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Spinal α2-Adrenoceptor-mediated Analgesia in Neuropathic Pain Reflects Brain-derived Nerve Growth Factor and Changes in Spinal Cholinergic Neuronal Function

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ABSTRACT

Introduction: Spinal α2-adrenoceptor stimulation produces analgesia in neuropathic pain states, and this effect in animals is blocked by the inhibitors of brain-derived neurotrophic factor (BDNF) function. In rats, α2-adrenoceptor stimulation normally inhibits acetylcholine release, but it excites release after nerve injury. The authors examined the roles of BDNF and excitatory Gs-protein in this change.

Methods: Male rats underwent L5–L6 spinal nerve ligation (SNL), and their lumbar spinal dorsal horns with or without spinal BDNF infusion were used for either synaptosome preparation for acetylcholine release or immunostaining for choline acetyltransferase.

Results: SNL did not alter spontaneous release from synaptosomes or choline acetyltransferase immunoreactivity in the spinal dorsal horn, but it reduced KCl-evoked acetylcholine release. Dexmedetomidine inhibited KCl-evoked acetylcholine release in synaptosomes from normal rats, but it excited KCl-evoked release in synaptosomes from SNL rats, and both effects were blocked by the α2-adrenoceptor antagonist idazoxan. Spinal infusion of an antibody to BDNF reduced choline acetyltransferase immunoreactivity in the spinal dorsal horn in both normal and SNL rats and abolished facilitation of KCl-evoked acetylcholine release by dexmedetomidine in SNL rats. Dexmedetomidine facilitation of acetylcholine release was also blocked by the inhibitors of Gs function.

Discussion: The increased reliance of spinal α2 adrenoceptors on cholinergic stimulation to cause analgesia after nerve injury reflects in part a shift from direct inhibition to direct excitation of spinal cholinergic neurons. The authors' results suggest that this shift relies on an interaction with Gs-proteins and BDNF.

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What We Already Know about This Topic

- ❖ Spinal α2-adrenoceptor agonists cause analgesia in neuropathic pain by stimulating acetylcholine release
- ❖ Nerve injury causes a shift from direct inhibition to excitation of spinal acetylcholine release by α2-adrenoceptor agonists

What This Article Tells Us That Is New

- ❖ In rats, the shift from direct inhibition to excitation of spinal acetylcholine release by α2-adrenoceptor agonists depends on brain-derived neurotrophic factor and on an interaction with the stimulatory G-protein, Gs

BETTER treatment for chronic neuropathic pain has been sought for decades. However, only a few drugs, including oral gabapentin and monoamine reuptake inhibitors and epidural clonidine, have been approved to treat chronic neuropathic pain. These drugs all share a common mechanism that involves the stimulation of spinal α2 adrenoceptors, which in turn results in the spinal release of acetylcholine to relieve neuropathic pain.[1–7] We have demonstrated previously that inhibitory M2 subtype muscarinic receptors are up-regulated in primary sensory afferents after nerve injury.[8] Thus, the activation of a spinal noradrenergic–cholinergic interaction is a key strategy to treat neuropathic pain.

Activation of α2 adrenoceptors directly reduces pain transmission by reducing the release of pronociceptive transmitter including substance P and glutamate from primary afferent terminals[9] and by hyperpolarizing spinal interneurons *via* G-protein-mediated activation of potassium channels.[10] This occurs in both normal and neuropathic animals. In contrast, the α2-adrenergic–cholinergic circuit predominates as a mechanism for analgesia after peripheral nerve injury. We previously demonstrated that clonidine inhibits acetylcholine release in spinal cord slices and synaptosomes in normal rats, consistent with the classic inhibitory effect of the G-protein-coupled α2 adrenoceptors.[3] Surprisingly, after peripheral nerve injury, clonidine enhances rather than inhibiting acetylcholine release from these preparations in rats with a peripheral nerve injury model of neuropathic pain,[3] consistent with behavioral observations that intrathecal atropine abolishes the analgesic effect of intrathecal clonidine in nerve-injured rats, but not in normal rats.[5,6]

α2 Adrenoceptors normally activate inhibitory, pertussis toxin-sensitive Gi/o-proteins to reduce cyclic adenosine monophosphate production and reduce neurotransmitter release.[11] Under some conditions, α2 adrenoceptors have been shown to couple with stimulatory Gs-proteins,[12,13] which activate voltage-gated Ca^{2+} channels and could enhance Ca^{2+}-dependent neurotransmitter release. We have shown previously, using quantitative ligand binding, that the total number of α2 adrenoceptors in the spinal cord is unchanged after nerve injury, but that the efficacy of G-protein coupling from spinal α2 adrenoceptors increases.[14] One goal of the current study was to test whether α2-adrenoceptor-mediated facilitation of acetylcholine release after nerve injury requires activation of Gs-proteins.

