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Nicotinic Acetylcholine Receptor Regulation of Spinal Norepinephrine Release

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Background: **Neuronal nicotinic acetylcholine receptor (nAChR) agonists produce antinociception in animals. nAChRs exist almost exclusively on presynaptic terminals in the central nervous system and stimulate neurotransmitter release. This study tested whether nAChR agonists stimulate spinal release of the neurotransmitter norepinephrine either by direct actions on noradrenergic terminals or indirectly by stimulating release of other neurotransmitters to induce norepinephrine release.**

Methods: **Adult male rats were anesthetized and microdialysis probes inserted in the L2–L4 dermatomes of the spinal cord. Probes were perfused with artificial cerebrospinal fluid containing nicotine, the specific $\alpha_4\beta_{2^*}$ nAChR agonist metanicotine, or nicotine plus nAChR antagonists and norepinephrine measured in the microdialysates. The effects of specific glutamate receptor antagonists and nitric oxide synthase inhibitors were also examined. To determine direct effects on noradrenergic terminals, synaptosomes were prepared from spinal cord and incubated with nAChR agonists and antagonists.**

Results: **Both nicotine and metanicotine induced norepinephrine release in spinal microdialsyates, an effect reduced by nicotinic antagonists but not glutamate antagonists or nitric oxide synthase inhibitors. Both of the nicotinic agonists stimulated norepinephrine release in synaptosomes, and the effect of metanicotine was blocked at lower concentrations of $\alpha_4\beta_{2^*}$- than α_{7^*}-preferring nAChR antagonists.**

Conclusion: **These results suggest that one mechanism by which nAChR agonists act for analgesia is to stimulate spinal norepinephrine release. They do so by actions on $\alpha_4\beta_{2^*}$ nAChRs, and perhaps other subtypes, most likely located on noradrenergic terminals, rather than by indirectly stimulating norepinephrine release through glutamate release or nitric oxide synthesis.**

NICOTINE has been known for many years to produce analgesia by an action in the central nervous system.[1] Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric proteins consisting of α (of which there are eight) and β (of which there are four) subunits. Distinct receptor subtypes, consisting of various combinations of α and β subunits, have been shown to coexist in many different brain areas.[2] Pharmacologic studies indicate that nAChR agonists produce analgesic effects predominantly through α_4 subunit–containing receptors.[3,4] This is further supported by lack of antinociception from nicotinic agonists in mice lacking the α_4 subunit protein[5] or in rats with antisense-induced acute knockdown of α_4 subunit protein.[6]

The mechanisms by which nAChR agonists produce analgesia are unknown. We previously observed that antinociception after intrathecal administration of a selective $\alpha_4\beta_{2^*}$ nAChR agonist, metanicotine, is partially reversed by phentolamine,[7] suggesting mediation *via* norepinephrine release. The vast majority of nAChRs in the central nervous system, if not all, are presynaptic, and their activation results in terminal depolarization and neurotransmitter release.[8] Nicotine and nicotinic nAChR agonists such as metanicotine and epibatidine induce norepinephrine release in brain, as measured by microdialysis *in vivo*[9,10] and hippocampus slice perfusion *in vitro*.[11] In addition, nicotine stimulates release of norepinephrine in cultured fetal rat neurons from the locus coeruleus,[12] the major source of noradrenergic innervation in the spinal cord.

