunit composition. Multiple neurobehavioral changes and effects have been attributed to nicotinic receptors in the CNS (Jacob et al., 2013; Dani and Bertrand, 2007; Hurst et al., 2013), including analgesia, allodynia, and pathological pain (Lawand et al., 1999; Bartolini et al., 2011; Umana et al., 2013; Hurst et al., 2013).

Various nicotinic agonists, e.g. epibatidine and related compounds, are potent analgesics acting at the spinal and supraspinal level (Khan et al., 1998, 2001; Bannon et al., 1998; Damaj et al., 1998). Substances such as epibatidine and ABT-594 have been known for quite some time to be equally or more potent analgesics than morphine, depending on the assay (Bannon and Jarboe, 1978). Nicotinic agonist antinociceptive effects have also been shown in animal models of postoperative (Rowley et al., 2008) and of neuropathic pain (Di Cesare et al., 2013; Abdin et al., 2006; Pacini et al., 2010). To date, several types of nAChRs have been implicated in mediating these effects, namely receptors containing the subunits α4 and β2 (Marubio et al., 1999; Khan et al., 2001), α3 (Young et al., 2008; Albers et al., 2014), α5 (Jackson et al., 2010) and α7 (Feuerbach et al., 2009). *In vivo* evidence for receptors containing the above subunits has been provided by the use of receptor-selective agonists and antagonists, as well as with mice carrying deletions of distinct nAChR subunit genes. Based on

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molecular modeling, desensitization of α4β2α5 receptors has recently been proposed as the mechanism which mediates the analgesic effect of nicotinic agonists (Zhang et al., 2012). Paradoxically, positive allosteric modulation using novel compounds acting on various nAChRs have also been shown to have potent effects in animal behavioral studies (Uteshev, 2014; Pandya and Yakel, 2013; Rode et al., 2012). For example, the positive α4β2 allosteric modulator NS-9283 can potentiate the analgesic efficacy of the epibatidine analogue ABT-594 (Zhu et al., 2011). Although analgesic effects have to date most often been reported to be due to action at α4β2 containing receptors, recent studies suggest that this subunit combination can be deemed as necessary but not necessarily sufficient to produce analgesia (Gao et al., 2010).

A number of studies have furthermore suggested that nAChRs are directly involved in the pain processing of noxious stimuli and in neuropathic pain. Hence, deletion of the β2 subunit lowers the mechanical and thermal nociceptive thresholds in β2-KO mice (Yalcin et al., 2011), knockdown of α5-containing receptors by intrathecal antisense oligonucleotides moderately reduces allodynia (Vincler and Eisenach, 2005), and hyperalgesia in a nicotine withdrawal model is lost in α7-KO mice (Jackson et al., 2008). After spinal nerve ligation in rats, spinal α5 receptor upregulation has also been reported (Vincler and Eisenach, 2004; Young et al., 2008).

Our work focuses on further studying the role of α5-containing receptors in neuropathic pain and in mediating the analgesic effects of nicotine. α5 is considered an accessory subunit as it can only form functional receptors when co-expressed with a principal subunit (such as α2, α3, or α4) and one complementary subunit (β2 or β4, e.g. as α4β2α5 or α3β4α5 receptors) (Wang et al., 1996; Gerzanich et al., 1998; Ramirez-Latorre et al., 1996). Recent studies using specific antibodies have localized the α5 subunit in various CNS regions, including the substantia nigra pars compacta, medial habenula, interpeduncular nucleus (IPN), striatum, thalamus, prefrontal cortex, hippocampus, and the spinal cord in both rats and mice (Mao et al., 2008; David et al., 2010; Grady et al., 2009; Scholze et al., 2012; Beiranvand et al., 2014). α5 assembles into α3β4 receptors in the superior cervical ganglion (SCG) (Mao et al., 2006; David et al., 2010), whereas in CNS regions such as the hippocampus, the striatum, the cerebral cortex, or the thalamus, α5 is found in combination with the subunits α4 and β2 (Mao et al., 2008). In the habenula, α5 co-assembles with both β2 and β4 to form the α3α5β4β2 complex (Grady et al., 2009; Scholze et al., 2012), while in the IPN α5 subunits co-assemble with β2, but not β4 (Grady et al., 2009; Beiranvand et al., 2014). The presence of α5 can profoundly impact the overall pharmacological and physiological properties of the receptor complex. Effects include altered calcium permeability, increased sensitivity to allosteric modulators, altered receptor desensitization, altered single-channel properties, or altered agonist-mediated responses such as effects on the potency and efficacy of agonists (Ciuraszkiewicz et al., 2013; Tapia et al., 2007; Kuryatov et al., 2008). Two tests for thermal sensitivity testing involving spinal and supraspinal mechanisms show that effects of nicotine are largely reduced in α5-KO mice (Jackson et al., 2010).

In the current study, we test whether α5-KO mice differ from their WT littermates in two well-established models of neuropathic pain and in their responses to analgesic doses of nicotine. We furthermore measure the overall number of hetero-pentameric nAChRs and the expression of distinct receptors containing the subunits β2-, β4-, and α5 by means of immunoprecipitation in the lumbar spinal cord, thalamus, hippocampus, habenula, striatum, and the IPN after peripheral nerve injury. We found no differences in the development of neuropathic pain between WT and α5-KO mice, and only minor changes in the expression of nicotinic receptors after peripheral nerve injury. The thermal analgesic effects of acute nicotine administration were also only marginally different. However, when tested in unoperated mice, WT animals developed tolerance to nicotine-induced analgesia to a larger extent than α5-KO mice.

2. Materials and methods

2.1. Animals

For behavioral experiments (see exception below) and all biochemical assays, adult male littermate WT mice and mice with a deletion of the α5 nAChR subunit gene (α5-KO) (Wang et al., 2002) were used. Mice used in this study were backcrossed into C57Bl/6J background for at least 7 generations after germ line transmission. For most of the experiments, KO and WT mice were littermates from heterozygous breeding pairs and genotyped at weaning (18 days after birth). When probing for nicotine tolerance, some experiments were, in addition, performed on "cagemate" mice (pooled at weaning from litters of the two homozygous breeding pairs). Experiments were performed within the age range of 2–5 months. All mice were bred in-house and kept in Type IIL cages (~553 cm^2) at a density of 4–6 per cage. Animals were maintained and tested in state-of-the-art temperature and humidity controlled housing facilities and behavioral testing rooms set at 20–24 °C, 40–60% humidity, 12 h light/dark cycle, and food and water provided *ad libitum*. Experiments were always performed during the light cycle between the hours of 10AM–6PM.

Experimental procedures were approved by the Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (BMWF). Extra care was taken to minimize animal suffering and to limit the number of animals used for experiments.

2.2. Models of neuropathic pain

For the partial sciatic nerve ligation (PSNL) model, mice received an injury to the left sciatic nerve, according to a standard method previously reported (Malmberg and Basbaum, 1998) which is based on the Seltzer model (Seltzer et al., 1990). Mice were kept anaesthetized using a gaseous mixture of nitrous oxide (~25%), oxygen (~75%), and 1.5% isoflurane (~5% for induction). Under sterile conditions, the sciatic nerve was exposed at thigh level and carefully freed from the surrounding connective tissue. Using a fine curved needle, the nerve was pierced at the midline and one tight ligature using a G-6 (8.0 mm 3/8c) silk suture (Ethicon, Vienna, Austria) was applied to ligate half the nerve. The wound was closed with 7-0 Prolene® polypropylene sutures (Ethicon, Vienna, Austria) which were applied to both muscle and skin. In the sham-operated mice, only the skin and the muscle were carefully freed, while the sciatic nerve was left intact. Nitrofurazone ointment was applied topically on the wound to prevent infection. Animals were housed individually after surgery and monitored for any motor deficits or abnormalities in the days after surgery (less than 3% of animals).

For the chronic constriction injury (CCI) model, a similar procedure as above was followed except that the nerve was not pierced but instead carefully ligated in its entirety with three loose CATGUT® chrome absorbable surgical suture (SMI AG; St. Vith, Belgium) based on the Bennett model (Bennett and Xie, 1988). Double knots were used to prevent knot slippage and ligature tightness was regulated to prevent ischemia. Animals were also housed individually after surgery, and any animal showing severe motor deficits was excluded (less than 5% of animals).

2.3. Behavioral tests

Prior to any behavioral tests, animals were acclimatized to the non-sterile holding rooms for at least two weeks. They were then habituated to the behavioral testing facilities, testing equipment, and to the experimenter for at least three days prior to any experimentation. In the neuropathic pain models, littermate WT and α5-KO mice were tested up to 21 days after PSNL, and up to 29 days after CCI nerve injury or sham surgery at time intervals indicated in the figures ($n = 9$ per group). The order of testing was always first for mechanical, followed by cold, and lastly heat sensitivity, with at least one hour of habituation time between each test modality. The contralateral (unoperated side) hindpaw was tested first, followed by the ipsilateral hindpaw (operated side). WT and α5-KO animals were assigned randomly into treatment and control groups, and these were tested in parallel. All experiments were performed by an experimenter who was blinded to the genotype of the animals. Genotyping was also confirmed at the end of the experiments in randomly chosen animals.

