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A tropomyosine receptor kinase inhibitor blocks spinal neuroplasticity essential for the anti-hypersensitivity effects of gabapentin and clonidine in rats with peripheral nerve injury

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Abstract

Spinally released brain derived nerve growth factor (BDNF) after nerve injury is essential to anatomic and functional changes in spinal noradrenergic and cholinergic systems which are engaged or targeted by commonly used treatments for neuropathic pain. Since BDNF signals via tropomyosine receptor kinases (trks), we tested whether trk blockade by repeated spinal injection of the trk inhibitor K252a would reduce anatomical (spinal noradrenergic and cholinergic fiber density), functional (α 2-adrenoceptor-mediated direct stimulation of spinal cholinergic terminals), and behavioral (antihypersensitivity from systemic gabapentin and spinal clonidine) plasticity which depends on BDNF. Spinal K252a treatment did not alter hypersensitivity from spinal nerve ligation (SNL), but blocked the SNL-associated increase in dopamine- β -hydroxylase (DBH) fiber density in the spinal cord dorsal horn while reducing spinal choline acetyltransferase (ChAT)-immunoreactivity. K252a treatment also abolished the facilitatory effect of dexmedetomidine on KCl-evoked acetylcholine release in spinal cord synaptosomes and reduced the anti-hypersensitivity effects of oral gabapentin and spinal clonidine. These results suggest that spinal trk signaling is essential for the anatomic and functional plasticity in noradrenergic and cholinergic systems after nerve injury and consequently for the analgesia from drugs which rely on these systems.

Perspective—Many drugs approved for neuropathic pain engage spinal noradrenergic and cholinergic systems for analgesia. This study demonstrates that spinal trk signaling after nerve injury is important to neuroplasticity of these systems which is critical for the analgesic action of common treatments for neuropathic pain.

Keywords

Neuropathic pain; noradrenergic; cholinergic; spinal cord; tyrosine kinase receptor; K252a

Introduction

Spinal processing and transmission of sensory signals are modulated by local circuits and supraspinal neurons which project to the spinal cord.²⁰ Bulbosplinal noradrenergic pathways inhibit pain and hypersensitivity after peripheral nerve injury in animals and many approved

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treatments for neuropathic pain, including gabapentin, the noradrenaline-mimetic clonidine, and noradrenaline re-uptake inhibitors, engage or mimic this mechanism to relieve neuropathic pain.^{9, 12, 21–23, 25, 32} Although noradrenergic inhibition can be demonstrated in the normal animal peripheral nerve injury fundamentally alters the structure and function of this system, and these alterations are essential to efficacy of this pathway after nerve injury. For one, noradrenergic fibers in the spinal cord dorsal horn sprout at dermatomes surrounding the site of input, allowing for great and more anatomically extensive release of norepinephrine when this pathway is activated.¹⁴ For another, spinally released norepinephrine, which normally inhibits spinal cholinergic interneurons, excites them after nerve injury, and this release acetylcholine is critical to analgesic effects of spinal norepinephrine release.²¹ Gabapentin, commonly used to treat neuropathic pain, activates neurons in the brainstem to induce spinal noradrenaline release which stimulates $\alpha 2$ -adrenoceptors and subsequent release of acetylcholine for analgesia in rodents with neuropathic hypersensitivity.^{11, 32} A combination of gabapentin with cholinesterase inhibitors produces synergistic analgesia which is dependent on spinal muscarinic receptors,^{11, 12, 32} and these muscarinic receptors are themselves up-regulated in primary sensory afferents after nerve injury.¹³ The clinical relevance of these laboratory observations is underscored by demonstration in patients with chronic pain that oral administration of gabapentin, in a dose that produces postoperative analgesia, increases noradrenaline concentrations in cerebrospinal fluid.⁹ These clinical and laboratory data validate spinal noradrenergic-cholinergic circuits as important targets for treatment of neuropathic pain.