Brain-derived neurotrophic factor (BDNF) may provide the stimulus for the shift in action of α2-adrenoceptor agonists on cholinergic neurons. We recently demonstrated that peripheral nerve injury increases descending noradrenergic axon density *via* BDNF-dependent mechanisms in rats.[15] Interestingly, spinal infusion of BDNF antibody not only blocked the increase in noradrenergic axon density but also reduced the analgesic efficacy of the spinally administered clonidine after nerve injury.[15] A final goal of the current study was to test whether BDNF is involved in the shift in α2-adrenoceptor agonist effect on acetylcholine release after nerve injury and in the expression of the synthetic enzyme for acetylcholine, choline acetyltransferase (ChAT) in the spinal dorsal horn.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 180–280 g were used. All experiments were approved by the Animal Care and Use Committee at Wake Forest University (Winston Salem, North Carolina). Animals were housed under a 12-h light–dark cycle, with free access to food and water.

Surgical Preparations

Spinal Nerve Ligation. As described previously,[16] animals were anesthetized with 2% isoflurane in oxygen, the right L6 transverse process was removed, and the right L5 and L6 spinal nerves were tightly ligated using 5-0 silk suture. Animals were allowed to recover for 3–14 days.

Anti-BDNF Treatment. Animals were anesthetized with 2% isoflurane, and intrathecal catheterization was performed as described previously.[17] A small puncture was made in the atlanto-occipital membrane of the cisterna magnum, and a 7.5-cm polyethylene catheter (ReCathCO LLC, Allison Park, PA) was inserted, so that the caudal tip reached the lumbar enlargement of the spinal cord. Animals were allowed at least 5 days to recover from the surgery. As described previously,[15] a sheep polyclonal antibody to BDNF (5 μg/day, AB1513p; Chemicon, Temecula, CA) or control sheep IgG (5 μg/day; Sigma Chemical CO., St. Louis, MO) was delivered in sterile saline *via* osmotic minipump (0.5 μl/h; model 2002; Alzet, Cupertino, CA). One day before spinal nerve ligation (SNL) surgery, animals were anesthetized, and the osmotic minipump was implanted subcutaneously on the back of the rat and connected to the intrathecal catheter. After 11 days of spinal infusion, animals were used for synaptosome studies or immunohistochemistry.

Acetylcholine Release from Synaptosomes

Crude synaptosomes from the lumbar spinal dorsal horn were prepared as described previously[3] with minor modifications. During deep anesthesia with 5% isoflurane, animals were killed by decapitation, and a 1-cm length of the spinal cord containing the lumbar enlargement, measured by ruler, was quickly removed and placed in oxygenated (with 95% O_2–5% CO_2) ice-cold Krebs buffer containing 124 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose, pH 7.35. The dorsal quadrants of the spinal cord ipsilateral and contralateral to SNL were removed and separately homogenized in ice-cold sucrose (0.32 M)–HEPES (10 mM) buffer, pH 7.4. Each synaptosome preparation contained four dorsal quadrants of the spinal cord, either unilateral from four SNL rats or bilateral from two normal rats. The initial homogenate was centrifuged at 1,000*g* for 5 min, and the resulting supernatant was centrifuged again at 10,000*g* for 12 min. The supernatant was discarded, and the pellet was resuspended in Krebs buffer. Acetylcholine release from synaptosomes was determined after loading with [^{3}H]choline, which results in rapid uptake of choline, acetylation to acetylcholine, and release of [^{3}H]acetylcholine with depolarization.[18,19] However, we did not confirm in the current experiments that released radioactivity was completely in the form of [^{3}H]acetylcholine. After incubation with 1 μM choline chloride (combination of both triturated and unlabeled choline) for 20 min at 37°C, the synaptosome-containing solution was centrifuged at 10,000*g* for 5 min, and the pellet was resuspended in Krebs buffer. Each preparation was divided into six equal aliquots and placed on Whatman filters in temperature-controlled perfusion chambers (SF-12; Brandel, Gaithersburg, MD). Synaptosomes were perfused with Krebs buffer (0.67 ml/min) for 20 min to remove free radioactivity, and then fractions were collected every 5 min for 20 min. Antagonists for α2 adrenoceptors (1 μM idazoxan) or Gs function (10 μM suramin or 10 μM NF449) were present during perfusion, including the washout period. After a 10-min baseline collection, synaptosomes were perfused with dexmedetomidine alone for 2 min and then stimulated with 12 mM KCl-Krebs buffer (115 mM NaCl, 12 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose, pH 7.35) containing dexmedetomidine for 3 min. An inhibitor of acetylcholine transporter hemicholinium-3 (10 μM) was present during perfusion to inhibit reuptake of acetylcholine. [^{3}H]Acetylcholine release from synaptosomes in each fraction was measured by a liquid scintillation counter (LS6500; Beckman Coulter Inc., Fullerton, CA). In some experiments, total radioactivity in each preparation was calculated from radioactivity present in the collected fractions and filters from six chambers. All chemicals were purchased from Sigma Chemical CO., except [^{3}H]choline (PerkinElmer, Waltham, MA) and NF449 (Tocris Bioscience, Ellisville, MO). Dexmedetomidine, idazoxan, suramin, and NF449 were dissolved in distilled water and then diluted with Krebs buffer.

