Molecular mechanism for analgesia involving specific antagonism of $\alpha9\alpha10$ nicotinic acetylcholine receptors

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$\alpha9\alpha10$ nicotinic acetylcholine receptors (nAChRs) have been identified in a variety of tissues including lymphocytes and dorsal root ganglia; except in the case of the auditory system, the function of $\alpha9\alpha10$ nAChRs is not known. Here we show that selective block (rather than stimulation) of $\alpha9\alpha10$ nAChRs is analgesic in an animal model of nerve injury pain. In addition, blockade of this nAChR subtype reduces the number of choline acetyltransferase-positive cells, macrophages, and lymphocytes at the site of injury. Chronic neuropathic pain is estimated to affect up to 8% of the world's population; the numerous analgesic compounds currently available are largely ineffective and act through a small number of pharmacological mechanisms. Our findings not only suggest a molecular mechanism for the treatment of neuropathic pain but also demonstrate the involvement of $\alpha9\alpha10$ nAChRs in the pathophysiology of peripheral nerve injury.

Conus | lymphocytes | neuropathic pain | RgIA | Vc1.1

Neuropathic pain is a prolonged, debilitating state characterized by allodynia (pain produced by previously innocuous stimuli), hyperalgesia (an increased or exaggerated response to painful stimuli), and spontaneous pain. Neuropathic pain is often refractory to conventional pain therapeutics such as opioids and nonsteroidal antiinflammatory agents and, therefore, represents a large, unmet clinical need. Neuropathic pain can be triggered in a variety of ways; injury to a peripheral nerve is one of the most common causes.

The involvement of nicotinic acetylcholine receptors (nAChRs) in pain has been suggested by a number of experimental observations, and the administration of nAChR agonists reduces pain-related behaviors in several animal models (1–5). nAChRs are pentameric ligand-gated ion channels composed of α ($\alpha1$–$\alpha10$) and non-α ($\beta1$–$\beta4$, ε, γ, and δ) subunits. The $\alpha2$–$\alpha6$ and $\beta2$–$\beta4$ subunits form heteromeric channels consisting of a combination of α and β subunits (6). Homomeric channels can be formed by $\alpha7$ or $\alpha9$ subunits; the $\alpha10$ subunit will only form functional receptors when it is expressed with the $\alpha9$ subunit (6). Many of the nAChRs show widespread patterns of neuronal and nonneuronal distribution; $\alpha9$ and/or $\alpha10$ subunits have been reported within hair cells of the inner ear (7), sperm (8), dorsal root ganglion neurons (9), skin keratinocytes (10), the pars tuberalis of the pituitary (11), and lymphocytes (12). The function of $\alpha9\alpha10$ nAChRs in the auditory system has been well characterized (13), but little is known regarding the function of $\alpha9\alpha10$ nAChRs in other tissues. Here we demonstrate that the highly selective antagonist of $\alpha9\alpha10$ nAChRs, RgIA, is analgesic and reduces migration of macrophages, lymphocytes, and acetylcholine (ACh)-producing cells into the area of nerve injury.

Results

RgIA Is Antinociceptive. Chronic constriction injury (CCI) produced mechanical hypersensitivity within 7 days of sciatic nerve ligation (Fig. 1). Paw withdrawal thresholds (PWTs) were reduced from 122 ± 5 g to 26 ± 5 g 7 days after CCI. The i.m. administration of the $\alpha9\alpha10$-selective *Conus* peptide, RgIA, increased PWTs ipsilateral to CCI within 3–4 h [$F(2, 10) = 9.5$, $P < 0.01$] in a dose-dependent manner (Fig. 1). Notably, the highest dose of RgIA administered completely reversed CCI-induced mechanical hypersensitivity. This analgesia was observed 3–4 h after injection on each day of testing; there was no significant difference when comparing effects among treatment days ($P > 0.05$). No adverse effects of RgIA administration were noted. RgIA did not alter normal locomotion (gait) or the stepping reflex, indicating a lack of activity at muscle subtype nAChRs.