In both normal and neuropathic pain states, $\alpha 2$ -adrenoceptors are coupled to Gi/o-proteins and Gi/o signaling by these metabotropic receptors results in antinociception in part by reducing release of pronociceptive neurotransmitters including substance P and glutamate from primary afferent terminals,^{20, 24} and in part by hyperpolarizing spinal neurons via activation of potassium channels.³⁰ We have previously demonstrated that activation of $\alpha 2$ -adrenoceptors by clonidine or dexmedetomidine inhibits KCl-evoked acetylcholine release in spinal cord slices and lumbar dorsal horn synaptosomes in normal rats, consistent with this classical Gi/o-mediated effect of $\alpha 2$ -adrenoceptors.^{10, 21} Interestingly, after peripheral nerve injury, activation of $\alpha 2$ -adrenoceptors results in Gs-mediated facilitation rather than Gi/o-mediated inhibition of acetylcholine release from synaptosomes,¹⁰ consistent with behavioral data that analgesia from intrathecal clonidine is blocked by intrathecal atropine in nerve injured rats, but not in normal rats.^{23, 25}

Brain-derived neurotrophic factor (BDNF) plays an important role in spinal noradrenergic neuroplasticity after nerve injury. BDNF can be released from the terminals of primary afferents and resident glia,^{3, 7, 14, 29} and primarily acts on its high-affinity receptor, tropomyosine receptor kinase B (trkB), to regulate survival and differentiation of neurons during development and in adulthood.¹⁹ We recently demonstrated that peripheral nerve injury increases BDNF content and dopamine- β -hydroxylase-immunoreactive (D β H-IR) axon density in the spinal dorsal horn, that spinal infusion of BDNF antibody blocks this increase in noradrenergic axon density in rats after nerve injury, and that intra-spinally injected BDNF in normal rats increases noradrenergic axon density.¹⁴ Additionally, spinal infusion of BDNF antibody also reduces choline acetyltransferase (ChAT)-IR in the dorsal horn and abolishes the shift from inhibition to excitation by dexmedetomidine of KCl-evoked acetylcholine in dorsal horn synaptosomes after nerve injury.¹⁰ Finally, spinal infusion of BDNF antibody reduces antihypersensitivity from intrathecal clonidine in nerve injured rats in parallel with its effect on anatomic and functional changes in the spinal cord from injury.¹⁴ These results suggest that spinal BDNF drives spinal noradrenergic-cholinergic neuroplasticity and hence efficacy for analgesia from drugs which rely in part on engagement of this pathway, after nerve injury.

The current study extends these observations by determining the role of trk signaling in this plasticity. Efficacy of spinal treatment with the anti-BDNF antibody to prevent this plasticity assumes that this antibody acts specifically and solely to sequester BDNF and experiments using this tool do not elucidate the mechanisms by which BDNF acts. For these reasons, we tested whether spinal infusion of the trk inhibitor, K252a, would block anatomic and functional neuroplasticity of spinal noradrenergic systems after nerve injury and behavioral anti-hypersensitivity from two clinically used treatments for neuropathic pain which are presumed to rely on this plasticity.

Materials and Methods

Animals

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

Animal surgery and K252a treatment

Animals were anesthetized with 2% isoflurane in oxygen and intrathecal catheterization was performed as previously described.³⁵ A small puncture was made in the atlanto-occipital membrane of the cisterna magnum and a polyethylene catheter (ReCathCo LLC, Allison Park, PA), 7.5 cm, was inserted so that the caudal tip reached the lumbar enlargement of the spinal cord. At least 5 days after intrathecal catheterization, L5–L6 SNL was performed as previously described.¹⁷ Briefly, under anesthesia with 2% isoflurane, the right L6 transverse process was removed and the right L5 and L6 spinal nerves were tightly ligated using 5–0 silk suture. For the behavior and synaptosome studies, animals received intrathecal injection of saline or K252a (Sigma Chemical CO., St. Louis, MO, 2 µg in 10 µl of saline) followed by 10 µl of saline 15 min prior to the surgery, and then saline and K252a were repeatedly injected at 1, 3, 5, 7, 9, 11, and 13 days after surgery. Other animals for immunohistochemistry were also treated with saline and K252a but their spinal cord tissues were collected at 10 days after surgery.