Immunohistochemistry

Immunostaining for ChAT was performed in the sheep immunoglobulin G (IgG) or BDNF antibody-infused spinal cord of normal and SNL rats, as described previously[20] with minor modifications. During deep anesthesia with 100 mg/kg pentobarbital, animals were perfused intracardially with cold phosphate-buffered saline containing 1% sodium nitrite and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The L4–L6 spinal cord was removed, postfixed in the same fixative for 3 h, and cryoprotected with 30% sucrose in 0.1 M phosphate buffer for 48 h at 4°C. Tissues were then sectioned on a cryostat at 40-μm thickness. After being pretreated with 0.3% hydrogen peroxide and 1.5% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), the sections were incubated for 24 h at 4°C in a goat anti-ChAT antibody (1:500, AB144P; Millipore, Billerica, MA) in 1.5% normal donkey serum. Subsequently, the sections were incubated in biotinylated donkey anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA), processed using Elite Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions and then developed by the standard glucose oxidase-nickel method.

For quantification of ChAT immunoreactivity, four to five L4–L6 spinal cord sections were randomly selected from each rat. Images of both ipsilateral and contralateral dorsal horns of SNL or normal rats were captured using a digital charge-coupled device camera. By using image analysis software (SigmaScan Systat Software, San Jose, CA), pixels of ChAT-immunoreactive objects within the area of the dorsal horn containing lamina I–IV were quantified based on a constant threshold of optical density. Data are expressed as a percentage of ChAT-immunoreactive pixels in total pixels of the quantified area. The person performing image analysis was blinded to treatment.

Statistical Analyses

Data were normally distributed and are presented as mean ± SE. Differences among groups were determined using one- or two-way analysis of variance (ANOVA) as appropriate. A P value of less than 0.05 was considered significant. SigmaStat, version 3.0 (Systat Software) was used for data analysis.

Results

Synaptosome Study

Total [^{3}H]choline uptake (sum of released radioactivity and that retained in filters) in the synaptosome preparation did not differ between normal rats (78,000 ± 1,500 cpm, n = 14) and SNL rats 14 days after SNL surgery (SNL-ipsilateral, 79,000 ± 2,700 cpm; SNL-contralateral, 76,000 ± 2,900 cpm, n = 16). SNL also did not alter spontaneous [^{3}H]acetylcholine release from synaptosomes compared with normal (total radioactivity: normal, 1.69 ± 0.17%; SNL-ipsilateral, 1.70 ± 0.15%; SNL-contralateral, 1.74 ± 0.21%).

In synaptosomes from normal animals, KCl increased [^{3}H]acetylcholine release in a concentration-dependent manner (fig. 1A). At 14 days after SNL in synaptosomes ipsilateral to surgery, the concentration response of KCl on