2.3.1. Heat hypersensitivity to measure pathological pain

The Hargreaves plantar test (Hargreaves et al., 1988) was used to test for thermal hyperalgesia in both hindpaws. Mice were placed individually inside Plexiglas cylinders (~7 cm diameter) on the glass floor of an Analgesiometer apparatus (Stoelting Co, Wood Dale, IL, U.S.A.) and habituated for at least 20–30 min prior to testing. Thermal stimulation of the hindpaws was performed by aiming the radiant heat source positioned beneath the glass floor to the center of the plantar hindpaw. The hindpaw withdrawal latency (PWL), i.e. the time until the first clear nociceptive reaction directed to the hindpaw (withdrawal, flinching, licking), was manually

recorded using a digital timer. This procedure was repeated 3 times for each hindpaw, with an interval of 10 min for the CCI and PSNL time course experiments, and every 5 min for the nicotine injection experiments. The light beam intensity was adjusted to measure a baseline PWL between 11 and 13 s. A cut-off of 20 s was used to prevent tissue damage. Consistency within experiments for radiant heat intensity applied was verified using a radiometer (Ugo Basile, Varese, Italy).

2.3.2. Mechanical hypersensitivity to measure pathological pain

Calibrated von Frey Hairs (Anesthesio®; Ugo Basile, Varese, Italy) were used to measure responses to mechanical stimulation. Mice were placed on a metal mesh inside Plexiglas cylinders (~7 cm diameter). A series of nylon monofilaments (0.07 g, 0.16 g, 0.40 g, 0.60 g, 1.0 g, 1.4 g, and 2.0 g) were applied to the plantar surface of the hindpaw in ascending order. Each stimulation was exerted until the filament bent gently, held for approximately 3 s, and was repeated 5 times per filament with a minimum 5 s interval. The threshold was determined when a monofilament induced 3 out of 5 positive nociceptive responses (hindpaw withdrawal, flinching, or licking) not related to general movement or grooming.

2.3.3. Cold hypersensitivity to measure pathological pain

To assess the development of cold hyperalgesia, a modified acetone test was used (Choi et al., 1994). Mice were again placed individually on a wire mesh inside Plexiglas cylinders (~7 cm diameter) and allowed to habituate for about 20 min. Using a pipette, 40 μl of acetone was then carefully applied to the plantar surface of the hindpaw. The total duration of nociceptive behaviors (e.g. flinching, elevation, licking) in response to the cooling effect of acetone was recorded manually for 2 min via the use of a stopwatch. This process was repeated 3 times with an interval of 10 min.

2.4. Nicotine antinociception experiments in pathological pain

The antinociceptive effect of nicotine on thermal hyperalgesia in WT and α5-KO mice was measured using the Hargreaves test. Following mechanical and cold hypersensitivity testing, and after having established the heat thermal baseline thresholds, mice were injected subcutaneously with either vehicle (saline) or 2 mg/kg nicotine ((*[-]-1-Methyl-2-[3-pyridyl]-pyrrolidine*, liquid free base; Sigma–Aldrich), freshly prepared in saline, and tested every 5 min for 60 min thereafter. The effects of nicotine were probed once at day 4 after surgery in mice having undergone PSNL (Sham: $n = 7–10$; PSNL: $n = 8–11$), and three times in the CCI model (4, 11, and 29 days after surgery; Sham: $n = 9$; PSNL: $n = 9$).

2.5. Nicotine tolerance experiments

The static hotplate test (Bioseb, Vitrolles, France) was used in experiments to measure tolerance to nicotine. This consisted of a temperature-regulated metal plate measuring 16 cm × 16 cm with a clear Plexiglas surrounding. The animals were habituated to the apparatus for 10 min at room temperature for two days prior to the start of the experiment. For testing, the temperature was set to 55 °C and the animal was gently placed on the plate. A cutoff of 40 s was used to prevent tissue damage. The latency to the first clear nociceptive behavior directed to the hindpaw (hindpaw stamping, licking, or elevation) was manually recorded using a digital timer. Jumping was seen in less than 3% of animals.

Littermate α5-KO and WT mice: Thermal analgesic tolerance to repeated nicotine injections was compared in littermate α5-KO and WT mice according to a previously reported dosing protocol (Galeote et al., 2006) involving three subcutaneous injections per day. Animals (not subjected to previous surgery) received 2, 3, or 4 mg/kg nicotine injections three times a day (10 a.m., 2 p.m., and 6 p.m.) for 10 days. The hotplate test was performed after the first morning injection on days 1, 3, 5, 7, 9, and 11. Testing was performed at 5, 10, 15, 20, 25, 35, and 45 min post-injection. Groups tested were α5-KO mice and WT littermates at the three nicotine doses ($n = 7–9$ per group).

"Cagemate" α5-KO and WT mice: In order to increase the number of observations, we also tested "cagemate" WT and α5-KO mice. After weaning, these WT and α5-KO mice (pooled from litters of homozygous breeding pairs) were housed together as "cagemates" in a random manner.

2.6. Tissue extraction and immunoprecipitation

2.6.1. Tissue extraction

At the end of the behavioral time course experiments, mice with sham, PSNL, or CCI surgeries and their respective unoperated littermates were sacrificed by deep CO_2 anesthesia, followed by cervical dislocation and subsequent decapitation. The brain and spinal cord were rapidly exposed, dissected from the surrounding tissue, and transferred to a plate with Ca^{2+}-free Tyrode's solution (4 °C cooled on ice; pH 7.4) composed of 150 mM NaCl, 4 mM KCl, 2.0 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. The lumbar region (L3–L6) of the spinal cord was isolated and subsequently divided into ipsilateral and contralateral sides. The IPN, habenulae, hippocampi, striatae as well as thalami of the brain were then dissected. These areas are well identified by their anatomical landmarks and are known to be involved in pain perception and/or are rich in α5-containing nAChRs. Samples were placed in Eppendorf tubes and centrifuged for 1 min at 16,000 *g* and the supernatant was then removed. Tissues were then flash-frozen with liquid nitrogen and stored at −80 °C until processing.

2.6.2. Subunit-specific antibodies

All antibodies used in this study were raised against the cytoplasmic loop domain of the respective mouse nAChR subunit (α5, β2, and β4). The antibodies were first described by our group in David et al., 2010 and have since been used in most of our recent publications (Scholze et al., 2011, 2012; Beiranvand et al., 2014). The specificity and immunoprecipitation efficacy of these antibodies has been tested extensively (David et al., 2010; Scholze et al., 2012). Additional information on these antibodies is provided in Supplementary Table 1.

2.6.3. Immunoprecipitation (IP) of [3H]-epibatidine-labeled receptors

For immunoprecipitation, tissue samples were solubilized in 2% Triton X-100 lysis buffer (pH 7.4) and with one complete mini protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA) per 10 ml buffer. Subsequently, samples were sonicated on ice for 5 s and incubated at 4 °C for 2 h. They were then centrifuged at 16,000 *g* for 15 min at 4 °C. The supernatant (containing the solubilized proteins) was collected and the pellet was discarded. An aliquot of 50 μl was collected and stored at −20 °C for protein quantification. 130 μl lysate was incubated with 20 μl 10 nM [3H]-epibatidine and 7 μg antibody in 30 μl phosphate-buffered saline (pH = 7.4) on a shaker overnight at 4 °C. Non-specific binding was defined by the addition of an excessive amount of nicotine (300 μM) before the addition of radioactive epibatidine to half of the samples.

Heat-killed, formalin-fixed *Staphylococcus aureus* cells (Standardized Pansorbin-cells; Calbiochem; San Diego, CA, USA) were centrifuged at 2300 *g* for 5 min at 4 °C. Resulting Pansorbin-pellets were washed twice with IP-High (50 mM Tris–HCl, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100; pH 8.3) and once with IP-Low (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100; pH 8.0), and then re-suspended with IP-Low. A volume of 20 μl of the washed Pansorbin was added to the samples which were subsequently incubated on a shaker for 2 h at 4 °C. After the incubation with Pansorbin, the samples were centrifuged at 2300 *g* for 5 min at 4 °C. The supernatant was then discarded, and the pellet which contained the complex Pansorbin-nAChR-[3H] Epibatidine was washed twice with IP-High and once with IP-Low with the samples being centrifuged once more at 2300 *g* for 1 min at 4 °C after each wash. To re-suspend the pellets, 200 μl 1 M NaOH was added and the suspensions were then transferred into 6 ml Mini Vial Sarstedt tubes. A volume of 2 ml scintillation cocktail (Rotszint Eco Plus, Carl Roth GmbH, Karlsruhe, Germany) was added per tube and liquid scintillation counting was then performed. Bicinchoninic acid assays were performed to determine the total protein concentration according to the manufacturer's instructions using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

To obtain enough protein for one assay, 2 animals were required for hippocampi, thalami, and striatae, whereas 4 animals were required for IPNs, habenulae, and lumbar spinal cords ($n = 3–5$ assays per group). Immunoprecipitation experiments for as many treatment groups as possible were performed in parallel. The inter-assay reproducibility was verified by different experimenters.