Behavioral testing

Hypersensitivity to light touch following SNL was assessed using calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied to the plantar surface of the paw. Filaments were applied to the bending point for 5 s, and a brisk paw withdrawal was considered a positive response. Withdrawal threshold was determined using an up–down statistical method.² At 10 days after SNL, gabapentin solution (Neurontin® 50 mg/ml, Parke-Davis, New York, NY) was diluted in 0.5% carboxymethylcellulose solution and orally administered by a feeding tube (100 mg/5 ml/kg) 2 h prior to measurement of withdrawal threshold in saline- and K252a-treated animals (n=6 in each group). At 12 days after SNL, these same animals received intrathecal injection of clonidine (Sigma Chemical CO., 15 µg in 10 µl of saline) followed by 10 µl of saline 1 h prior to the measurement. At 14 days after SNL, spinal cord tissues from those animals were used for the synaptosome study. The person who performed behavior testing was blinded to chronic treatment (repeated saline or K252a).

Immunohistochemistry

At 10 days after SNL, saline- or K252a-treated animals (n=6 in each group) were deeply anesthetized with 100 mg/kg pentobarbital and perfused intracardially with cold phosphate buffered saline (PBS) containing 1 % sodium nitrite followed by 4 % paraformaldehyde in

0.1 M phosphate buffer (pH 7.4). Age-matched unoperated rats (n=5) were also perfused. 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 a 40 µm thickness. After being pretreated with 0.3 % hydrogen peroxide and 1.5 % normal donkey serum (NDS, 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) or a mouse monoclonal anti-DβH antibody (1:1,000, MAB308, Chemicon International Inc., Temecula, CA) in 1.5 % NDS. Subsequently, the sections were incubated in biotinylated donkey anti-goat or anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA), processed using Elite Vectastain ABC kit (Vector) according to the manufacturer instructions, and then developed by the standard glucose oxidase-nickel method.

For quantification of DβH- and ChAT-IR, 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 CCD camera. Using image analysis software (SigmaScan, Jandel Scientific Inc., San Rafael, CA), pixels of DβH- or ChAT-immunoreactive objects within the area of the dorsal horn containing lamina I to IV were quantified based on a constant threshold of optical density. Data are expressed as percentage of DβH- or ChAT-IR pixels above threshold compared to the total number of pixels in the area. The person performing image analysis was blinded to chronic treatment or group.

Acetylcholine release from synaptosomes

Crude synaptosomes from the lumbar spinal dorsal horn were prepared as previously described.¹⁰ At 14 days after SNL, saline- or K252a-treated animals (n=6 in each group) were killed by decapitation under deep anesthesia with 5% isoflurane, and a 1 cm length of the spinal cord containing the lumbar enlargement, measured by the ruler, was quickly removed and placed in oxygenated (with 95% O₂–5% CO₂) ice-cold Krebs buffer containing 124 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 1.25 mM KH₂PO₄, 25 mM NaHCO₃, and 10 mM glucose, pH 7.35. The dorsal quadrants of the spinal cord ipsi- 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 3 unilateral dorsal quadrants of spinal cord from 3 SNL 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 [³H]-choline which is taken up into synaptosomes, acetylated to acetylcholine in those which contain ChAT, and released as [³H]-acetylcholine with depolarization.^{16, 27} We did not, however, confirm in the current experiments that the released radioactivity was completely in the form of [³H]-acetylcholine. After incubation with 1 µM choline chloride (combination of both trituated and unlabeled choline) for 20 min at 37°C, the synaptosome-containing solution was centrifuged at 10,000XG 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. After a 10 min baseline collection, synaptosomes were perfused with 10 nM dexmedetomidine alone for 2 min, then stimulated with 12 mM KCl-Krebs buffer (in mM: 115 NaCl, 12 KCl, 2 MgSO₄, 2 CaCl₂, 1.25 KH₂PO₄, 25 NaHCO₃, and 10 glucose, pH 7.35) containing dexmedetomidine for 3 min. Concentrations of KCl and dexmedetomidine were determined from our previous studies.^{10, 21} An inhibitor of the acetylcholine transporter, hemicholinium-3 (10 µM), was present during perfusion to prevent reuptake of

acetylcholine. [³H]-Acetylcholine release from synaptosomes in each fraction was measured by a liquid scintillation counter (LS6500, Beckman Coulter Inc., Fullerton, CA). All chemicals were purchased from Sigma Chemical Co. except [³H]-choline (Perkin Elmer, Boston, MA).