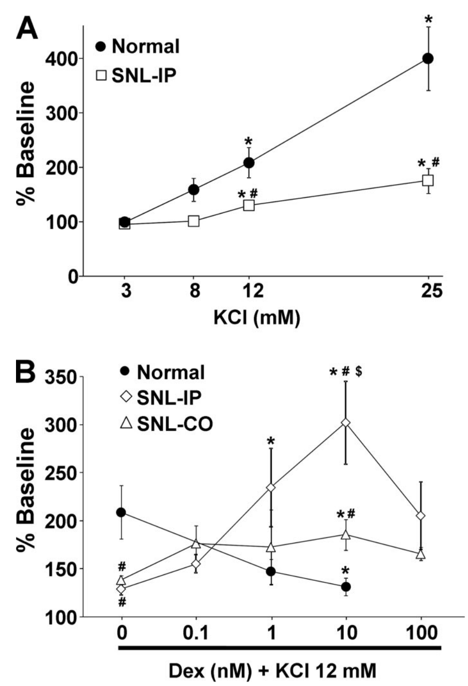

Fig. 1. Spinal nerve ligation (SNL) reduced potassium chloride (KCl)-evoked acetylcholine release and altered dexmedetomidine (Dex) action in the spinal dorsal horn synaptosomes. Data are presented as percentage of baseline release. SNL synaptosomes were prepared at 14 days after surgery. (*A*) Concentration response of KCl on acetylcholine release from synaptosomes ipsilateral to SNL (SNL-intraperitoneal, n = 6–18) significantly differs from the normal (n = 9–14) by two-way analysis of variance (ANOVA) ($P < 0.001$). * $P < 0.05$ *versus* 3 mM KCl by one-way ANOVA. # $P < 0.05$ *versus* normal by two-way ANOVA. (*B*) Concentration response of Dex on 12 mM KCl-evoked acetylcholine release from SNL synaptosomes ipsilateral to surgery (SNL-intraperitoneal, n = 9–18) significantly differs from those of the normal (n = 6–12, $P < 0.01$) and contralateral synaptosomes (SNL-CO, n = 9–18, $P < 0.05$) by two-way ANOVA. * $P < 0.05$ *versus* vehicle by one-way ANOVA. # $P < 0.05$ *versus* normal and $ $P < 0.05$ *versus* SNL-CO by two-way ANOVA. SNL-CO = spinal nerve ligated-contralateral; SNL-IP = spinal nerve ligated-ipsilateral.

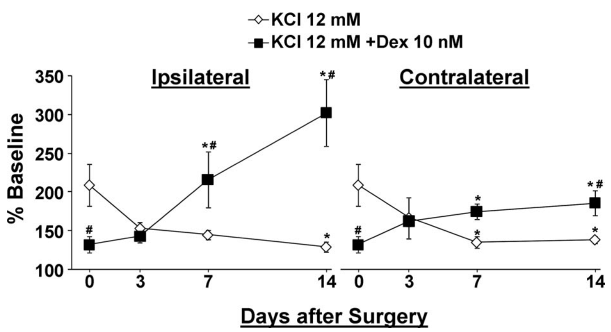

Fig. 2. Time course change of synaptosomal acetylcholine release after nerve injury in response to potassium chloride (KCl) with or without dexmedetomidine (Dex). Synaptosomes were prepared from normal (day 0) or spinal nerve ligation (SNL) rats (days 3, 7, and 14) and treated with 12 mM KCl (n = 6–18) or a combination of 12 mM KCl with 10 nM Dex (KCl + Dex, n = 6–12). Data are presented as percentage of baseline release. Time course in acetylcholine release in response to KCl + Dex in synaptosomes ipsilateral to SNL differs from that of contralateral synaptosomes by two-way analysis of variance (ANOVA) ($P < 0.05$). * $P < 0.05$ *versus* day 0 by one-way ANOVA. # $P < 0.05$ *versus* KCl by two-way ANOVA.

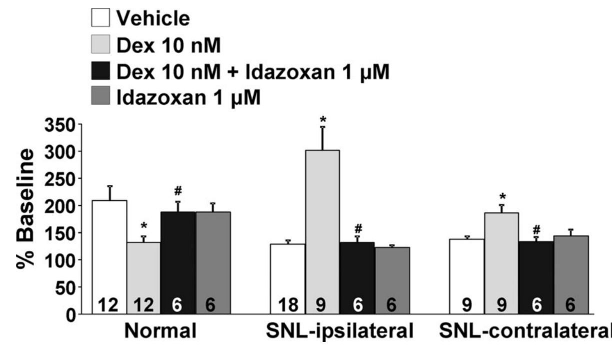

Fig. 3. Idazoxan blocked facilitatory and inhibitory effects of dexmedetomidine on potassium chloride (KCl)-evoked acetylcholine release from normal and spinal nerve ligation (SNL) synaptosomes. Data are presented as percentage of baseline release. SNL spinal dorsal horn synaptosomes were prepared at 14 days after surgery. Synaptosomes were treated with 12 mM KCl in the presence of vehicle, dexmedetomidine (Dex), idazoxan, or their combination (Dex + Idazoxan). * $P < 0.05$ *versus* vehicle and # $P < 0.05$ *versus* Dex by one-way analysis of variance.