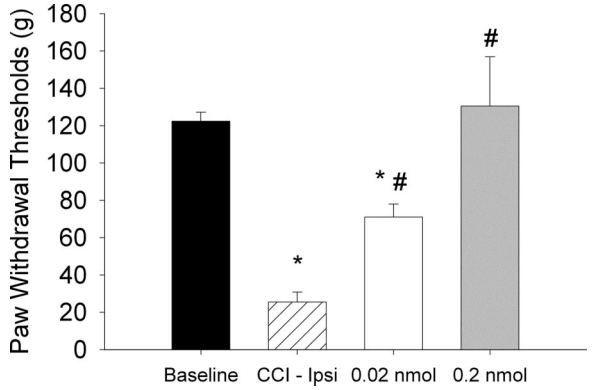

Fig. 1. RgIA exhibits acute antinociception in CCI rats. The $\alpha9\alpha10$-selective peptide, RgIA, increased PWT ipsilateral to CCI 3–4 h after administration. Data shown are the mean PWT in grams ± SEM. *, $P < 0.01$ compared with baseline PWTs; #, $P < 0.05$ compared with PWTs ipsilateral to CCI (CCI − Ipsi); $n = 8$.

In addition to the acute antinociceptive effects of RgIA, repeated i.m. injections of 0.2 nmol of RgIA, once daily, produced a sustained analgesic effect (Fig. 2). Ipsilateral PWTs in CCI rats were increased 40 ± 15% 24 h after the initial administration of RgIA. No change in PWTs was observed on the contralateral side (data not shown). The sustained analgesic effect was observed after each subsequent administration.

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Conflict of interest statement: B.M.O. has been a consultant and officer of Cognetix. J.M.M. has been a consultant and officer of Cognetix.

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Abbreviations: ACh, acetylcholine; nAChR, nicotinic ACh receptor; CCI, chronic constriction injury; PWT, paw withdrawal threshold; ChAT, choline acetyltransferase; PBS+T, 0.01 M PBS with 0.15% Triton X-100.

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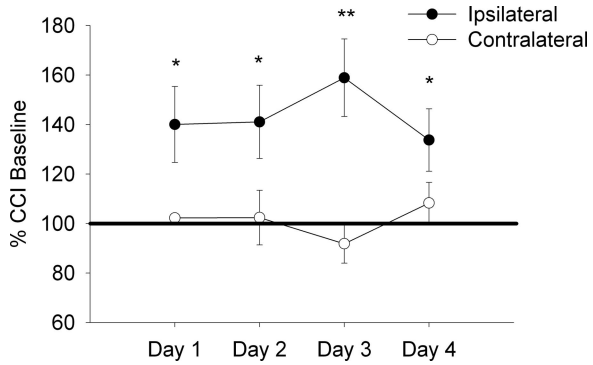

Fig. 2. Repeated administration of RgIA significantly decreased CCI-induced mechanical hypersensitivity. The graph depicts the mean percent change ± SEM of PWTs of the ipsilateral hind paw of CCI rats 24 h after each once-daily injection of 0.2 nmol of RgIA. *, $P < 0.01$; **, $P < 0.005$ compared with baseline, post-CCI PWTs; $n = 6–8$.

Vc1.1 Administration Produces Antinociception. Consistent with previous reports (14), i.m. administration of 0.036 μg (0.018 nmol) or 0.36 μg (0.18 nmol) of Vc1.1 in CCI rats produced 34 ± 18% and 89 ± 20% increases in PWTs, respectively (data not shown). This analgesic effect was consistently observed across 7 days of administration. Thus, similar to RgIA, Vc1.1, with repeated once-daily administration, produced an apparent decrease in CCI-induced mechanical hypersensitivity. After 7 days of repeated Vc1.1 administration, baseline PWTs (measured 24 h after Vc1.1 administration) were significantly increased by 61 ± 4% (0.36-μg dose) and 13 ± 7% (0.036-μg dose) compared with pretreatment values for Vc1.1. This decrease in injury-induced mechanical hypersensitivity is consistent with the observations of Satkunanathan *et al*. (14).