The aforementioned studies support the possibility that a major mechanism of nicotinic agonist analgesia may be stimulation of norepinephrine release in the spinal cord. However, the effects of nAChR activation in the spinal cord need not be identical to that in the brain, and the only study of direct relevance to the spinal cord was performed in cultured fetal cells, which may differ in important ways from the adult. In addition, microdialysis and slice perfusion studies cannot distinguish between direct effects of nAChR activation on noradrenergic terminals from indirect effects. For example, nicotinic agonists stimulate glutamate release and nitric oxide synthesis in the spinal cord,[11,13,14] and both of these agents can induce norepinephrine release.[11,15] Indeed, norepinephrine release induced by nicotinic agonists in the hippocampus is thought to reflect glutamate release by α_7-containing nAChRs and subsequent stimulation of norepinephrine release by glutamate.[16] Therefore, the purpose of the current investigation was to examine the effect of nAChR activation on norepinephrine release, using both microdialysis perfusion *in vivo*, in which neuronal circuits are in place, and synaptosome release *in vitro*, where direct synaptic connections are lacking. The role of glutamate receptors and nitric oxide synthesis on norepinephrine release from nAChR stimulation was also examined.

Methods

Surgical Preparation and Procedures

Experiments were performed on male Sprague-Dawley rats (weight, 220–300 g). The protocols were approved

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by the Institutional Animal Care and Use Committee of the Wake Forest University School of Medicine (Winston-Salem, NC), and experimental procedures adhered to the Guide for the Care and Use of Laboratory Animals (US Public Health Service). For microdialysis experiments, anesthesia was induced with halothane in an induction chamber and maintained with α-chloralose (50–60 mg/kg administered intraperitoneally, followed by 20–25 mg/kg as indicated by hemodynamic changes) and sodium pentobarbital (20 mg/kg administered intraperitoneally). Adequate depth of anesthesia was verified by the absence of responses to noxious pinch of the paw. Supplemental doses of α-chloralose (20–25 mg/kg administered intravenously) were administered at hourly intervals. The trachea was cannulated, and respiration was controlled using 100% oxygen and a rodent ventilator. Paralysis was provided with 1 mg/kg intravenous pancuronium bromide. The left carotid artery was cannulated, and the arterial blood pressure was measured with a pressure transducer. A femoral vein was cannulated for intravenous injection of drugs. Body temperature was maintained in the range of 37–38°C with a heating lamp. The spinal cord was then exposed at the L5–L6 level *via* an 8-mm^2 laminotomy. A pin-shape commercial microdialysis probe (CMA-12; CMA/Microdialysis AB, Stockholm, Sweden) was inserted into spinal cord dorsal horn with a 45° angle to the dorsolateral surface to a depth of 2 mm. Animals were killed at the end of experiments by an intravenous injection of sodium pentobarbital.

Microdialysis

Microdialysis was performed with artificial cerebrospinal fluid using a syringe pump and a flow rate of 2 μl/min. After 30 min of recovery after implantation, two 20-min baseline samples were collected, followed by drug application by replacing the perfusion fluid in the pump syringe with artificial cerebrospinal fluid containing various drugs. Each drug application was applied for 40 min, comprising two 20-min samples. Each sample was collected into a vial containing 4 μl of 1 M perchloric acid.

Three series of experiments were performed. In the first, concentration responses to nicotine, metanicotine, and glutamate were determined. In the second, the stability of drug-induced effect was determined by perfusion with 10^{-2} and 10^{-4} M nicotine and metanicotine for 120 min, with sampling at 20-min intervals. In the third, the effect of 10^{-2} M nicotine alone or in the presence of nAChR antagonists, mecamylamine, methyllycaconitine (α_{7*}-preferring antagonist), and dihydro-β-erythroidine ($\alpha_2\beta_{4*}$-preferring antagonist), the *N*-methyl-D-aspartate antagonist 2-amino-5-phosphopentanoic acid (AP-5), the AMPA–kainate antagonist 6-cyano-7-nitroquinoxaline-2,3-disodium (CNQX), or the nitric oxide synthase inhibitor (1)-(2-triflurometyl-phenyl)imidazol (TRIM). The effects of AP-5 and CNQX on glutamate-induced norepinephrine release were also examined. Experiments were performed in 5–7 animals per group.