2.7. Statistical analysis

Results of the behavioral tests were analyzed using a two-way repeated measures (with time as repeated factor) ANOVA followed by a Bonferroni's multiple comparison posthoc test to assess the effects of nicotine and/or genotypes. *P* values below 0.05 were considered as significant. Immunoprecipitation experiments were analyzed using one-way independent measures ANOVA for each subtype and tissue region individually, followed by Bonferroni's Multiple Comparison posthoc test to compare groups to controls and to shams. In order to take the duration of nicotine analgesia into account, the antinociceptive response was also calculated as the percentage of the maximal area under the curve (% of maximal AUC) in some nicotine experiments (Figs. 3 and 4), with the appropriate pre-nicotine baseline value applied for each individual animal. The maximal effect was set assuming that the analgesia had reached cutoff (20 s), and the maximal AUC thus results from integrating the area between the maximal effect and baseline for the indicated time (0–15, 0–60, and 15–60 min). The % of maximal AUC was thereafter calculated by dividing the AUC (obtained by applying the AUC analysis supplied by GraphPad Prism to the measured effects) by the maximal AUC, followed by multiplication by 100. A two-way repeated measures ANOVA (side of foot as repeated factor) followed by Bonferroni's test was then used to calculate effects due to genotype in each time period individually. A two-way repeated measures ANOVA (with time as repeated factor) followed by Bonferroni's multiple comparison posthoc test was also used for tolerance experiments for each treatment day and nicotine dose individually. For statistical analysis at the 5-min timepoint (Fig. 6G and H), the time difference (seconds) from baseline was determined, and a one-sample Student's *t*-test was applied to assess whether the values were significantly different from 0. All data are presented as mean ± standard error of the mean (SEM). Graphs and statistical analyses were conducted using the software GraphPad Prism version 6.01 for Windows GraphPad Software, San Diego, California, USA, www.graphpad.com.

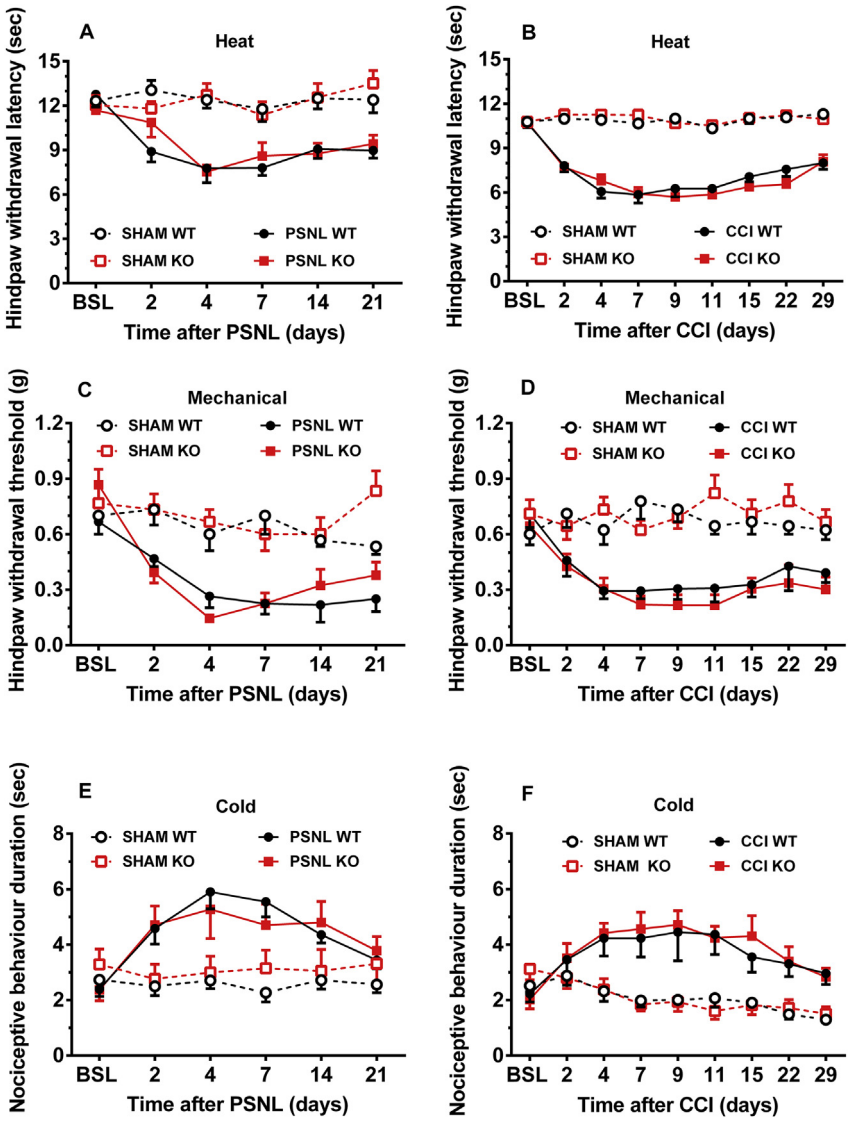

Fig. 1. Time course for mechanical, heat, and cold hyperalgesia in two models of neuropathic pain in WT and α5-KO mice. Both partial sciatic nerve ligation (A, C, E) and chronic constriction injury (B, D, F), but not sham surgery, induced significant heat hyperalgesia (Hargreaves test) (A, B) mechanical hyperalgesia (von Frey test) (C, D), and cold hyperalgesia (acetone test) (E, F) on the ipsilateral hindpaw. During the time course of three weeks, we did not observe significant differences between α5-KO and WT animals in the development or maintenance of neuropathic pain symptoms ($P > 0.05$). (2-way repeated measures ANOVA with the 4 groups as shown per figure and with time as the repeated factor, followed by Bonferroni's posthoc test to compare WT vs KO and baseline vs timepoints after surgery; n = 9 per group). Abbreviations: BSL, baseline; WT, wild-type; KO, α5-knockout; PSNL, partial sciatic nerve ligation; CCI, chronic constriction injury.

3. Results

3.1. Development of neuropathic pain behaviors is not altered in mice with deletions of the α5 nicotinic receptor subunit

Throughout all experiments, WT and α5-KO animals could not be distinguished by the blinded experimenter and did not show any differences in weight gain. Furthermore, prior to any procedure, control WT and α5-KO mice did not significantly differ in their reaction to heat, cold, or mechanical stimuli (Fig. 1). The development and maintenance of neuropathic pain was compared in α5-KO animals and their WT littermates in two different, widely used animal models: chronic constriction injury (CCI) and partial sciatic nerve ligation (PSNL). Heat, mechanical, and cold hypersensitivity was measured in both models and in the corresponding sham groups in both the ipsilateral (operated) and contralateral (non-operated) hindpaws. For both PSNL and CCI analyses, two-way repeated measures ANOVA revealed a significant effect of group, time, and interaction for all modalities in ipsilateral hindpaw analyses. There were no significant changes after nerve injury in the contralateral hindpaw measurements in either genotype or at any timepoints (data not shown).

Both WT and α5-KO mice developed neuropathic pain in the PSNL model in heat (time factor $F_{5,\ 160} = 7.02$; $P < 0.0001$; Fig. 1A), mechanical stimuli (time factor, $F_{5,\ 100} = 14.54$; $P < 0.0001$; Fig. 1C) and cold (time factor, $F_{5,\ 160} = 8.36$; $P < 0.0001$; Fig. 1E). Posthoc analysis comparing groups revealed differences only between PSNL and sham groups, but not any differences between PSNL WT *vs* PSNL α5-KO at any timepoint ($P > 0.05$).

As in the PSNL model, CCI induced neuropathic pain in WT as well as α5-KO mice in heat hyperalgesia (time factor, $F_{8,\ 256} = 25.31$; $P < 0.0001$; Fig. 1B), mechanical hyperalgesia (Fig. 1D) (time factor, $F_{8,\ 256} = 3.75$; $P < 0.0001$) and cold hyperalgesia (Fig. 1F) (time factor, $F_{8,\ 256} = 4.97$; $P < 0.0001$). Further posthoc analysis comparing groups revealed significant differences only between sham and CCI groups, but no differences between CCI WT *vs* CCI α5-KO at any timepoint ($P > 0.05$).