RgIA Alters the Peripheral Immune Response to Nerve Injury. The behavioral effects of the $\alpha 9\alpha 10$ nAChR antagonist RgIA strongly suggest that endogenous ACh plays a role in nerve injury-induced pain. Therefore, we examined the number of ACh-producing cells at the site of nerve injury in CCI rats. As shown in Fig. 3, CCI significantly increased the number of choline acetyltransferase (ChAT)-immunoreactive cells in the ipsilateral ligated sciatic nerve (neural) and its immediate vicinity (perineural) compared with the contralateral side ($P < 0.001$).

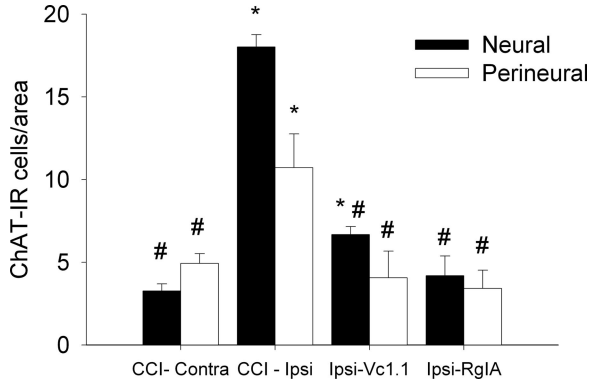

Fig. 3. The mean numbers of ChAT-immunoreactive (ChAT-IR) cells ± SEM are shown both ipsilateral (CCI-Ipsi) and contralateral (CCI-Contra) to sciatic nerve ligation. CCI rats treated with Vc1.1 (0.2 nmol) or RgIA (0.2 nmol) for 5–7 days showed reduced numbers of ChAT-IR cells ipsilateral to CCI. *, $P < 0.05$ compared with those contralateral to sciatic nerve ligation (CCI-Contra); #, $P < 0.05$ compared with those ipsilateral to sciatic nerve ligation (CCI-Ipsi).

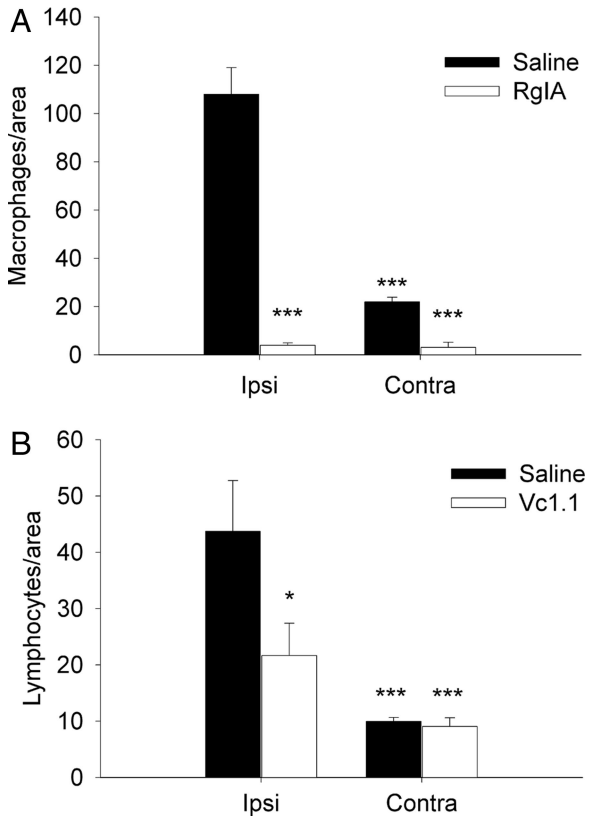

Fig. 4. Immune cells are reduced in CCI rats after 5 days of RgIA or Vc1.1 treatment. (*A*) The mean number of macrophages per area ± SEM is shown in CCI rats ipsilateral (Ipsi) and contralateral (Contra) to nerve ligation. RgIA treatment (0.2 nmol) is compared with control saline treatment. (*B*) The mean number of T cells per area ± SEM is shown in CCI rats. Vc1.1 treatment (0.2 nmol) is compared with control saline treatment. ***, $P < 0.001$; *, $P < 0.05$ compared with CCI rats ipsilateral to nerve ligation (CCI-Ipsi) treated with saline.