Microdialysis probes were handled and maintained according to company guidelines and were used for a total of two to three experiments, then discarded. The recovery rate of microdialysis was determined by *in vitro* experiments and *in vivo* norepinephrine reverse microdialysis in spinal cord on the day after each experiment. The recovery rate for the probes was 7.5 ± 2% (n = 20). At the end of experiments, the position of the probe was verified by gross inspection, then removal of the probe and sectioning of the cord. Data were used only from probes located in the dorsal horn of the spinal cord.

Norepinephrine Analysis

Samples (20 μl) were injected using an autosampler onto a 150 × 2.1-cm C18 column at a flow rate of 0.4 ml/min with a mobile phase consisting of 0.1 M phosphate, pH 3.8, with 4% methanol and 600 mg/l 1-octanesulfonic acid. Norepinephrine was determined using an electrochemical detector at 620 mv and 1.0 nA. The detection limit for norepinephrine is 0.2 pg/20-μl sample.

Synaptosomes

After induction of anesthesia with 1.5–2% inhalational halothane, animals were killed by decapitation, and the lumbar part of the spinal cord was quickly removed and placed in aerated (with 95% O_2–5% CO_2) ice-cold modified Krebs-bicarbonate buffer containing 118 mM NaCl, 3.3 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO4, 25 mM $NaHCO_3$, 10 mM HEPES, 5 mM ascorbic acid, 11.5 mM glucose, 30 μM EDTA, and 10 μM pargyline. The dorsal half of the spinal cord was selected and homogenized in 8 ml ice-cold 0.32 M sucrose. A crude synaptosomal pellet (P_2) was prepared by differential centrifugation at 2,000*g* followed by 20,000*g*.[17]

The crude P_2 pellet was resuspended into 4 ml modified Krebs buffer, loaded with norepinephrine in a 50-nM final concentration containing 20% [^{3}H]norepinephrine and incubated at 37°C for 5 min. Free norepinephrine was then removed by centrifugation at 15,000*g* for 10 min. The synaptosomal pellet was again suspended into 4.5 ml modified Krebs buffer, and 150 μl of the suspension was aliquoted into each test tube with 850 μl Krebs buffer containing desipramine at final concentrations of 0 or 10^{-5} M and similar concentrations of protein. The test tubes were then incubated for 10 min at 37°C in a 1-ml volume. At the end of incubation, the amount of [^{3}H] remaining in synaptosomes was determined by rapid filtration through GF/C glass filters presoaked for 30 min or more in 0.1% (vol/vol) polyethylenimine to reduce nonspecific binding. This was followed by three times 4-ml washes with ice-cold buffer in which glucose was substituted for NaCl. The bound (retained) radioac-

tivity was determined 24 h later (to reduce variability and increase counts) by scintillation counting. [^{3}H]norepinephrine release induced by nicotine was calculated from the amount of [^{3}H]norepinephrine remaining in the synaptosome after vehicle (100 μl buffer) compared with treatment with experimental compound. Fractional release was calculated as: (buffer − nicotine)/buffer.

Two types of experiments were performed. In the first, the effects of nicotine and metanicotine, 10^{-7} to 10^{-3} M, on [^{3}H]norepinephrine release were determined in the absence or presence of desipramine to prevent norepinephrine reuptake into the synaptosomes. In the second, we compared [^{3}H]norepinephrine release evoked by 10^{-4} M metanicotine alone to metanicotine plus dihydro-β-erythroidine or methyllycaconitine, 10^{-5} to 10^{-2} M. Each experiment was performed in duplicate, and each study consisted of at least five determinations.