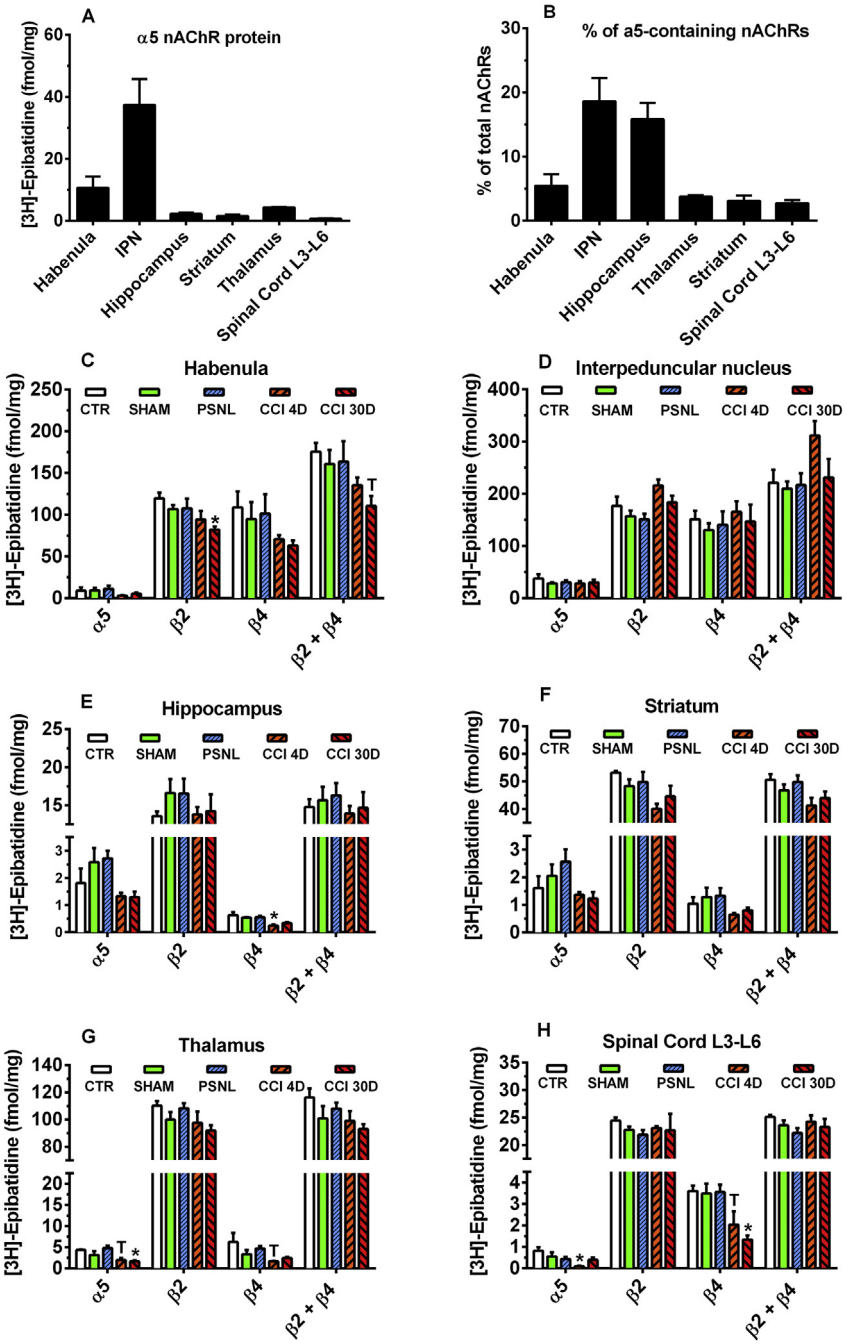

Fig. 2. Levels of α5-, β2-, and β4-containing and total (β2 + β4) receptors in control animals and after peripheral nerve injury. Levels of α5-, β2-, and β4-containing receptors were assessed with immunoprecipitation using subunit-specific antibodies. (A) Absolute and (B) relative levels (shown as a percentage of overall hetero-oligomeric receptors precipitated by the combined use of anti-β2 and anti-β4 antibodies) of α5-containing receptors in the indicated CNS structures. There was a significant decrease in β2-containing receptors in the habenula at the 30-day CCI timepoint (C). In the IPN (D) and striatum (F), no significant differences were seen after sham, PSNL, or CCI surgeries. In the hippocampus (E), a significant decrease in β4-containing receptors was seen at the 4-day CCI timepoint. In the thalamus (G), a significant decrease was detected in α5-containing receptors at the 30-day CCI timepoint. In the ipsilateral lumbar spinal cord (H), significant decreases were detected in α5-containing receptors at the 4 day CCI timepoint, and in β4-containing receptors at the 30-day CCI timepoint. (1-way ANOVA for the 5 groups in every subtype and every tissue, followed by Bonferroni's posthoc test to compare treatment groups to CTR; n = 3–5 per group; *P < 0.05 as compared to CTR; T (trend) P < 0.1 as compared to CTR). Abbreviations: CTR, control; CCI 4D, chronic constriction injury at 4 days post-injury; CCI 30D, chronic constriction injury at 30 days post-injury.

3.2. Minor reductions of distinct nAChRs after CCI, but not PSNL, in various CNS regions

We determined the total number of hetero-oligomeric receptors, and the number of receptors containing the subunits β2, β4, and α5 using immunoprecipitation with subunit-specific antibodies. The total number of hetero-oligomeric receptors was determined by combining anti-β2 and anti-β4 antibodies for immunoprecipitation (David et al., 2010). We quantified these receptors in sham (4 and 30 days after surgery), PSNL (5 days after injury), CCI (4 days and 30 days after injury), and control animals (unoperated WT littermates) in tissues obtained from the habenula, IPN, hippocampus, striatum, thalamus, and ipsilateral lumbar spinal cord. α5-containing nAChRs were detected at measurable levels

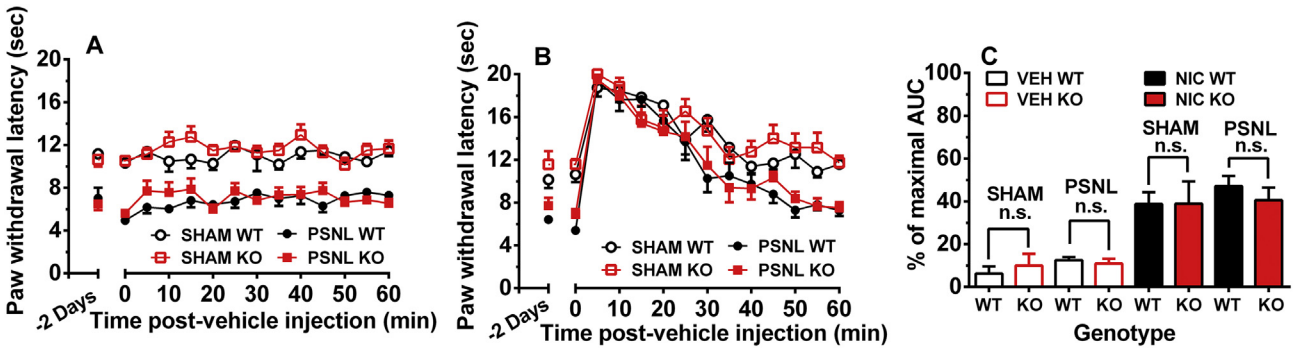

Fig. 3. Antinociceptive response to nicotine after PSNL in WT and α5-KO mice. PSNL resulted in significant heat hyperalgesia as measured with the Hargreaves test in both WT and α5-KO mice. At 4 days post-injury, subcutaneous vehicle injection (A) induced minor and transient increases in PWL in α5-KO mice, whereas subcutaneous nicotine (2 mg/kg) (B) induced significant increases in the PWL of all groups (sham and PSNL). There were no significant differences between WT and α5-KO mice in neither vehicle or nicotine injected mice. Calculation of % of maximal AUC revealed no significant differences between WT and α5-KO animals in neither sham nor PSNL groups (C). (in A and B, 2-way repeated measures ANOVA with 4 groups as shown per figure and time as repeated factor, followed by Bonferroni's posthoc test to compare WT vs KO and time 0 vs timepoints after vehicle or nicotine injection; in C, 1-way independent measures ANOVA for all groups shown, followed by Bonferroni's posthoc test to compare WT vs KO; n = 7–11 per group; n.s. = not significant with $P > 0.05$). Abbreviations: VEH, vehicle; NIC, nicotine.

in these CNS regions. The absolute levels (expressed as fmol radioactive ligand per mg tissue protein) were highest in the IPN, followed by the habenula (Fig. 2a), whereas relative levels (expressed as % of the total number of nAChRs) were highest in the IPN, followed by the hippocampus (Fig. 2B).

One-way independent groups ANOVA consisting of control, sham, 5-days post PSNL, and 4 and 30 days post CCI WT mice was used to individually analyze the total number of receptors and of receptors containing the subunits α5, β2, β4. Posthoc comparisons were then made to compare the control group with the groups of operated animals. Subtle decreases in expression were found mainly after CCI in the habenula (Fig. 2C) for β2-containing receptors ($F_{4, 19} = 3.47$; $P < 0.05$) and a non-significant trend for the sum of β2-and β4-containing receptors ($F_{4, 19} = 2.49$; $P < 0.1$); in the hippocampus (Fig. 2E) for receptors containing the β4 subunit ($F_{4, 18} = 3.29$; $P < 0.05$); in the thalamus (Fig. 2G) for receptors containing the α5 subunit ($F_{4, 14} = 6.51$; $P < 0.01$) and a non-significant trend for β4-containing receptors ($F_{4, 14} = 2.71$; $P < 0.1$); and in the ipsilateral lumbar spinal cord (Fig. 2H) for receptors containing the subunits α5 ($F_{4, 14} = 3.36$; $P < 0.05$) and β4 ($F_{4, 14} = 5.53$; $P < 0.01$). Similar trends were seen in the contralateral spinal cord (data not shown). There were no significant differences in levels of expression in the IPN (Fig. 2D) or in the striatal (Fig. 2F) regions.

3.3. No major differences in the antinociceptive response to nicotine after PSNL or sham surgery in WT or α5-KO mice

We measured the thermal antinociceptive responses to subcutaneous nicotine (Hargreaves test) over a 60 min time course in WT and α5-KO animals which had undergone PSNL or sham surgery. Both WT and α5-KO animals receiving PSNL surgery, but not shams, showed a highly significant reduction in the PWL (Figs. 1A and 3A) of the ipsilateral hindpaw 2 and 4 days after injury. Vehicle injection resulted in just transient increases in the PWL of PSNL WT and α5-KO (possibly due to handling stress) animals at single timepoints (Fig. 3A), whereas large analgesic effects were seen in nicotine-injected animals (two-way repeated measures ANOVA: effect of time ($F_{12, 348} = 63.43$; $P < 0.0001$), group ($F_{3, 29} = 8.72$; $P < 0.001$), and interaction ($F_{36, 348} = 2.22$; $P < 0.0001$) (Fig. 3B). However, the increased PWL was not significantly different when comparing WT and α5-KO within both sham and PSNL groups ($P > 0.05$). Raw data was then converted to % of maximal AUC, and a one-way ANOVA was performed comparing all groups (sham or PSNL, WT or α5-KO) treated with vehicle or nicotine. A significant group effect was found ($F_{7, 61} = 10.77$; $P < 0.0001$). Individual comparisons revealed significant differences only between vehicle and nicotine injection groups but no significant differences due to genotype or sham/injury within either the vehicle or the nicotine-treated groups ($P > 0.05$) (Fig. 3C).