Upon daily administration of RgIA for 5 days, the number of ChAT-immunoreactive cells present within the ipsilateral sciatic nerve and perineural area was significantly reduced ($P < 0.001$). Seven days of i.m. administration of the related peptide Vc1.1 (see below) also reduced the number of ChAT-positive cells.

CCI also increased the number of ED1-immunoreactive macrophages and CD2-immunoreactive T cells at the site of nerve injury, which was consistent with previous reports (Fig. 4). This increase was countered by administration of RgIA (0.2 nmol) once daily for 5 days, which significantly ($P < 0.001$) reduced the number of ED1-immunoreactive macrophages in CCI rats both ipsilaterally and contralaterally. The smaller number of macrophages present near the contralateral sciatic nerve was also reduced. The reason for this reduction is unknown and was not further investigated.

RgIA and Vc1.1 Are both Analgesic and Have Nanomolar Affinity for $\alpha 9\alpha 10$ nAChRs. Although it is generally nAChR agonists that are reported to be analgesic, α-conotoxin Vc1.1, also known as ACV1, a compound in human clinical trials by Metabolic (Melbourne, Australia), is a nAChR antagonist that previously has been shown to be analgesic. Peripheral application of this peptide blocks the vascular inflammatory response to electrical stimulation of C fibers and is analgesic in CCI and partial nerve ligation models of human neuropathic pain (14, 15). The previously reported subtype specificity of this analgesic peptide is for $\alpha 3(\alpha 5)\beta 2$ and $\alpha 3(\alpha 5)\beta 4$ nAChRs with micromolar IC_{50}