[^{3}H]acetylcholine release significantly differed from that in synaptosomes from normal rats (fig. 1A; $P < 0.001$). Dexmedetomidine inhibited KCl-evoked [^{3}H]acetylcholine release in a concentration-dependent manner in synaptosomes from normal rats, but it enhanced evoked release in synaptosomes ipsilateral to SNL surgery (fig. 1B). KCl-evoked [^{3}H]acetylcholine release was also decreased in synaptosomes contralateral to SNL surgery (fig. 1B, $P < 0.05$), and dexmedetomidine also increased KCl-evoked [^{3}H]acetylcholine release in synaptosomes contralateral to surgery, but to a lesser extent than in those ipsilateral to SNL surgery (fig. 1B; $P < 0.05$). Dexmedetomidine-induced enhancement of evoked [^{3}H]acetylcholine release peaked at 10 nM and decreased at 100 nM. On the basis of these results, we selected 12 mM KCl and 10 nM dexmedetomidine for the subsequent studies.

We next examined the time course of changes in KCl-evoked [^{3}H]acetylcholine release and the effect of dexmedetomidine on this release after SNL surgery. KCl-evoked [^{3}H]acetylcholine release decreased in a time-dependent manner in synaptosomes from both sides of the spinal cord after SNL surgery (fig. 2). The effect of dexmedetomidine on KCl-evoked [^{3}H]acetylcholine release shifted from inhibition in normal animals (day 0) to no effect at 3 days after SNL surgery to enhancement by 7 days after surgery in synaptosomes from dorsal spinal cord ipsilateral to injury (fig. 2; $P < 0.05$). A similar pattern, although of lesser magnitude, was observed in the spinal cord tissue contralateral to injury (fig. 2; $P < 0.05$).

The selective α2-adrenoceptor antagonist idazoxan (1 μM), which did not affect KCl-evoked [^{3}H]acetylcholine release alone, abolished the inhibitory effect of dexmedetomidine on KCl-evoked [^{3}H]acetylcholine release in normal rats and its facilitation after SNL in both ipsilateral and contralateral to surgery (fig. 3). Dexmedetomidine alone, in the absence of KCl, slightly but significantly increased [^{3}H]acetylcholine release in synaptosomes ipsilateral and contralateral to SNL (fig. 4; $P < 0.05$). This direct excitatory effect of dexmedetomidine on basal [^{3}H]acetylcholine release was also blocked by idazoxan (fig. 4). Perfusion of synaptosomes with the selective Gs inhibitors, suramin (10 μM) and NF449 (10 μM), neither of which affected KCl-evoked [^{3}H]acetylcholine release alone, blocked the facilitatory effect of dexmedetomidine on KCl-evoked [^{3}H]acetylcholine release from synaptosomes ipsilateral to surgery (fig. 5). In synaptosomes contralateral to surgery, suramin treatment significantly decreased dexmedetomidine effect ($P < 0.05$).

To examine whether blockade of BDNF signaling affects the facilitatory effect of dexmedetomidine on KCl-evoked

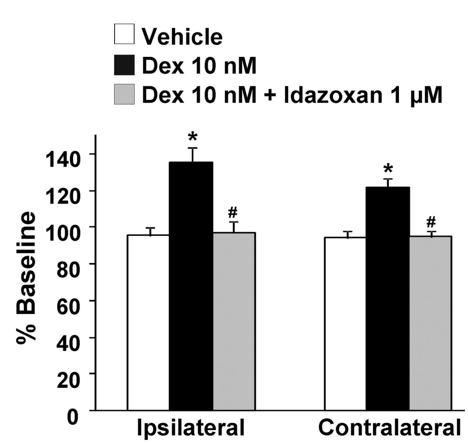

Fig. 4. Dexmedetomidine (Dex) increased basal acetylcholine release from spinal nerve ligation (SNL) synaptosomes. Data are presented as percentage of baseline release. SNL spinal dorsal horn synaptosomes were prepared at 14 days after surgery. Synaptosomes (n = 6 in each group) were treated with vehicle, Dex, or Dex and Idazoxan. * $P < 0.05$ *versus* vehicle and # $P < 0.05$ *versus* Dex by one-way analysis of variance.