3.4. α5-KO differ from WT animals by showing reduced thermal antinociceptive responses to nicotine 4 days after CCI

Thermal analgesic responses to subcutaneous nicotine (Hargreaves test) were also measured in WT and α5-KO animals which had undergone ipsilateral CCI or sham surgery. Both WT and α5-KO animals receiving CCI surgery, but not ipsilateral shams, showed a significant reduction in the ipsilateral PWL at 4, 11, and 29 days post injury (Figs. 1 and 4A, B, C; $P \leq 0.001$), but not in the contralateral PWL (data not shown).

Using two-way repeated measures ANOVA with group (sham, CCI, WT, KO) and time post-injection as the repeated factor, the different timepoints after CCI or sham surgery (d4, d11, and d29) were analyzed. At 4 days post-injury (Fig. 4A), ANOVA revealed a significant effect of group ($F_{3, 384} = 10.73$; $P < 0.0001$). Posthoc comparisons between WT *versus* α5-KO showed no significant differences at any timepoints for the sham groups ($P > 0.05$), but a significant reduction in antinociception for the CCI α5-KO as compared to the CCI WT group at the 20 min timepoint ($P < 0.05$), and trends for a difference at the 35 and 40 min timepoints ($P < 0.1$). At 11 days post-injury (Fig. 4B), two-way repeated measures ANOVA also showed a significant effect of group ($F_{3, 384} = 7.24$; $P < 0.001$). Between-group posthoc comparisons between α5-KO *versus* WT showed a significantly enhanced analgesic effect of nicotine in sham α5-KO as compared to sham WT at the 5 min timepoint and a significantly reduced effect of nicotine in CCI α5-KO mice as compared to CCI WT at the 45 min timepoint ($P < 0.05$). However, at 29 days post-injury (Fig. 4C), ANOVA showed only a non-significant trend for the group factor ($F_{3, 384} = 2.55$; $P < 0.1$) and no significant differences were detected between α5-KO *versus* WT shams or CCI at any timepoints ($P > 0.05$).

In order to also take the duration of nicotine analgesia into account, the area under the curve for the PWL after nicotine injection was calculated for all data by a comparison with the pre-injection values at 4, 11, and 29 days after sham operation or CCI injury. This was done in both the ipsilateral and contralateral hindpaws for the entire 60 min post-nicotine injection time period, as well as

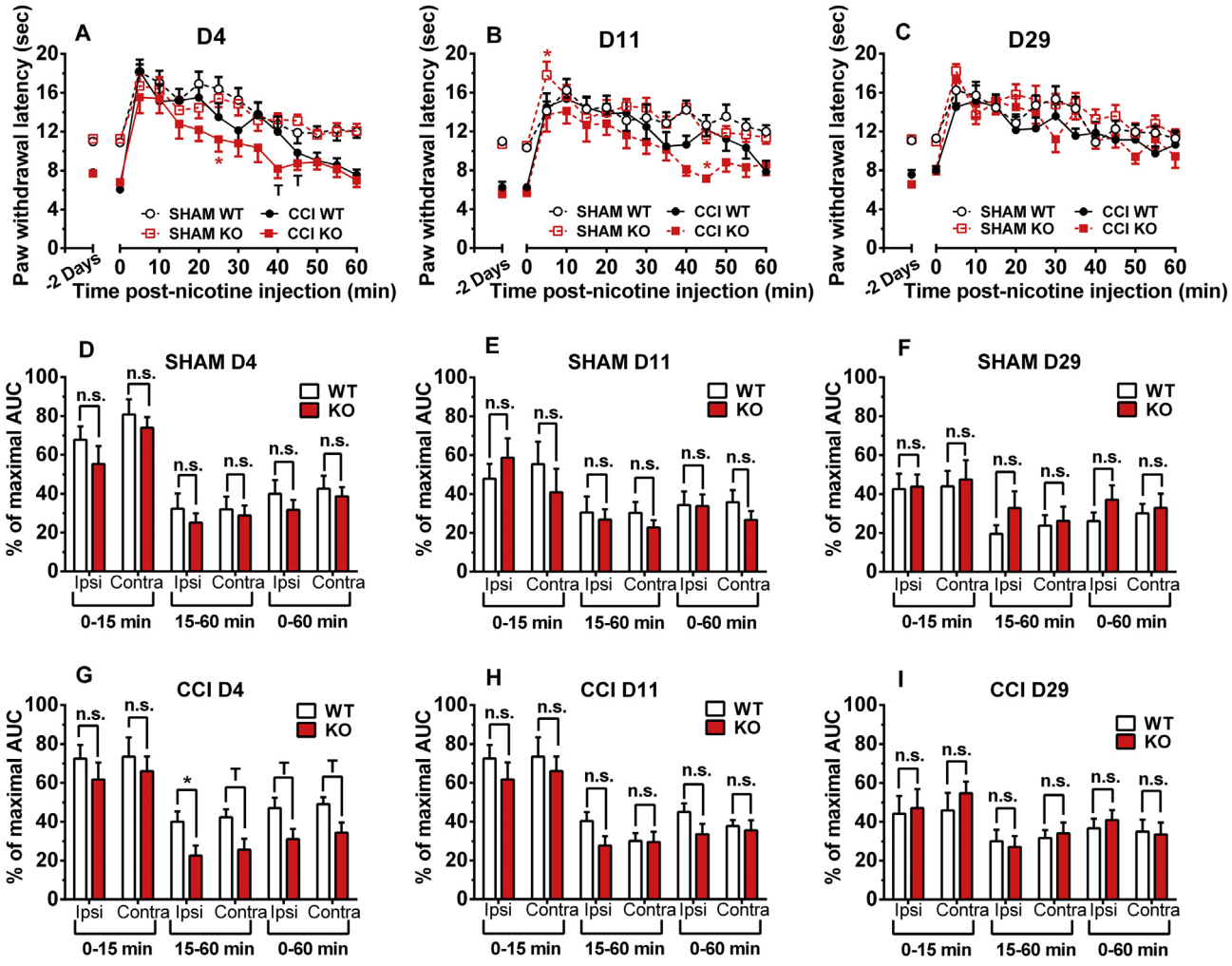

Fig. 4. Antinociceptive response to nicotine at different timepoints after CCI in WT and α5-KO mice. CCI resulted in significant heat hyperalgesia as measured by the Hargreaves test in both WT and α5-KO mice. Subcutaneous nicotine injection (2 mg/kg) induced significant increases in the PWL at 4 days (A), 11 days (B), and 29 days (C) post-injury. At 4 days post-CCI, significant decreased PWL in α5-KO CCI mice as compared to WT CCI mice was seen at 25 min post-injection. At 11 days post-CCI, significant decreased PWL in α5-KO CCI mice as compared to WT CCI mice was seen at 45 min post-injection and increased PWL in α5-KO sham as compared to WT sham at 5 min post-injection. There were no significant differences between groups at day 29 post-CCI. Calculation of % of maximal AUC divided in 0–15, 15–60, and 0–60 min time periods revealed no significant differences between WT and α5-KO for the sham group at neither 4 day (D), 11 day (E), nor 29 days (F) post-surgery. For the CCI groups, a significantly decreased % of maximal AUC was found for the α5-KO group as compared to the WT group at 4 days post-CCI on the ipsilateral hindpaw for the 15–60 min time course (G). There was no significant difference in % of maximal AUC between groups in sham animals or at 11 days (H) and 29 days (I) post-CCI. (in A–C, 2-way repeated measures ANOVA with the 4 groups as shown per figure and with time as the repeated factor, followed by Bonferroni's posthoc test to compare WT vs KO and time 0 vs timepoints after vehicle or nicotine injection; in D–I, 1-way independent measures ANOVA for all groups shown, followed by Bonferroni's posthoc test to compare WT vs KO; n = 9 per group; *P < 0.05 WT vs KO, T (trend) P < 0.1 as compared to time 0 or WT, n.s. = not significant with P > 0.05). Abbreviations: D4, D11, D29, days after CCI.

separately for both the 0–15 and 15–60 min post-nicotine injection time periods. The % of maximal possible AUC was individually calculated for each time period and each animal. Separate two-way repeated measures ANOVA (with hindpaw side as the repeated factor) for each timepoint (d4, d11, and d29) and for each time period (0–15 min, 15–60 min, and 0–60 min) were used to compare the following groups: ipsilateral WT, ipsilateral KO, contralateral WT, and contralateral KO.

In the sham groups, two-way repeated-measures ANOVA revealed no significant effect of hindpaw side or genotype at any of the time periods analyzed (0–60 min, 0–15 min, or 15–60 min) and at neither 4, 11, or 29 days after sham operation (Fig. 4D, E, and F). However, in the CCI animals at 4 days post-injury, there was a significant effect of genotype detected in the 0–60 min time period ($F_{1,\ 16} = 8.15$; $P < 0.05$) and the 15–60 min time period ($F_{1,\ 16} = 10.04$; $P < 0.01$) (Fig. 4G), but not in the 0–15 min time period. Posthoc analysis showed a significant difference between WT and α5-KO mice on the ipsilateral side for the 15–60 min time period ($P < 0.05$), a trend for significance on the contralateral side for the 15–60 min time period ($P < 0.1$), and a trend for significance for both the ipsilateral and contralateral side for the 0–60 min time period ($P < 0.1$). At 11 days post-injury, similar trends were seen for α5-KO CCI animals but these were not statistically significant (Fig. 4H). At 29 days post-injury, there were no trends or statistically significant differences between WT and α5-KO groups at any time periods (Fig. 4I). In summary, analysis by % AUC only revealed differences between WT and α5-KO at 4 days post-injury.