Materials

L-[2,5,6-^{3}H]norepinephrine (2294 GBq) was purchased from New England Nuclear (Wilmington, DE). Bio Safe II scintillation cocktail was obtained from Research Product International Corp. (Mount Prospect, IL). $MgSO_4$, ascorbic acid, KCl, and glucose were obtained from Fisher Scientific (Fair Lawn, NJ). (−)Nicotine was purchased from Research Biochemical Incorporation (Natick, MA). Metanicotine was gift from RJR Nabisco (Winston-Salem, NC). Methyllycaconitine was obtained from Latoxan (Valence, France). AP-5, dihydro-β-erythroidine, CNQX, mecamylamine, TRIM, and the remaining chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Microdialysis samples after drug were compared with the average of the two baseline samples and converted to percent change for depiction in figures. However, analysis was performed on raw data, which were log transformed before analysis. Data are presented as mean ± SE. Concentration response curves and were analyzed by one-way analysis of variance, and time course experiments were analyzed by one-way repeated-measures analysis of variance. Antagonist–agonist combination data were compared with agonist alone using one-way analysis of variance. $P < 0.05$ was considered significant.

Results

Microdialysis: Concentration Response and Time Course

Norepinephrine in microdialysis perfusion in the absence of drugs was 2.1 ± 0.6 pg/50 μl (mean ± SD; range, 1.2–3.6 pg/50 μl). Perfusion of all three agonists in the spinal cord dorsal horn microdialysis fiber induced norepinephrine release in the dialysates (fig. 1). The

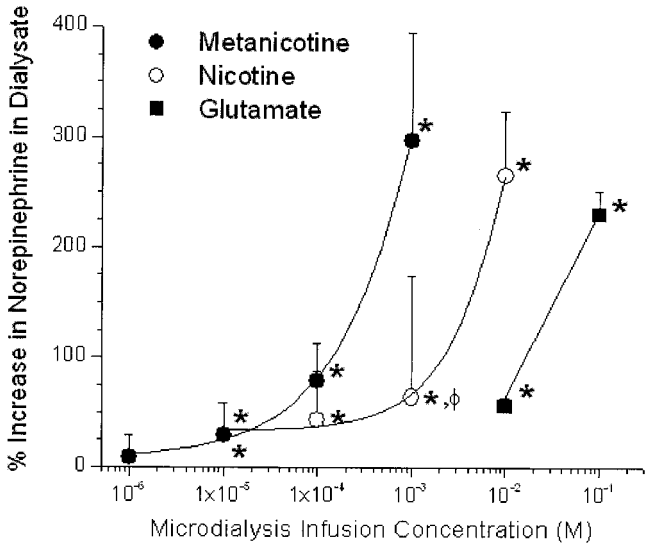

Fig. 1. Norepinephrine release induced by metanicotine (closed circles), nicotine (open circles), or glutamate (closed squares) perfused in microdialysis probes in the spinal cord dorsal horn. Each point represents the mean ± SE of five to six animals. *$P < 0.05$ compared with predrug infusion by one-way analysis of variance on the raw data. $\phi P < 0.05$ compared with metanicotine.

threshold concentration for norepinephrine release was less for nicotine and metanicotine (10^{-5} M) than it was for glutamate (10^{-2} M). Pilot data examining concentrations less than 10^{-5} M for nicotine or less than 10^{-3} M glutamate showed no norepinephrine release (data not shown). Concentration–response relations demonstrated a rank order of potency for norepinephrine release of metanicotine > nicotine > glutamate, with the potency of each agonist separated by the next by a factor of 10–20 (fig. 1). None of the treatments resulted in changes in either heart rate or blood pressure in the anesthetized animals.

There was a difference in the time course of norepinephrine release from continuous exposure between nicotine and metanicotine when they were perfused at 10^{-2} M (fig. 2). In this time course experiment, there was a greater initial release of norepinephrine from nicotine than metanicotine, unlike what was observed with the single, 40-min exposures used for the concentration response in figure 1. Metanicotine resulted in stable norepinephrine release over the 2-h exposure in this time course study, whereas norepinephrine release from nicotine exposure was significantly reduced at times beyond 1 h of continuous exposure (fig. 2). Infusion of lower concentrations of nicotine (or metanicotine) resulted in stable norepinephrine release over a 2-h period (fig. 2).