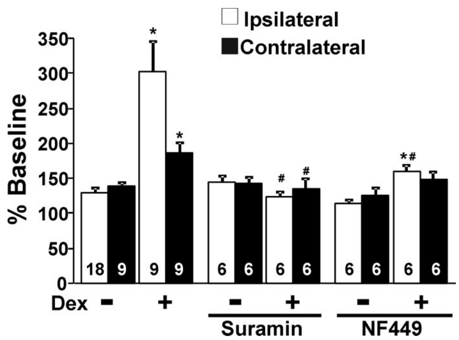

Fig. 5. Gs (stimulatory G-protein) inhibitors blocked facilitatory effect of dexmedetomidine (Dex) on potassium chloride (KCl)-evoked acetylcholine release from spinal nerve ligation (SNL) synaptosomes. Data are presented as percentage of baseline release. SNL synaptosomes were prepared at 14 days after surgery and treated with 12 mM KCl or a combination of 12 mM KCl with 10 nM Dex in the presence or absence of suramin (10 μM) or NF449 (10 μM). Suramin and NF449 were present throughout the perfusion. * $P < 0.05$ *versus* KCL alone and # $P < 0.05$ *versus* Dex alone by one-way analysis of variance.

[^{3}H]acetylcholine release in synaptosomes after nerve injury, animals received continuous intrathecal infusion of BDNF antibody (5 μg/day, n = 8) or control IgG (5 μg/day, n = 8) starting from 1 day before SNL and synaptosome experiments were performed at 10 days after the surgery. Because we only made two synaptosome preparations in each group, we did not compare total [^{3}H]choline uptake and basal [^{3}H]acetylcholine release between treatments. In IgG-treated animals, dexmedetomidine significantly enhanced KCl-evoked [^{3}H]acetylcholine release bilaterally compared with KCl alone (fig. 6). In BDNF antibody-treated animals, KCl-evoked [^{3}H]acetylcholine release did not differ from IgG-treated animals, but dexmedetomidine enhancement of release was absent.

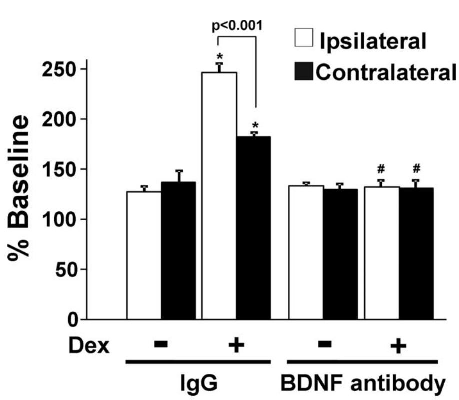

Fig. 6. Spinal infusion of brain-derived neurotrophic factor (BDNF) antibody blocked facilitatory effect of dexmedetomidine (Dex) on potassium chloride (KCl)-evoked acetylcholine release from spinal nerve ligation (SNL) synaptosomes. SNL animals were treated with a spinal infusion of BDNF antibody (5 μg/day, n = 8) or immunoglobulin G (IgG) (5 μg/day, n = 8), as detailed in Materials and Methods. SNL spinal dorsal horn synaptosomes were prepared at 10 days after surgery and treated with 12 mM KCl or a combination of 12 mM KCl with 10 nM Dex (n = 6 in each group). * $P < 0.05$ *versus* KCL alone and # $P < 0.05$ *versus* IgG by one-way analysis of variance.