Statistical analyses

Data were normally distributed and are presented as mean $\pm$ SE. Differences among groups were determined using one or two-way ANOVA as appropriate. $P < 0.05$ was considered significant.

Results

Effect of K252a on hypersensitivity after SNL

Animals tolerated repeated intrathecal injection of saline and K252a with no change in spontaneous behavior. At 10 and 12 days after SNL, pre-drug withdrawal threshold values in the ipsilateral paw were significantly lower than pre-surgery values in both saline- and K252a-treated animals (Fig. 1, $p < 0.05$). K252a treated animals showed slightly higher pre-drug withdrawal threshold values compared to saline treated animals. However, there was no significance in pre-drug values between saline- and K252a-treated groups ($p = 0.52$ at Day 10, $p = 0.46$ at Day 12), consistent with previous observations that repeated spinal injection of K252a did not prevent development of hypersensitivity in rats after peripheral nerve injury.³¹ In saline treated animals, orally administered gabapentin (100 mg/kg) and intrathecal clonidine (15 μ g) significantly increased withdrawal threshold in the paw ipsilateral to SNL compared to pre-drug values ($p < 0.05$). In K252a treated animals, neither gabapentin nor clonidine showed significant antihypersensitivity effects. There was a significant difference in post-drug withdrawal threshold values between saline- and K252a-treated groups ($p < 0.05$).

Immunohistochemistry for D β H- and ChAT-IR

Figure 2A, B and C depict D β H-IR axons in the lumbar spinal dorsal horn in SNL rats treated with saline or K252a, and in unoperated rats. In these photomicrographs, numerous D β H-IR axons and axonal varicosities along these axons were observed throughout the dorsal horn. As we previously reported,¹⁴ the density of D β H-IR axons was increased bilaterally in the spinal dorsal horn in saline treated SNL animals compared to tissue from unoperated controls (Fig 2D). Spinal K252a treatment dramatically reduced D β H-IR axon density in the spinal dorsal horn of SNL rats compared to saline treatment in SNL rats.

Figure 3A, B and C depict ChAT-IR in the lumbar spinal dorsal horn in SNL rats treated with saline or K252a and in unoperated rats. ChAT-IR in the spinal dorsal horn was found mainly in axons but a few ChAT-positive cells were also observed in both unoperated and SNL animals. In saline treated SNL animals, there was no difference in ChAT-IR density in the spinal dorsal horn compared to the unoperated controls (Fig. 3D). In contrast, SNL rats treated with K252a exhibited a decreased ChAT-IR in the spinal dorsal horn compared to saline treatment in SNL rats and unoperated controls ($p < 0.05$).

Acetylcholine release from synaptosomes

In saline treated SNL animals, [³H]-acetylcholine release increased about 30 % from the baseline by 12 mM KCl and dexmedetomidine significantly enhanced KCl-evoked [³H]-acetylcholine release compared to KCl alone in synaptosomes both ipsilateral and contralateral to SNL surgery (Fig. 4; $p < 0.05$). However, there was a significant difference in the facilitatory effect of dexmedetomidine on KCl-evoked [³H]-acetylcholine release between synaptosomes ipsilateral and contralateral to SNL injury in saline treated animals,

(Fig. 4; $p < 0.05$) consistent with our previous report.¹⁰ In K252a treated SNL animals, KCl-evoked [³H]-acetylcholine release did not differ from saline treated SNL animals, but dexmedetomidine's enhancement of release was abolished. Since we only made two synaptosome preparations in each group, we did not compare total [³H]-choline uptake and spontaneous [³H]-acetylcholine release between treatments.