Microdialysis: Pharmacology of Norepinephrine Increase

To determine the role of nitric oxide synthesis and ionotropic glutamate receptors in norepinephrine re-

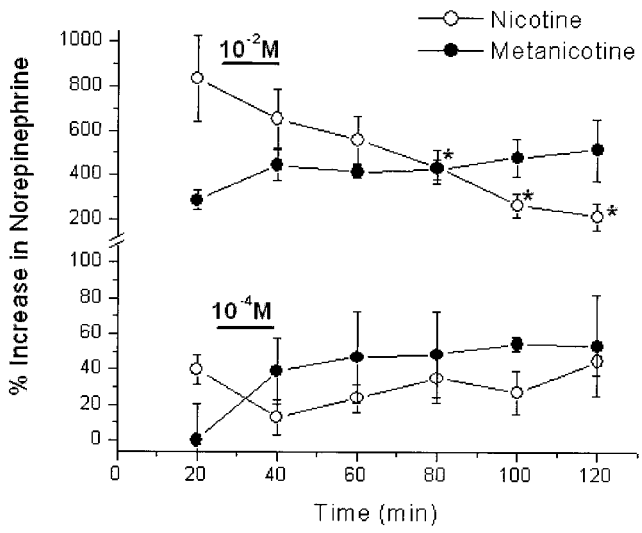

Fig. 2. Time course of norepinephrine release induced by 10^{-2} M (*top*) or 10^{-4} M (*bottom*) nicotine (open circles) or metanicotine (closed circles), continuously perfused in microdialysis probes in the spinal cord dorsal horn. Each point represents the mean ± SE of five to seven animals. *$P < 0.05$ compared with peak drug effect by predrug infusion by one-way repeated-measures analysis of variance on the raw data.

lease induced by nicotine, microdialysis perfusion was performed with nicotine alone (10 mM) or in the presence of the *N*-methyl-D-aspartate antagonist AP-5 (10 mM) or the nitric oxide synthase inhibitor TRIM (10 mM). Because of poor water solubility, it was not possible to achieve a high concentration of the AMPA–kainate antagonist CNQX for microdialysis perfusion. In this case, CNQX was administered intravenously in a dose demonstrated to block AMPA–kainate receptors (1 mg/kg). There was no difference in the percent increase in norepinephrine from 10 mM nicotine alone (470 ± 89%) compared with nicotine plus AP-5 (600 ± 140%), CNQX (390 ± 110%), or TRIM (430 ± 85%). In contrast, AP-5 and CNQX reduced norepinephrine release from perfusion of microdialysis probes with glutamate (10^{-2} M) from 56 ± 8% release in the absence of the antagonists to 22 ± 10% in their presence ($P < 0.05$), confirming their effect on spinal glutamate receptors at these doses.

Norepinephrine release from nicotine exposure (10^{-2} M) was reduced to a similar degree by three nAChR antagonists, mecamylamine (31 ± 8.5% inhibition), methyllycaconitine (39 ± 14% inhibition), and dihydro-β-erythroidine (43 ± 9.2% inhibition), when the antagonists were perfused at a concentration of 10^{-4} M. Because of the decrease in norepinephrine release from nicotine exposure beyond 1 h in microdialysis experiments, necessitating the study of only one concentration of antagonist per animal, further concentration–responses for antagonists were performed with synaptosomes, rather than microdialysis.