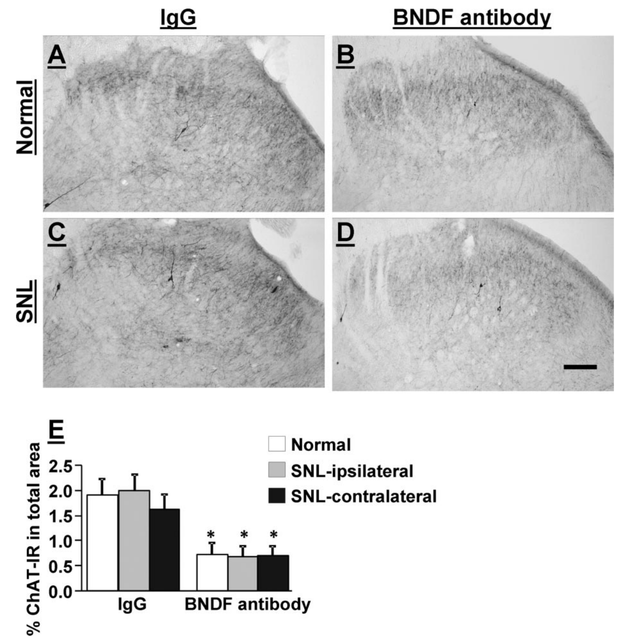

Fig. 7. Spinal infusion of brain-derived neurotrophic factor (BDNF) antibody reduced choline acetyltransferase immunoreactivity (ChAT-IR) in the spinal dorsal horn. (*A–D*) Photomicrographs depict ChAT-IR in the right L5 spinal dorsal horns from normal (*A*, *B*) and spinal nerve ligation (SNL) (*C*, *D*) rats treated with a spinal infusion of immunoglobulin G (IgG) (5 μg/day) or BDNF antibody (5 μg/day) for 11 days. (*E*) Quantification of ChAT-IR in the L4–L6 spinal dorsal horn of normal and SNL rats (n = 4 in each group). Scale bar = 100 μm. * $P < 0.05$ *versus* IgG by one-way analysis of variance.

Immunohistochemistry for ChAT

Figures 7A–D depict ChAT immunoreactivity in the L5 spinal dorsal horn in normal and SNL rats treated with a spinal infusion of IgG (5 μg/day) or BDNF (5 μg/day) for 11 days. ChAT immunoreactivity in the L5 spinal dorsal horn was found mainly in the axons, but a few ChAT-positive cells were also found in both normal and SNL animals. In IgG-treated groups (n = 4), there was no difference between tissues from normal and SNL rats in ChAT immunoreactivity in the spinal dorsal horn (fig. 7E). In BDNF antibody-treated groups (n = 4), ChAT immunoreactivity in the spinal dorsal horn significantly decreased similarly in both normal and SNL rats compared with the IgG-treated group ($P < 0.05$).

Discussion

Several lines of evidence support a key role for spinal $\alpha 2$ adrenoceptors and spinal cholinergic activation in analgesia from various pharmacologic interventions in animals with hypersensitivity from nerve injury and in humans with neuropathic pain.[21] Although drugs may target multiple mechanisms for analgesia, gabapentin, antidepressants, and clonidine clearly reduce hypersensitivity in animals with peripheral nerve injury by direct or indirect activation of $\alpha 2$ adrenoceptors and subsequent acetylcholine release in the spinal cord.[1–7] The current study confirms our previous observation[3] of a shift from direct inhibition to direct enhancement of evoked acetylcholine release from a preparation that includes spinal cholinergic terminals when exposed to $\alpha 2$-adrenoceptor agonists. We extend these observations by determining the time course of this effect, its bilateral nature, and probing its mechanisms, which likely involve a shift in G-protein species interacting with these receptors and in a direct or indirect action of BDNF.

In the current study, [^{3}H]choline uptake in synaptosomes and ChAT immunoreactivity in the spinal dorsal horn did not differ between normal and SNL animals, suggesting a lack of anatomic plasticity of cholinergic neurons and fibers in this model of neuropathic pain. However, SNL resulted in a bilateral decrease in KCl-evoked acetylcholine release in synaptosomes, which is consistent with other studies that peripheral nerve injury reduced KCl-evoked acetylcholine release in the spinal cord slices and *in vivo* microdialysis in the spinal cord.[22,23] These data suggest that cholinergic terminals may be less excitable after SNL and that the tonic inhibitory cholinergic tone on sensory processing shown in normal animals[24] may be reduced in this state of hypersensitivity. The current study does not determine the source of cholinergic terminals affected by SNL whether from descending neuronal terminals, interneurons, or the central terminals of primary afferents.[9,22] We did previously observe, however, that unilateral peripheral nerve injury results in a bilaterally decreased KCl-induced Ca^{2+} response in dorsal root ganglion neurons,[8] consistent with a reduction in evoked acetylcholine release from afferent terminals in the spinal cord. Further studies would be required to determine the relative contribution of each of the cholinergic terminal sources to this change after peripheral nerve injury.

Activation of $\alpha 2$ adrenoceptors typically inhibits voltage-gated Ca^{2+} channels to reduce neurotransmitter release *via* pertussis toxin-sensitive Gi/o-protein-dependent mechanisms,[11] and this mechanism was likely responsible for the decreased in KCl-evoked acetylcholine release by dexmedetomidine in synaptosomes from normal animals. This direct inhibition of acetylcholine release may partially underlie behavioral studies, which show that antinociception in normal rats from intrathecal clonidine is not reversed by intrathecal injection of the muscarinic antagonist atropine or by a selective toxin to cholinergic neurons.[5,6] Thus, acetylcholine probably plays little or no role in antinociception from spinal $\alpha 2$-adrenoceptor agonists in normal states.