Discussion

Among oral and intrathecal drugs to treat neuropathic pain, gabapentin and clonidine, respectively are commonly used and both reduce allodynia and hyperalgesia as well as spontaneous pain in patients.^{6, 28} The current study supports previous observations in animals and humans which indicate a role for spinal acetylcholine release as an important mechanism of action of the anti-hypersensitivity effects of these drugs,^{5, 12, 32} and extends these observations by showing that trk signaling in the spinal cord is permissive for this mechanism to be active. Three phenomena following nerve injury – increased D β H-IR fiber density, novel and direct augmentation of acetylcholine release by α 2-adrenoceptor stimulation, and the anti-hypersensitivity effect of these drugs – were all prevented by prolonged spinal administration of a trk inhibitor. Although it is unclear whether increased D β H-IR fiber density is essential to the anti-hypersensitivity effects of oral gabapentin and spinal clonidine, the novel and direct stimulation of acetylcholine appears to be, since the cholinergic antagonist, atropine, blocks their anti-hypersensitivity effects.^{12, 23, 25, 32}

Unlike postganglionic sympathetic noradrenergic neurons, which express trkA and respond to nerve growth factor, spinal noradrenergic axons express trkB and respond to BDNF,¹⁸ supported by our previous observations that spinal infusion of BDNF antibody blocked the increase in noradrenergic axons in nerve injured rats and that intra-spinal injection of BDNF increased D β H-IR fiber density in the spinal dorsal horn in normal rats.¹⁴ In the present study, inhibition of trk signaling by K252a blocked the increase of D β H-IR fiber density in the spinal dorsal horn after nerve injury and reduced anti-hypersensitivity from gabapentin, which relies on spinal noradrenaline release to relieve such hypersensitivity.¹¹ These results suggest that activation of BDNF-trkB signaling can be essential for the plasticity of spinal noradrenergic axons and gabapentin analgesia after peripheral nerve injury.

In normal conditions, activation of α 2-adrenoceptors inhibits voltage-gated Ca^{2+} channels to reduce neurotransmitter release via pertussis toxin-sensitive Gi/o protein-dependent mechanisms,²⁴ consistent with the inhibitory effect of dexmedetomidine on KCl-evoked acetylcholine release from synaptosomes in normal rats.¹⁰ However, under some conditions, α 2-adrenoceptors have been shown to couple with stimulatory Gs-proteins,^{4, 8} which activate voltage-gated Ca^{2+} channels and could enhance Ca^{2+} -dependent neurotransmitter release. After peripheral nerve injury, efficiency of G-protein coupling with spinal α 2-adrenoceptors increases¹ and α 2-adrenoceptor-mediated facilitation of acetylcholine release is reversed by Gs inhibitors,¹⁰ consistent with a shift from Gi/o to Gs signaling by these receptors. In the present study, α 2-adrenoceptor agonists neither enhanced KCl-evoked acetylcholine release nor produced analgesia in K252a treated animals, consistent with our recent observation that nerve injury-induced transformation of dexmedetomidine's effect on acetylcholine release from inhibition to enhancement was abolished by spinal infusion of a BDNF antibody.¹⁰ These results suggest that activation of trk signaling in the spinal cord after nerve injury, most likely by BDNF, is necessary for the shift in α 2-adrenoceptor-mediated effects on spinal acetylcholine release in the spinal cord. Further study is required to determine whether BDNF-trkB signaling directly or indirectly affects expression and/or Gs-coupling of α 2-adrenoceptors on cholinergic terminals.