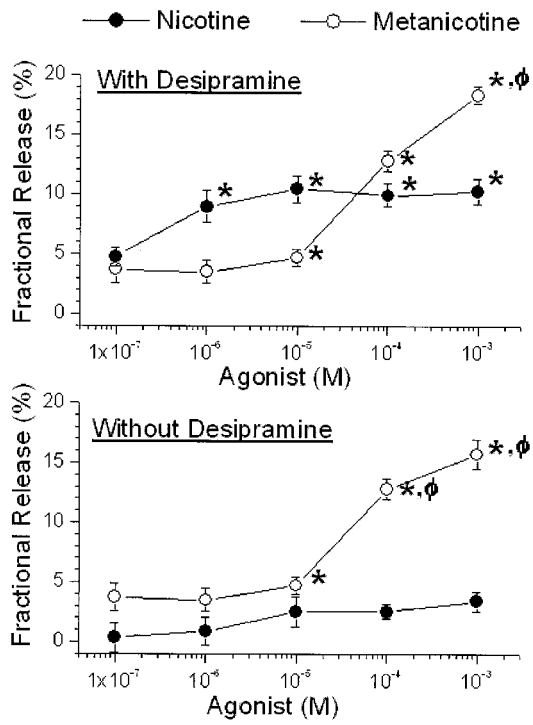

Fig. 3. Nicotine (open circles) and metanicotine (closed circles) induced release of norepinephrine from synaptosomes prepared from dorsal spinal cord in the presence (*top*) or absence (*bottom*) of the norepinephrine reuptake inhibitor desipramine (10^{-5} M). Each point represents the mean ± SE of seven experiments in duplicate. *$P < 0.05$ compared with control without nicotine. ϕ$P < 0.05$ compared with nicotine.

Synaptosome Experiments: Concentration Response and Effect of Desipramine

When administered alone to synaptosomes, metanicotine, but not nicotine, induced norepinephrine release (fig. 3, bottom). Based on the decrease in norepinephrine release over time from nicotine, but not metanicotine, observed in the microdialysis experiments (fig. 2), we reasoned that the lack of effect of nicotine could reflect rapid desensitization after exposure of high drug concentrations in the synaptosomes, allowing time for the initial burst of norepinephrine release to be taken back up into the synaptosome by the norepinephrine transporter. When the norepinephrine transporter inhibitor desipramine was added to the synaptosome preparation, norepinephrine release from nicotine exposure was easily demonstrable at lower concentrations than metanicotine (fig. 3, top).

Synaptosome Experiments: Pharmacology of Norepinephrine Release

Metanicotine-induced norepinephrine release was blocked in a concentration-dependent manner by the $\alpha_4\beta_{2*}$-preferring antagonist dihydro-β-erythroidine, with a threshold of 10^{-4} M (fig. 4). In contrast, only the highest concentration of the α_{7*}-preferring antagonist methyllycaconitine that was studied (10^{-2} M) reduced metanicotine-induced norepinephrine release (fig. 4).

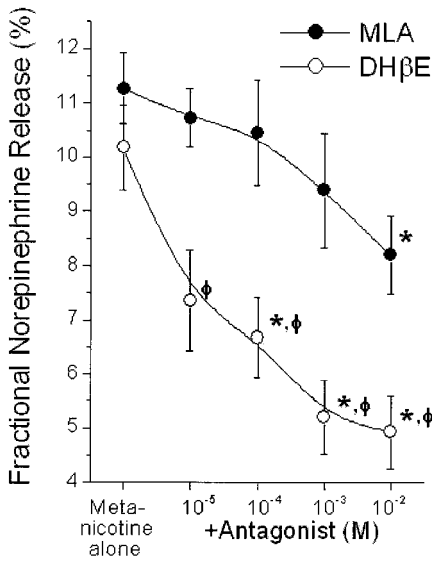

Fig. 4. Norepinephrine release from synaptosomes prepared from dorsal spinal cord by metanicotine alone (10^{-4} M) or in the presence of the α_{7*}-preferring antagonist methyllycaconitine (MLA; open circles) or the $\alpha_2\beta_{4*}$-preferring antagonist dihydro-β-erythroidine (DHβE; closed circles). Each point represents the mean ± SE of five to seven experiments. *$P < 0.05$ compared with metanicotine alone. $\phi P < 0.05$ compared with DHβE.

Discussion

Although analgesic actions of noradrenergic and cholinergic agonists have long been recognized, the receptor subtypes, mechanisms of action, neuronal circuits, and pharmacologic interactions between these systems remain unclear. The current results provide new information regarding interactions at the spinal level that carry important implications both for nicotinic and noradrenergic analgesia.