3.5. Tolerance to the analgesic effects of nicotine is more pronounced in WT than in α5-KO animals following repeated nicotine administrations

3.5.1. Experiments on littermate WT and α5-KO mice

WT and α5-KO animals that had not undergone any nerve ligation surgery were tested over a 11-day time period on the hotplate test after repeated nicotine treatments (three daily

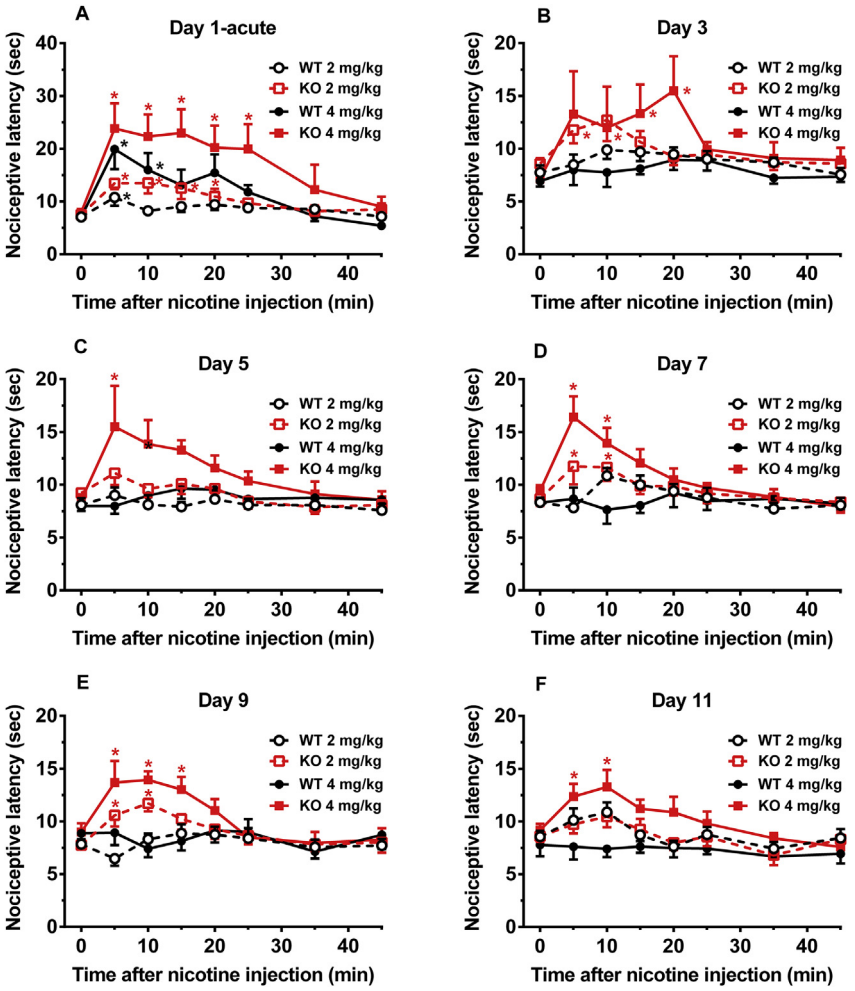

Fig. 5. Heat analgesic tolerance with repeated nicotine treatment in WT and α5-KO mice: 2 and 4 mg/kg. On day 1 (previously untreated animals), subcutaneous nicotine (2 mg/kg and 4 mg/kg) increased nociceptive latencies (measured by the hotplate test) to a similar extent in WT and α5-KO mice (A). Repeated injections (3 times daily for 10 days) resulted in reduced nociceptive latency (or tolerance) to nicotine over time, particularly in WT animals. Significant differences between WT and α5-KO mice were not seen upon first administration or on day 3 (B) but were found for the lower dose at day 9 (E) and for the higher dose (2 mg/kg) on day 5 (C), day 7 (D), day 9 (E), and day 11 (F). All experiments were done in unoperated animals. (2-way repeated measures ANOVA with each dose separately with WT and KO as shown per figure, time as repeated factor, and followed by Bonferroni's posthoc test to compare time 0 vs timepoints after nicotine injection; n = 8 in 2 mg/kg groups, n = 6–9 in 4 mg/kg groups).

subcutaneous injections of 2, 3, or 4 mg/kg nicotine). On day 1, the analgesic effects of nicotine were probed in animals that did not receive nicotine injections before. Two-way repeated measures ANOVAs using genotype as the group factor and the times before and after injection as repeated factors were performed for each dose and timepoint (days 1, 3, 5, 7, 9, and 11) separately to compare the post-injection PWL latencies between WT and α5-KO animals. Pre-injection PWL baseline values remained stable over the 11-day nicotine time course (Fig. 5A–F). The experimenter remained blind to the genotype throughout the experimental time course. Visible nicotine withdrawal symptoms such as paw tremor or head shakes (Damaj et al., 2003) were not observed in either a5-KO or WT animals.

Results for the 2 and 4 mg/kg dose of nicotine treatment are jointly presented in Fig. 5 and show significant time effects for both doses on all days tested: 2 mg/kg ($F_{7, 98} = 9.29$) and 4 mg/kg ($F_{7, 98} = 11.08$) on day 1, 2 mg/kg ($F_{7, 98} = 4.42$) and 4 mg/kg ($F_{7, 91} = 2.30$) on day 3, 2 mg/kg ($F_{7, 98} = 3.11$) and 4 mg/kg ($F_{7, 91} = 2.69$) on day 5, 2 mg/kg ($F_{7, 98} = 4.06$) and 4 mg/kg ($F_{7, 84} = 5.99$) on day 7, 2 mg/kg ($F_{7, 98} = 4.50$) and 4 mg/kg ($F_{7, 84} = 4.70$) on day 9, and 2 mg/kg ($F_{7, 98} = 5.71$) and 4 mg/kg ($F_{7, 77} = 4.53$) on day 11. Posthoc tests revealed that on day 1 (Fig. 5A), 2 mg/kg nicotine analgesia persisted for at least 20 min in KO and at least 5 min in WT mice, whereas 4 mg/kg nicotine analgesia persisted for at least 25 min in KO and at least 20 min in WT mice. On day 3 (Fig. 5B), only KO mice showed responses for at least 5 min with the 2 mg/kg nicotine dose and a up to the 20 min timepoint with the 4 mg/kg nicotine dose. On day 5 (Fig. 5C), only the 4 mg/kg nicotine dose showed analgesia in KO mice for at least 10 min. On days 7 (Fig. 5D) and 9 (Fig. 5E), both the 2 and 4 mg/kg nicotine doses showed analgesia in KO mice for at least 10 min. Finally, on day 11 (Fig. 5F), only the 4 mg/kg nicotine dose showed analgesia in KO mice for at least 10 min. With the higher nicotine dose, brief seizures lasting seconds could occasionally be seen immediately after nicotine injections which resulted in the death of three animals in the WT group (on day 3, 5, and 11) but none in the α5-KO group, previously reported to be less sensitive to nicotine-induced seizures (Salas et al., 2003).

We thus added experiments with an intermediate dose of 3 mg/kg nicotine. Results are shown in Fig. 6A–F. On day 1 (Fig. 6A), repeated-measures 2-way ANOVA showed a highly significant time effect ($F_{7, 98} = 20.80$; $P < 0.0001$) with the posthoc test indicating that both WT and α5-KO groups had significantly increased nociceptive latencies (compared to baseline) for at least 20 min post