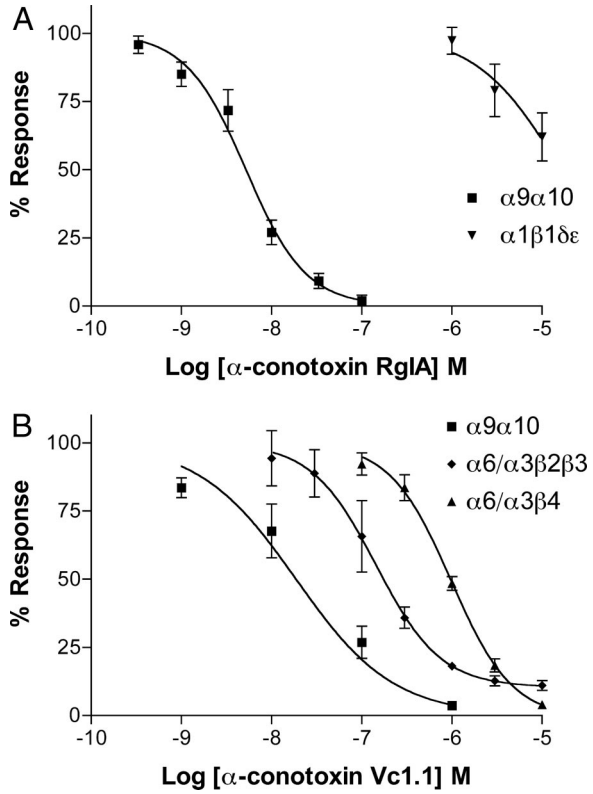

Fig. 5. Activity of conotoxins on nAChRs. Ligands were bath-applied to oocytes expressing the indicated nAChR subtypes, and then the response to a 1-s pulse of ACh was measured as described in *Materials and Methods*. (*A*) RgIA blocks $\alpha9\alpha10$ and $\alpha1\beta1\delta\varepsilon$ nAChRs with IC_{50} values (and 95% confidence intervals) of 5.2 (3.9–6.9) nM and 16 (5.4–48) μM and Hill slopes of 1.3 $\pm$ 0.057 and 0.93 $\pm$ 0.40, respectively. (*B*) Vc1.1 blocks $\alpha9\alpha10$, $\alpha6/\alpha3\beta2\beta3$, and $\alpha6/\alpha3\beta4$ nAChRs with IC_{50} values (and 95% confidence intervals) of 19 (14–38), 14 (91–230), and 980 (750–1,280) nM and Hill slopes of 0.81 $\pm$ 0.089, 1.2 $\pm$ 0.27, and 1.30 $\pm$ 0.16, respectively. See Table 1 for comparison with activity at other nAChR subtypes. Error bars show the SEM for the Hill slopes.

values (16). However, potent antagonists that block these subtypes are not analgesic. Furthermore, if block of $\alpha3(\alpha5)\beta2$ and $\alpha3(\alpha5)\beta4$ nAChRs were the analgesic mechanism of action, one might expect the drug to produce hypotension through antagonism of autonomic ganglionic receptors, a side effect not reported in animal or human clinical trials (18). Given these inconsistent observations, the mechanism of analgesia for Vc1.1 was reinvestigated; we show Vc1.1 to be similar to RgIA in a number of ways.

As previously reported, RgIA potently blocks ACh-induced current in heterologously expressed $\alpha9\alpha10$ nAChRs. As shown in Fig. 5, Vc1.1 is also a potent antagonist of $\alpha9\alpha10$ nAChRs, although, in contrast to RgIA, which is highly selective for the $\alpha9\alpha10$ nAChR, Vc1.1 has comparatively significant activity at $\alpha6$-containing nAChRs (Table 1). Both peptides show very low activity at muscle cholinergic receptors, and no changes in motor function were observed after i.m. administration (data not shown). Consistent with previous reports (14), we also confirmed that Vc1.1, like RgIA, is an effective analgesic in the CCI model. Additionally, Vc1.1, like RgIA, reduces the number of ACh-producing cells at the nerve injury site (Fig. 3).

Discussion

Treatment of neuropathic pain patients remains a significant clinical challenge. Currently available therapies include topical applications of lidocaine or capsaicin, antiepileptics, antidepressants, and opioids. Although topical applications have limited use in peripheral neuropathies such as postherpetic neuralgia, the antiepileptics, antidepressants, and opioids produce a host of unwanted side effects (for a review, see ref. 17). Several nicotinic agonists are analgesic and have entered phase I clinical trials, but these agonists have also suffered from a variety of side effects due to a lack of nAChR subtype specificity. Conversely, the nAChR antagonist Vc1.1 has produced analgesia in a variety of pain models in animals and has currently entered phase II clinical trials (18). The data presented here reveal a molecular target in the treatment of neuropathic pain, the $\alpha9\alpha10$ nAChR subtype.

Table 1. Selectivity of analgesic α-conotoxins

nAChR subtype	RgIA IC_{50}, nM	Vc1.1 IC_{50}, nM
$\alpha9\alpha10$	5.2*	19*
$\alpha1\beta1\delta\varepsilon$	16,000*	ND
$\alpha1\beta1\delta\gamma$	ND	>30,000‡
$\alpha2\beta2$	>10,000†	>10,000‡
$\alpha2\beta4$	>10,000†	>10,000‡
$\alpha3\beta2$	>10,000†	7,300‡
$\alpha3\beta4$	>10,000†	4,200‡
$\alpha4\beta2$	>10,000†	>30,000‡
$\alpha4\beta4$	>10,000†	>30,000‡
$\alpha6/\alpha3\beta2\beta3$	>10,000†	140*
$\alpha6/\alpha3\beta4$	>10,000*	980*
$\alpha7$	4,700†	>30,000‡

Values shown are from the following sources: *, this article; †, ref. 32; and ‡, ref. 16. ND, Not determined.