Systemic Nicotinic Analgesia: Interaction with Norepinephrine and Receptors Involved

Because the observations that epibatidine, a natural toxin secreted by South American poisonous frogs, and ABT-594, a synthetic analog, are both powerful analgesics and selective nAChR agonists,[18] the mechanisms by which these agents act have been extensively examined. Several interactions, including dopaminergic, serotonergic, γ-aminobutyric acid–mediated, noradrenergic, cholinergic, and glutamatergic, have been explored, primarily with systemic drug administration.[19–21]

Activation of descending spinal noradrenergic pathways plays the major role in analgesia from systemically administered nAChR agonists. Thus, antinociception from systemic or intracerebroventricular nicotine is blocked by destruction of noradrenergic neurons by the neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4)[22] or by adrenoceptor inhibition with yohimbine.[23] Similarly, antinociception from systemic or intracerebroventricular administration of selective $\alpha_4\beta_{2*}$ ligands are inhibited by DSP-4 treatment[22] and by intrathecal injection of an α-adrenergic antagonist.[24]

The current study suggests that one site of nAChR-induced spinal noradrenergic activation is nerve terminals in the spinal cord itself. Thus, nicotine stimulates spinal norepinephrine release from local spinal cord tissue administration *in vivo* (microdialysis) as well as in a preparation where local as well as descending circuits are disrupted (spinal synaptosomes). nAChR agonists are known to stimulate norepinephrine release in brain, which can occur by direct actions on noradrenergic neurons or fibers themselves or by indirect actions on glutamatergic terminals, whereby the released glutamate acts on noradrenergic terminals.[16] Results from the current study are most consistent with direct effects on noradrenergic terminals, since the effect *in vivo* was not blocked by glutamate receptor antagonists or by a nitric oxide synthase inhibitor. Similarly, the pharmacology of nAChR-induced norepinephrine release in the spinal cord is not consistent with α_{7*} receptor activation (see below), which is the cause of indirect norepinephrine release *via* glutamate in the brain.[16]

The two most prevalent nAChR subtypes in the central nervous system are the α_{7*} (α-bungarotoxin–sensitive) and the $\alpha_4\beta_{2*}$ types. The current study is most consistent with $\alpha_4\beta_{2*}$ subtypes producing spinal norepinephrine release. Although the relative potencies varied with experimental model and conditions in the current study, the $\alpha_4\beta_{2*}$-preferring agonist metanicotine was at least as potent as the nonselective agonist, nicotine, *in vivo* and *in vitro*. Similarly, the $\alpha_4\beta_{2*}$-preferring antagonist dihydro-β-erythroidine was more potent *in vitro* than the α_{7*}-preferring antagonist methyllycaconitine at blocking norepinephrine release from metanicotine. However, we recognize that dihydro-β-erythroidine could, at the concentrations used, also affect $\alpha_3\beta_4$ receptors.[25] These results are consistent with failure of methyllycaconitine to block antinociception from nicotine[22] (but also see Damaj *et al.*[26]), potent antinociception from $\alpha_4\beta_{2*}$-selective agonists,[18] and $\alpha_4\beta_{2*}$ pharmacology of norepinephrine release in the locus coeruleus,[27] a major source of spinal noradrenergic innervation.

Spinal Nicotinic Analgesia: Interaction with Norepinephrine and Receptors Involved

Spinally administered nAChR agonists are often reported to elicit behaviors consistent with increased nociception and hypersensitivity as well as antinociception, and these two effects differ in nAChR subtype pharmacology.[28] Pronociception has been speculated to reflect C-fiber activation, because nAChRs exist on capsaicin-sensitive primary afferents[29] and nAChR agonists stimulate neurotransmitter release from sensory afferent-derived cells in culture.[30] On the other hand, nAChR agonists produce antinociception in a variety of pain models after systemic or intrathecal injection, and the

net effect of spinal nAChR stimulation could differ from direct effects on isolated afferent neurons.