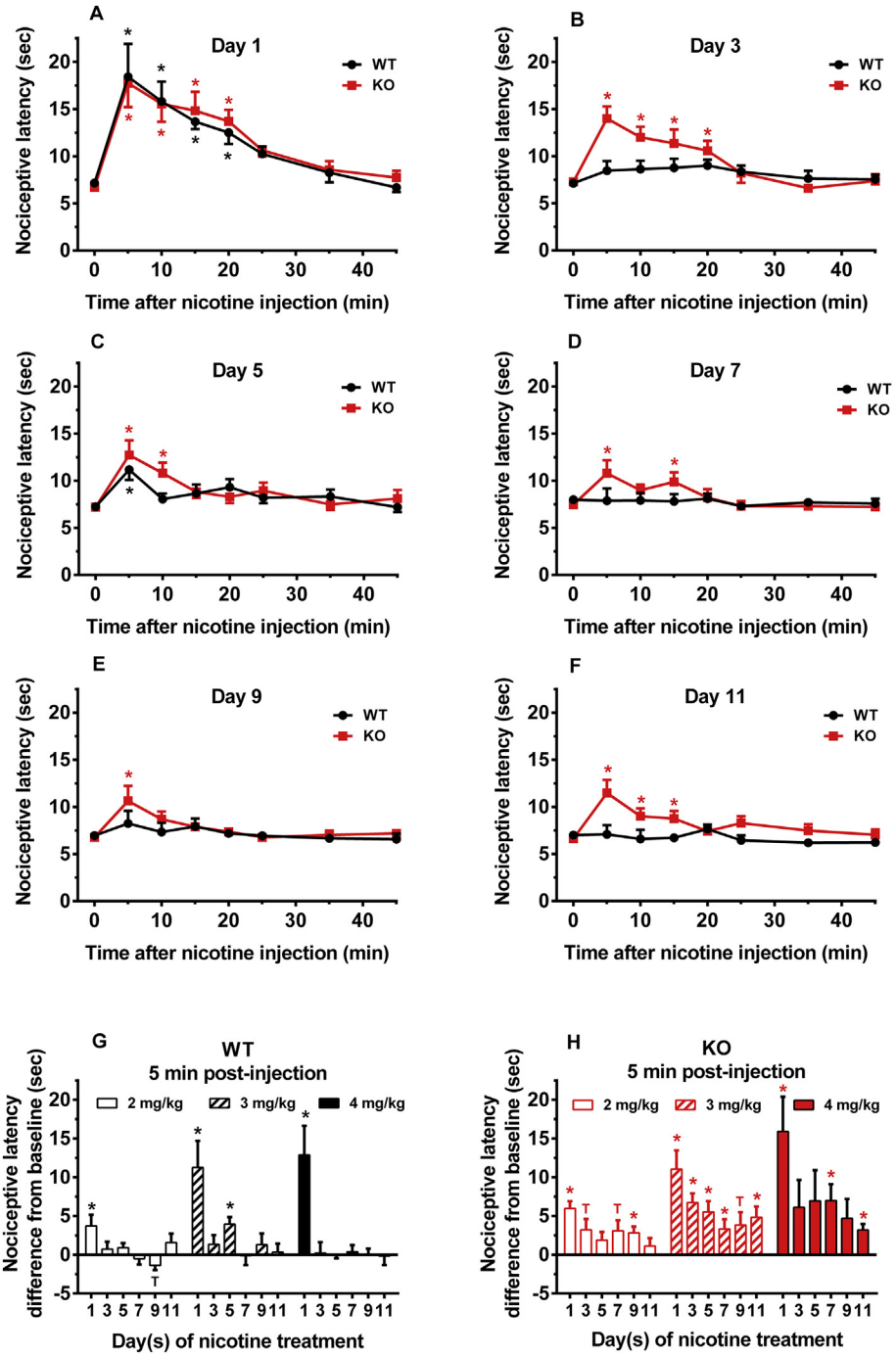

Fig. 6. Heat analgesic tolerance with repeated nicotine treatment in WT and α5-KO mice: 3 mg/kg. A–F: On day 1 (previously untreated animals), subcutaneous nicotine (3 mg/kg) increased nociceptive latency (measured by the hotplate test) to a similar extent in WT and α5-KO mice Following repeated injections (3 times daily for 11 days), nicotine significantly increased the nociceptive latency in WT mice thereafter only on day 5, whereas nociceptive latencies were significantly increased in α5-KO mice throughout the time course of the experiments (until day 11). All experiments were done in unoperated animals. (2-way repeated measures ANOVA with WT and KO as shown per figure, time as repeated factor, and followed by Bonferroni's posthoc test to compare time 0 vs timepoints after nicotine injection; n = 7–8; *P < 0.05). G, H: Baseline-subtracted nociceptive latencies 5 min after the injection of 2, 3, or 4 mg/kg nicotine. Nicotine had analgesic effects at all doses in both WT and α5-KO mice on day 1 (previously untreated animals). However, following chronic treatment with 3 daily doses of nicotine, analgesic effects were preserved in α5-KO in but not in WT mice. n = 6–10. T (trend) P < 0.1; *P < 0.05, Student's one-sample t-test for probing significant differences to 0.

injection ($P < 0.05$). Significant time effects were also found for day 3 ($F_{7, 98} = 8.79$), day 5 ($F_{7, 98} = 12.01$), day 7 ($F_{7, 98} = 2.86$), day 9 ($F_{7, 91} = 4.12$), and day 11 ($F_{7, 91} = 5.15$). On day 3 (Fig. 5B), only α5-KO mice showed significantly increased nociceptive latencies after nicotine injection for at least 20 min. On day 5 (Fig. 5C), WT mice showed increased latencies for at least 5 min whereas α5-KO mice showed this for at least 10 min. On day 7 (Fig. 5D), day 9 (Fig. 5E), and day 11 (Fig. 5F), only α5-KO showed increased nociceptive latencies after nicotine for at least 5–15 min.

In most of our experiments, the analgesic effects of nicotine peaked 5 min after injection. Fig. 6G (WT) and H (α5-KO) shows differences of nociceptive latencies measured between baseline

(before injection) and the effect of 2, 3, and 4 mg/kg nicotine at this 5 min post-injection timepoint. As shown in the figure, the initial analgesic effects of nicotine are mostly lost in WT but less so in α5-KO mice. These observations suggest that chronic treatment with nicotine caused analgesic tolerance to a larger extent in WT than in α5-KO animals.

3.5.2. Experiments on "cagemate" WT and α5-KO mice

In order to strengthen our results, we repeated the experiments with the 2 mg/kg and 4 mg/kg nicotine doses in mice which had been bred in distinct WT and α5-KO lines for several generations. After weaning, these WT and α5-KO mice (pooled from several litters) were housed together as "cagemates". None of the animals died due to the treatment with the 4 mg/kg dose in these groups. Nicotine at 2 mg/kg increased nociceptive latencies in α5-KO mice much more than in the littermate experiments mentioned above, and this effect lasted until day 9 after the start of nicotine injections (Supplementary Fig. 1A, C and E). In WT mice, on the other hand, 2 mg/kg had a small but significant effect on the first day of application only (Supplementary Fig. 1A, C and E). Likewise, treatment with 4 mg/kg had a sustained effect in α5-KO mice but was only effective on the first day of application in WT mice (Supplementary Fig. 1B, D and F).

4. Discussion

The use of nicotinic agonists as an alternative to conventional analgesics for the treatment of acute and pathologic pain is of major clinical interest. It is known that chronic pain patients have high smoking rates (Fishbain et al., 2013) and that smokers are more likely to show chronic pain disorders and have more intense pain (Palmer et al., 2003; Patterson et al., 2012). Studies conducted particularly in postoperative pain have shown mixed significant clinical analgesic effects of nicotine (Turan et al., 2008; Benowitz, 2008). Though analgesic and antinociceptive effects of nicotine have been demonstrated in animal models of acute pain (Anderson et al., 2004; AlSharari et al., 2012), post-operative pain (Rowley et al., 2008), and neuropathic pain (Di Cesare et al., 2013; Abdin et al., 2006), clinical trials have shown disappointing results, usually attributed to toxic side effects. Factors such as delivery method, pre-existing nicotine tolerance, and sex seem to affect results, as well as dosing regimens which may be particularly critical in triggering different degrees of positive *versus* adverse effects (Benowitz, 2008). In addition, a lack of understanding of the mechanisms of action - including the types of nicotinic receptors involved - have hampered the clinical use of nicotine and of nicotinic agonists (Umana et al., 2013; Flores, 2000; Rowbotham et al., 2009).

Here, we investigated the role of nicotinic receptors containing the α5 subunit. A previous study suggests that in mice lacking the α5 nAChR subunit, the analgesic effects of nicotine are greatly reduced in two models of acute pain (Jackson et al., 2010). Furthermore, α5-containing receptors may be up-regulated in the rat lumbar spinal cord after spinal nerve ligation (Vincler and Eisenach, 2004; Young et al., 2008), and mechanical hypersensitivity after this injury is partially reduced using spinally administered antisense oligonucleotide (Vincler and Eisenach, 2005). Taken together, these findings have suggested that α5-containing receptors – including those spinally-located - may play a role in the analgesic response to nicotine and in pathological pain. Using α5-KO animals and selective antibodies for the α5-nicotinic subunit, our results show, however, that unlike these reports, α5-containing nicotinic receptors do not play a significant role in neuropathic pain or in mediating the analgesic effects of nicotine. Of interest though, we discovered a notable role of the α5-nicotinic subunit in the tolerance to the thermal analgesic response of subcutaneous nicotine, administered intermittently over several days.

α5-containing nAChRs are prominently expressed in the habenulo-interpeduncular system (Scholze et al., 2012; Grady et al., 2009; Beiranvand et al., 2014), but also in several other brain regions, such as the prefrontal cortex, the hippocampus, thalamus, cerebellum, striatum, and the ventral tegmental area (Gotti et al., 2006; Chatterjee et al., 2013). A structure of particular interest is the medial habenula, not only because of the distinct collection of receptors expressed (relatively high levels of α5-, and α3β4-outnumbering α4β2-containing receptors; (Grady et al., 2009; Scholze et al., 2012), but also because of the key role it plays in nicotine withdrawal (Salas et al., 2009). The somatic signs for nicotine withdrawal (which include hyperalgesia, (Grabus et al., 2005; Salas et al., 2004)) depend on the presence of medial habenular α5-and β4-containing nAChRs (Salas et al., 2009, 2004). Furthermore, bilaterally injected epibatidine into the medial habenula has an analgesic effect in the hotplate test (Plenge et al., 2002). Interestingly, deletion of the β4 subunits also decreases the antinociceptive effects of nicotine in models of acute thermal pain (Semenova et al., 2012). Given the high expression level of β4-containing receptors in the medial habenula the authors hypothesized that "activation of α3α5β4 nAChRs expressed in the habenula or interpeduncular nucleus produces analgesia".