Our results demonstrate that RgIA, a highly selective antagonist of the $\alpha9\alpha10$ nAChR, produces an acute antinociceptive effect in peripheral nerve-injured rats similar to that previously reported for Vc1.1 (14). More importantly, repeated daily administration of this nAChR antagonist produced a stable decrease in injury-induced mechanical hypersensitivity. CCI caused a significant increase in the number of immune cells at the sciatic nerve, which was blocked by RgIA. Thus, the antinociceptive properties of RgIA may be related to the inhibition of immune cell buildup at the site of nerve injury.

Systemic administration of nicotinic agonists has long been known to produce analgesia, yet our findings and those of Satkunanathan *et al*. (14) reveal analgesic effects caused by nAChR antagonists. Satkunanathan *et al*. (14) showed that i.m. injection of Vc1.1 produced analgesia in CCI rats and hypothesized that Vc1.1 produced analgesia by antagonizing $\alpha3\beta4$ or $\alpha3\alpha5\beta4$ nAChRs expressed by injured and uninjured sensory nerves. However, the IC_{50} of Vc1.1 for $\alpha3\beta4$ nAChRs is 4.2 μM, a concentration that may be difficult to achieve with a 0.36-μg i.m. administration *in vivo*. In contrast, as described in *Results*, the affinity of Vc1.1 for the $\alpha9\alpha10$ nAChR is >100-fold higher (IC_{50} of 22.9 nM; see Table 1) and similar to that seen for RgIA. Thus, the present results are consistent with RgIA and Vc1.1 acting as analgesics by a common molecular mechanism, antagonism of the $\alpha9\alpha10$ nicotinic receptor subtype.

One way in which block of $\alpha9\alpha10$ receptors might cause antinociception in CCI is by antagonism of $\alpha9\alpha10$ receptors expressed in sensory nerves. Dorsal root ganglia have been reported to contain both the $\alpha9$ and $\alpha10$ nAChR subunits (9), but the expression of these subunits along the sciatic nerve is unknown. If $\alpha9$ and $\alpha10$ subunits are transported along the sciatic nerve, $\alpha9\alpha10$ nAChRs may accumulate at the site of nerve injury. Another plausible site of action for RgIA and Vc1.1 is $\alpha9\alpha10$ nAChRs expressed on lymphocytes (12).

Our conclusion that RgIA and Vc1.1 can significantly reduce the number of macrophages and T cells near the site of nerve

injury, coincident with a reduction in mechanical hypersensitivity, is consistent with previous observations implicating macrophages and T cells in CCI pain. Depletion of macrophages with clodronate alleviated thermal hyperalgesia and reduced Wallerian degeneration in partial sciatic nerve-ligated rats (19), and athymic nude rats, which lack mature T cells, developed significantly reduced mechanical and thermal hypersensitivity after CCI compared with heterozygous control animals (20). Reduced numbers of immune cells, demonstrated here, may also contribute to the increased rate of functional recovery observed previously in CCI rats after repeated Vc1.1 administration (14). However, it is unknown whether suppression of the responses of lymphocytes, macrophages, and T cells is a primary response in reversing the nociception or a consequence of the reduced nociception.

Antinociception by RgIA and Vc1.1 suggests that an endogenous source of ACh must play an important role in the neuropathic pain state. This source of ACh is likely ChAT-positive lymphocytes, which are known to possess the proteins necessary to produce and release ACh and are thought to play a significant role in the localized immune response (21). Our results demonstrate that injury to a peripheral nerve increases the number of cholinergic cells at the site of injury and suggest that these may provide a localized source of ACh. This locally released ACh seems to be a potent stimulator of the immune response and to participate in the maintenance of behavioral hypersensitivity.

Our results contrast with studies indicating the presence of a cholinergic antiinflammatory system mediated by the release of ACh from the vagus nerve acting on α7 nAChRs localized on macrophages (22). In the periphery, the proinflammatory cytokines IL-1α and TNF-α released by macrophages contribute to behavioral hypersensitivity after peripheral nerve injury (23–25). Stimulation of the vagus nerve reduces TNF-α production by macrophages in response to bacterial endotoxin in wild-type mice, but not in α7 nAChR knockout mice (22). Endogenous ACh has also been proven to suppress the immune response in models of sepsis, pancreatitis (26), and postoperative ileus (27) via stimulation of the α7 nAChR on macrophages (1–5). Together, these studies and our current results suggest complex interplay between α7 and α9α10 nAChR-mediated effects, where stimulation of the former, yet block of the latter, is antiinflammatory. Such information could prove essential for the rational development of nAChR-targeted analgesics; most previous ligands are unable to distinguish between α7 and α9α10 subtypes.