Activation of spinal noradrenergic terminals may play an important role in antinociception from intrathecally administered nAChR agonists. Thus, antinociception from intrathecal metanicotine is inhibited by the noradrenergic antagonist phentolamine,[7] and antinociception from the intrathecal carbachol is inhibited by noradrenergic terminal destruction by DSP-4.[31] The current study suggests that direct stimulation of spinal noradrenergic terminals by nicotinic agonists after intrathecal administration underlies their antinociceptive effects.

Spinal Noradrenergic Analgesia: Interaction with Nicotinic Acetylcholine Receptors and Glutamate

nAChR-induced spinal norepinephrine release is also relevant to noradrenergic analgesic mechanisms. Spinally released norepinephrine produces analgesia by acting on α_2-adrenergic receptors,[32] and intrathecal administration of α_2-adrenergic agonists produces analgesia.[33] Spinal α_2-adrenoceptor stimulation results in acetylcholine release in animals and humans,[34,35] and antinociception from intrathecal α_2-adrenergic agonists is partially inhibited by intrathecal mecamylamine in the setting of peripheral nerve injury.[36] Thus, we previously proposed the following spinal cascade in analgesia:

NE → α2-adrenoceptor → ACh →

nicotinic/muscarinic receptors

The current results indicating nAChR-induced norepinephrine release suggest that this cascade can result in a feed-forward mechanism, wherein norepinephrine release stimulates acetylcholine release, which in turn further stimulates norepinephrine release. Further characterization of this proposed mechanism and definition of its controls are currently being examined in our laboratory.

It has long been recognized that painful stimuli increase spinal release of norepinephrine and acetylcholine, both in animals and in humans.[37,38] It is conceivable that glutamate release from primary afferents during painful stimulation could underlie acetylcholine release, leading to norepinephrine release by an nAChR-mediated mechanism on noradrenergic terminals, as indicated in the current study, or directly by glutamate stimulating norepinephrine release.[15] We believe this is unlikely, because spinalization abolishes nociception-induced spinal norepinephrine release[38] and because glutamate was very ineffective at stimulating spinal norepinephrine release directly in the current study.

Nicotinic Acetylcholine Receptor Desensitization

Continuous exposure to nicotine results in complex changes in response. One can observe desensitization of receptors *in vitro* in milliseconds to seconds[39] and in minutes *in vivo*.[40] Chronic exposure over days to weeks results in up-regulation of receptor number, thereby maintaining response to agonist in the face of desensitization.[41] The reduction in norepinephrine release from nicotine exposure over 120 min *in vivo* and the lack of norepinephrine release by nicotine *in vitro* in the absence of norepinephrine transporter blockade likely represents desensitization. In contrast, we previously demonstrated that prolonged intrathecal injection of metanicotine results in enhanced, not decreased, response, likely reflecting receptor up-regulation.[7] In addition, it is conceivable that reduced desensitization from metanicotine compared with nicotine could have resulted in the apparent increased potency of metanicotine *in vivo* in the microdialysis experiments. Thus, whether prolonged intrathecal administration of nicotinic agonists would result in rapid dose escalation in humans is uncertain from these *in vitro* and *in vivo* studies in rodents.

In summary, local administration of nicotine or the $\alpha_4\beta_{2^*}$-preferring agonist metanicotine induces release of norepinephrine in rat spinal cord *in vivo* and *in vitro*. Antagonist studies are consistent with a direct effect on noradrenergic terminals of nicotine, most likely on an $\alpha_4\beta_{2^*}$ type of nAChR. There is some evidence that desensitization of the response to nicotine, but not metanicotine, occurs, although the relevance of this observation to prolonged systemic or intrathecal treatment with this class of analgesics is uncertain. These results further support mutually reinforcing effects of cholinergic and α_2-adrenergic mechanisms in the spinal cord that may be manipulated to provide analgesia.

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