Consistent with a previous study (Jackson et al., 2010), we were unable to find differences in the basal responses between α5-KO and WT mice measuring sensitivity to heat, mechanical, or cold stimuli. We were, however, also unable to detect significant differences between WT and α5-KO nerve-injured mice in mechanical, heat, or cold hypersensitivity in two different animal models of neuropathic pain. We went on analyzing the expression of nicotinic receptors by immunoprecipitation in a number of CNS structures known to be absolutely or relatively enriched with α5-containing receptors and/or being involved in pain processing (spinal cord, thalamus, and habenula). Nicotinic receptors are upregulated in the ventral posterolateral thalamic nucleus in rats with partial sciatic nerve ligation, as shown by increased binding of the ABT-594 analogue 5-Iodo-A-85380, and injection of 5-Iodo-A-85380 into this nucleus has a dose-dependent anti-allodynic effect (Ueda et al., 2010). A previous study (Vincler and Eisenach, 2004) based on immunohistochemistry also reported that following spinal nerve ligation, α3-and α5-containing receptors were upregulated in the rat lumbar spinal cord, whereas several other receptors (α4, α7, β2, β3 and β4) were unaffected. We detected only minor changes in receptor levels in habenula, thalamus, and hippocampus in our animal models of neuropathic pain. We further found that α5-containing receptors were reduced rather than increased in the spinal cord of mice with CCI. The reason for these differing observations is unclear but may be due to the different detection techniques (immunoprecipitation *versus* immunocytochemistry), differences in species (mice *versus* rats (Young et al., 2008; Vincler and Eisenach, 2004)), different models of neuropathic pain (PSNL and CCI in our study *versus* spinal nerve ligation (Young et al., 2008; Vincler and Eisenach, 2004)), or different timepoints for measuring the receptors (ten to fourteen days post-surgery in (Young et al., 2008; Vincler and Eisenach, 2004)). Experiments based on unconditioned gene deletion strategies always carry the risk of compensation during development, which may obscure effects of the gene of interests (Picciotto et al., 2000). For example, thermal hyperalgesia induced by complete Freund's adjuvant was potentiated in a $P2X_3$ mouse KO model (Souslova et al., 2000), whereas in rats, s.c. injection of the mixed $P2X_3/P2X_{2/3}$ antagonist A-317491 reversed mechanical hyperalgesia (Wu et al., 2004), and antisense oligonucleotide treatment (Honore et al., 2002) significantly decreased nociceptive behaviors in this model. Obviously, this

difference may not necessarily be caused by compensatory mechanisms but – as in our experiments – could also be due to species differences. Since α5-specific antagonists are currently not available we could not take advantage of the pharmacological approach.

Alternatively, the authors in a previous report (Vincler and Eisenach, 2004) could have been misled by commercial antibodies of uncertain specificity. It has previously been stressed that particular care must be taken regarding the specificity of anti-nAChR antibodies (Moser et al., 2007; Herber et al., 2004). The antibodies that we use regularly for immunoprecipitation are tested extensively for subunit specificity, including the use of materials taken from the respective knockout animals (Scholze et al., 2012; David et al., 2010).

Interestingly, our results show that WT and α5-KO mice differed in their analgesic response to nicotine 4 days post-CCI in the 15–60 min time period after nicotine injections, suggesting that α5-containing receptors are transiently affected in this model. Since early mechanisms after nerve constriction (CCI) involve neurogenic neuroinflammation (Xanthos and Sandkühler, 2014), and given the presence of α5-containing receptors in immune cells (Kawashima et al., 2012; Khan et al., 2003), it could be speculated that activation of these receptors contributes to the analgesic effects of nicotine in this model. It has been known for some time that nicotine is a potent anti-inflammatory agent (Lawand et al., 1999; Miao et al., 2004), though pro-inflammatory effects of nicotine due to the enhancement of evoked release of calcitonin gene-related peptide in the oral mucosa have been described as well (Dussor et al., 2003). Recent evidence also suggests an interaction between nicotinic and neurokinin signaling in the medial habenula in the response to nicotine. The intrinsic excitability of cholinergic neurons in the medial habenula is enhanced by acute nicotine, an effect that depends on the presence of α5-containing nAChRs and on intact neurokinin 1 (substance P) and neurokinin B signaling (Dao et al., 2014). Substance P is being released from nerve terminals in the dorsal horn in an activity-dependant manner (see Bannon et al., 1998), but may lose its modulatory role on spinal nicotinic receptors lacking the α5 subunit.

Besides effects in the CNS, early studies have shown that peripheral mechanisms contribute to nicotine-induced analgesia (Caggiula et al., 1995). Hence, the anti-allodynic effects of A-85380 in the spinal nerve ligation model were fully blocked by systemic injections of chlorisondamine, a quaternary nicotinic antagonist which does not readily pass the blood–brain barrier. Likewise, local infusion of A-85380 into the L5 ipsilateral dorsal root ganglion induced significant anti-allodynia. On the other hand, mecamylamine, but not chlorisondamine, fully antagonized A-85380-induced analgesia in acute thermal pain (Rueter et al., 2003). These data point to dorsal root ganglia as a site of action of nicotinic agonists in models of pathological pain. Sensory ganglia indeed express a full range of nicotinic receptors containing the subunits α2-7,9 and β2-4 (Bschleipfer et al., 2012; Albers et al., 2014; Genzen and McGehee, 2005; Flores et al., 1996; Boyd et al., 1991; Albers et al., 2014; McIntosh et al., 2009). It is thus possible that peripheral α5-containing receptors in DRGs contribute to nicotine analgesia, and that nAChRs in the DRG undergo plasticity in the CCI model. For example, complete Freund's adjuvant (CFA) inflammation of paw skin, which causes an increase in artemin in the skin, also increased the level of nAChR mRNAs in DRGs (Albers et al., 2014). Given our focus on the CNS we did, however, not analyze the expression of nAChRs in DRGs.

A number of studies have shown altered nicotine behavioral responses upon knockout of the α5 subunit. For example, α5-KO mice are reported to be less sensitive to nicotine when tested for locomotion (in the open field test), body temperature changes (Jackson et al., 2010), and nicotine aversion (Fowler et al., 2011). These mice are also less susceptible to the occurrence of nicotine-induced seizures (Salas et al., 2003; Kedmi et al., 2004) and lack almost entirely the thermal analgesic effects of nicotine in the hotplate test (Jackson et al., 2010). The latter study concluded that this was due to altered brain (but not spinal) α5-containing receptors which also displayed reduced neurotransmitter release in several brain structures investigated. Interestingly, although we used nicotine doses in a similar range, we could not replicate the loss of nicotine analgesia in unoperated α5-KO control or sham-operated mice, neither with the Hargreaves test, nor with the hotplate test. Our observations also covered the range between 5 and 60 min after the injection of nicotine, thus increasing the chances to detect possible differences in the analgesic effect of nicotine. The reason for this discrepancy is unclear, particularly because we have used the same α5-KO strain and hotplate parameters as investigators in the above mentioned publication (Jackson et al., 2010). Experimenter influence (Chesler et al., 2002), changes in the genetic background over generations, as well as potential epigenetic mechanisms (Bai et al., 2015; Li et al., 2014) may thus be more important than the absence or presence of α5-containing receptors.

A novel discovery from our study is that intermittent nicotine administration caused rapid tolerance to the analgesic effects of nicotine in the hotplate test in WT but less so in α5-KO mice. Whereas WT mice became fully tolerant to all doses of nicotine (2, 3, and 4 mg/kg subcutaneously) within 3 days, α5-KO mice were still showing nicotine analgesic responses after 10 days of nicotine administration. Analgesia induced by 3 or 4 mg/kg nicotine in KO mice persisted for at least 20 min on the first day of administration and still lasted for 10 min by day 11. Tolerance to effects of nicotine is a well-established phenomenon not only in rodents but also in humans (Marks, 2013). Mice strains differ in their disposition for tolerance, with mice most sensitive to the acute effects of nicotine (e.g. C57Bl/6) developing tolerance after subsequent treatments using lower nicotine doses than other mice strains that were less sensitive to the acute effects (Marks et al., 1991). However, previous *in vitro* experiments have shown that higher nicotinic agonist concentrations are required to desensitize α4β2α5 than α4β2 nAChRs (Wageman et al., 2014). It is also worth mentioning that the presence of the α5 subunit can prevent α4β2 receptor upregulation known to occur with continuous nicotine infusion (Mao et al., 2008). To our knowledge, we are first to report that tolerance to the analgesic effects of nicotine is affected by the presence of α5-containing receptors.

In animal models, continuous nicotine infusions have been shown to exacerbate neuropathic pain and lead to enhanced CNS neuroinflammation (Brett et al., 2007; Young et al., 2008). Although we did not use chronic nicotine in the animal models of neuropathic pain, it is possible that repeated intermittent nicotine injections in unoperated animals as administered in our study induced persistent hyperalgesia in the hours after injection. Nevertheless, baseline PWL did remain similar between groups on the days tested even though tolerance to nicotine had clearly developed.

In summary, here we show several novel findings concerning the role of α5-containing nicotinic receptors in neuropathic pain and nicotine analgesia. First, we demonstrate in two different mouse models of peripheral neuropathic pain that deletion of the α5 subunit does not affect nociception in three different modalities over a time course of one month. Second, we show by immunoprecipitation that the number of α5-containing receptors is not upregulated – but may even be downregulated – in these models. Third, we find that acute nicotine analgesia is reduced early (but not late) after CCI in animals lacking the α5 subunit. Fourth, the development of tolerance of nicotine analgesia is largely dependent on receptors containing the α5 subunit. In conclusion, our results

suggest that α5-containing nAChRs neither play a major role in pathological pain nor in mediating nicotine analgesia. Future experiments may show whether the reduced tolerance in α5 KO mice upon repeated administration is a general phenomenon or restricted to nicotine thermal analgesia.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2015.02.012.

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