In conclusion, our results suggest that the α9α10 nAChR is a critical mediator of peripheral nerve injury-induced immune cell buildup and mechanical hypersensitivity. The α9α10-selective nAChR antagonists, RgIA and Vc1.1, reduced CCI-induced mechanical hypersensitivity as well as buildup of macrophages and T cells. Therefore, pharmacological targeting of the α9α10 subtype of the nAChR represents a therapeutic strategy for the treatment of neuropathic pain. Recognition of the involvement of the α9α10 nAChR in the cross-talk between the injured nerve and the immune system and of the unprecedented selectivity of RgIA potentially opens a window to understanding the complex interactions that contribute to neuropathic pain.

Materials and Methods

Male Sprague–Dawley rats (200–300 g; Harlan, Indianapolis, IN) were used for these studies. All animals were housed in pairs and had free access to food and water. All experiments were performed in accordance with the regulations of the Wake Forest University School of Medicine Animal Care and Use Committee.

CCI. Rats underwent loose ligation of the sciatic nerve as described previously by Bennett and Xie (28) but with slight modification. Briefly, rats were anesthetized with halothane (2–3% halothane, remainder oxygen), the left sciatic nerve was exposed at midthigh level, and two 4-0 chromic gut sutures were loosely ligated around the sciatic nerve ≈1 mm apart. The incision was closed with 4-0 silk suture.

Behavioral Testing. All behavioral tests were conducted between 0900 and 1600 hours. No differences in baseline PWTs were noted during these hours. PWTs were determined for left and right hind paws by using the Randall–Selitto paw pressure technique (29). The Analgesy meter (Ugo Basile, Varese, Italy) applied a constant rate of increasing pressure (16 g per second) to the hind paws. The cutoff pressure was 250 g. For the Randall–Selitto test, animals were first subjected to four training sessions to stabilize baseline responses (30). Hind paws were alternately tested three times with a 5-min intertrial interval.

Seven days after CCI of the sciatic nerve, PWTs were measured to confirm the development of mechanical hypersensitivity. Mechanical hypersensitivity was defined as the presence of at least a 20% decrease in PWT compared with pre-CCI baselines. Rats not exhibiting mechanical hypersensitivity were discarded. Rats exhibiting mechanical hypersensitivity were i.m. injected with RgIA (0.02 or 0.2 nmol in 200 μl of physiological saline), and PWTs were measured hourly for 5 h and at 24 h after RgIA administration. This regimen was repeated daily for 5–7 days.

Immunohistochemistry. After behavioral testing on day 5 or 7 after RgIA administration, rats were deeply anesthetized with pentobarbital and perfused transcardially with 0.01 M PBS with 1% sodium nitrite followed by 4% paraformaldehyde (400 ml). The left (injured) and right (uninjured) sciatic nerves were removed and postfixed in 4% paraformaldehyde (2–3 h) followed by 30% sucrose (48–72 h). Tissue was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) and cut transversely at 16 μm on a Leica (Deerfield, IL) CM3000 cryostat.