This state of affairs is altered after nerve injury. We show here that dexmedetomidine enhancement of evoked acetylcholine release in synaptosomes occurs bilaterally in the spinal cord after nerve injury and that this depends on Gs-proteins. We previously showed that peripheral nerve injury increases the efficacy of G-protein coupling from exposure to $\alpha 2$-adrenoceptor agonists,[14] perhaps reflecting this novel interaction with Gs-proteins. *In vitro*, $\alpha 2$ adrenoceptors in transfected cells couple not only with Gi-proteins but also with Gs-proteins.[12] Although one can argue that this merely reflects the overexpression of G-protein species in these artificial cell lines, *in vivo* data also support an interaction between $\alpha 2$ adrenoceptors and Gs-proteins in some circumstances. For example, in the pregnant rat cervix, $\alpha 2$ adrenoceptors change their balance of Gi to Gs coupling depending on the day of pregnancy.[13] Whether peripheral nerve injury increases Gs coupling of $\alpha 2$ adrenoceptors in the spinal dorsal horn cholinergic terminals by an alteration in expression of Gs- or Gi-proteins or by posttranslational modification of either these proteins or the $\alpha 2$ adrenoceptors is under current study.

BDNF regulates survival and differentiation of neurons during development and in adulthood and can be released to induce plasticity in pathologic states. The most likely sources of BDNF in the spinal cord after peripheral nerve injury include the terminals of primary afferents and resident glia.[15,25–27] We and others have recently shown that peripheral nerve injury bilaterally increases BDNF content in the spinal dorsal horn.[15,28] BDNF plays important roles in synaptogenesis and plasticity in cholinergic motor neurons, as demonstrated by increased survival and axonal growth of cholinergic motor neurons in rats after spinal cord injury from spinal infusion of BDNF or overexpression of BDNF by gene transfer,[29,30] and prevention of cholinergic neuron loss in the rat brainstem after hypoglossal nerve transection by intracerebroventricular infusion of BNDF.[31] The current study demonstrates that tonic BDNF signaling may be important to regulate the density of cholinergic fibers in the normal spinal cord dorsal horn, because spinal infusion of BDNF antibody reduced ChAT immunoreactivity in the spinal cord dorsal horn. Interestingly, increased BDNF in the spinal cord after peripheral nerve injury does not further increase the density of cholinergic fibers. In addition, the increase in BDNF content and signaling in the spinal cord after peripheral nerve injury indirectly or directly affects the action of $\alpha 2$ adrenoceptors on cholinergic terminals, because injury-induced transformation of dexmedetomidine effect on acetylcholine release from inhibition to enhancement was abolished by spinal infusion of a BDNF antibody. These results coincide with a reduction in the antihypersensitivity effect of intrathecal clonidine after SNL injury from spinal infusion of a BDNF antibody.[15] These results suggest that BDNF is essential for maintenance of ChAT expression in the spinal cord dorsal horn and also for enhanced spinal acetylcholine release by $\alpha 2$-adrenoceptor stimulation after nerve injury. Conversely, spinal BDNF antibody infusion itself did not alter KCl-evoked acetylcholine release, suggesting that the regulation of $\alpha 2$-adrenoceptor function and depolarization-induced acetylcholine in the spinal dorsal horn cholinergic terminals reflects different mechanisms. However, the current study does not address whether BDNF affects expression and Gs-coupling of $\alpha 2$ adrenoceptors on the cholinergic terminals. Further study will be required to clarify these points.

In summary, peripheral nerve injury alters $\alpha 2$-adrenoceptor function on spinal cholinergic terminals from inhibition to facilitation *via* Gs-coupling. This shift requires BDNF, which plays a pivotal role in expression of ChAT in the spinal dorsal horn and α2-adrenoceptor-mediated facilitation of spinal acetylcholine release after nerve injury. These results suggest that the functional alterations of α2 adrenoceptors and actions of BDNF on cholinergic terminals after peripheral nerve injury are important for the analgesia induced by drugs commonly used to treat neuropathic pain, which rely in part on engagement of the spinal noradrenergic–cholinergic pathway.

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