Immunohistochemistry was performed by using standard biotin–streptavidin techniques. For all immunohistochemistry, sciatic nerve sections were washed in 0.01 M PBS with 0.15% Triton X-100 (PBS+T) and incubated in 0.3% H_2O_2 (15 min). After further washes in PBS+T, sections were incubated in 50% alcohol (45 min), washed in PBS+T, and blocked with 1.5% normal goat serum for 1 h. Sections were incubated with primary antibodies to CD2 (1:1,000; Serotec, Oxford, United Kingdom), CD68 (ED1) (1:1,000; Serotec), or ChAT (1:1,000; Chemicon, Temecula, CA) primary antibodies overnight at 4°C. Sections were washed in PBS+T, incubated in biotinylated goat anti-rabbit (ChAT) or anti-mouse (CD2 and ED1) antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature, washed in PBS+T, and incubated for 1 h in streptavidin-linked horseradish peroxidase (ABC Elite Kit, Vector Laboratories). Antibodies were visualized by using the enhanced glucose–nickel–diaminobenzidine method. Images were captured on a Zeiss (Oberkochen, Germany) AxioPlan2 light microscope at a magnification of ×10. Positively labeled objects were identified for counting by using SigmaScan Pro 5 (SPSS, Chicago, IL) at a preset intensity threshold. For sciatic nerve slices, four nonconsecutive slices were quantified for ChAT, CD2, and ED1 staining.

Electrophysiology. *Xenopus* oocytes were used to heterologously express cloned nAChR subtypes. The oocyte recording chamber was fabricated from Sylgard and was 30 μl in volume. Oocytes were gravity-perfused with ND96A (96.0 mM NaCl/2.0 mM KCl/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/5 mM Hepes, pH 7.1–7.5/1 μM atropine) or ND96A minus atropine (ND96) with or without toxin at a rate of ≈1 ml/min. ND96A was used for oocytes expressing all

nAChR subtypes except $\alpha7$ and $\alpha9\alpha10$; ND96 was used for these subtypes because atropine is an antagonist of $\alpha7$-like receptors (14). All solutions also contained 0.1 mg/ml BSA to reduce nonspecific adsorption of peptide. The perfusion medium could be switched to one containing peptide or ACh by use of a series of three-way solenoid valves (model 161T031, Neptune Research, Northboro, MA). All recordings were made at room temperature (≈22°C). Oocytes were harvested and injected with cRNA encoding rat neuronal and human muscle nAChR subunits as described previously (31, 32) except that Ala-278 of the $\alpha6/\alpha3$ chimera corrected to Val. ACh-gated currents were obtained with a two-electrode voltage-clamp amplifier (model OC-725B, Warner Instruments, Hamden, CT). The membrane potential was clamped at −70 mV, and the current signal, recorded through virtual ground, was low-pass-filtered (5-Hz cutoff) and digitized at a sampling frequency of 20 Hz.

To apply a pulse of ACh to the oocyte, the perfusion fluid was switched to one containing ACh for 1 s. This was automatically done at intervals of 1–2 min. The shortest time interval was chosen such that reproducible control responses were obtained with no observable desensitization. This time interval depended on the nAChR subtype being tested. The concentration of ACh was 200 μM for $\alpha7$, 10 μM for $\alpha1\alpha1\delta\varepsilon$ and $\alpha9\alpha10$, and 100 μM for all other subtypes. The ACh was diluted in ND96A for tests of all nAChR subtypes except $\alpha7$ and $\alpha9\alpha10$, in which case the diluent was ND96.

To measure conotoxin antagonism, peptide was bath-applied for 5 min before subsequent exposure to ACh. All ACh pulses contained no toxin, for it was assumed that little, if any, bound toxin would have washed away in the brief time (<2 s) it took for the responses to peak. The average peak amplitude of three control responses just preceding exposure to toxin was used to normalize the amplitude of each test response to obtain percent response as y axis values. Data were fitted by nonlinear regression to the equation $y = 100/(1 + [\text{toxin}]/\text{IC}_{50})^{n_H}$ with weighting by $1/y^2$ using Prism 3.0 for Macintosh (GraphPad, San Diego, CA). IC_{50} is the toxin concentration that produced half of the maximal block, and n_H is the Hill coefficient.

Statistical Analysis. Most data are presented as the mean ± SEM and were analyzed by using one-way ANOVA or Student's t test, where appropriate. Immunohistochemical data were analyzed by using Student's t test. The EC_{50} values for block of oocyte-expressed receptors were reported with their 95% confidence intervals.

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