BDNF-trkB signaling has been shown to enhance synaptogenesis and growth in cholinergic neurons. As such, spinal infusion of BDNF or over-expression of BDNF by gene-transfer increases survival and axonal growth of cholinergic motor neurons in rats after spinal cord injury.^{15, 34} Intracerebroventricular infusion of BDNF, but not the TrkA ligand, nerve growth factor, or the TrkC ligand, neurotrophin-3, prevents loss of cholinergic neurons in the rat brainstem after hypoglossal nerve transection.³³ We recently demonstrated that tonic BDNF signaling is important to maintain cholinergic innervation in the spinal cord dorsal horn, since spinal infusion of BDNF antibody reduced ChAT-immunoreactivity in the spinal dorsal horn in normal and nerve injured rats.¹⁰ The importance of trkB signaling for cholinergic neurons is further demonstrated in the present study by reduction in ChAT-IR in the spinal dorsal horn from chronic K252a administration. BDNF has been shown to act not only on trkB but also p75 receptors.¹⁹ However, since destruction of p75-expressing axons by a selective neurotoxin did not alter ChAT immunoreactivity and acetylcholine content in the spinal dorsal horn,²⁶ these data suggest that BDNF signaling via TrkB rather than p75 is important to the maintenance of cholinergic innervation in the spinal dorsal horn.

In summary, blockade of trk signaling by repeated spinal injection of K252a in rats with peripheral nerve injury inhibited the increase of spinal D β H-IR fiber density, reduced spinal ChAT fiber density, blocked enhancement of acetylcholine release by dexmedetomidine, and reduced the anti-hypersensitivity efficacy of systemic administered gabapentin and intrathecal clonidine. We conclude that trk signaling is important not only for spinal noradrenergic and cholinergic neuronal functions but also for analgesia from drugs which are commonly used to treat chronic neuropathic pain.

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Figure 1.

Repeated spinal injection of K252a reduced antihypersensitivity effects of gabapentin and clonidine in SNL rats. SNL rats were treated with repeated injection of intrathecal saline (n=6) or K252a (2 μ g, n=6) as detailed in the Materials and Methods. Effects of oral administered gabapentin (100 mg/kg) and intrathecal clonidine (15 μ g) were tested at 10 and 12 days after surgery, respectively. Pre-drug (Pre) withdrawal threshold values were measured at each experiment day. Post-drug (Post) withdrawal threshold values were measured 2 h following gabapentin and 1 h following clonidine administrations, respectively. *p<0.05 vs. Pre-SNL, #p<0.05 vs. Pre-drug (Pre), and \$ p<0.05 vs. Saline by two-way repeated measures ANOVA.

Figure 2.

Effect of spinal K252a treatment on DβH-IR axons in the spinal dorsal horn 10 days following SNL. (A, B and C) Photomicrographs of DβH-IR axons in the ipsilateral spinal dorsal horn of saline treated SNL (A), K252a treated SNL (B), and unoperated (C) rats. Scale bar =100 μm (D) Quantification of DβH-IR axons in the ipsilateral (Ip) and contralateral (Co) spinal dorsal horn from unoperated (n=5), SNL saline-treated (n=6), and SNL K252a-treated (n=6) animals. *p<0.05 vs. normal. #p<0.05 vs. saline.

Figure 3.

Effect of spinal K252a treatment on ChAT-IR axons in the spinal dorsal horn 10 days following SNL. (A, B and C) Photomicrographs of ChAT-IR axons in the ipsilateral spinal dorsal horn of saline treated SNL (A), K252a treated SNL (B), and unoperated (C) rats. Scale bar =100 μ m. (D) Quantification of ChAT-IR axons in the ipsilateral (Ip) and contralateral (Co) spinal dorsal horn from unoperated (n=5), SNL saline-treated (n=6), and SNL K252a-treated (n=6) animals. * p <0.05 vs. normal. # p <0.05 vs. saline.

Figure 4.

Repeated spinal injection of K252a blocked the facilitatory effect of dexmedetomidine on KCl-evoked acetylcholine release in spinal dorsal horn synaptosomes from rats after SNL. Data are presented as percentage of baseline release. SNL synaptosomes were prepared from saline- and K252a-treated animals at 14 days after surgery, and treated with 12mM KCl or a combination of 12 mM KCl with 10 nM dexmedetomidine (Dex). n=6 in each group.

*p<0.05 vs. KCl alone. #p<0.05 vs. saline. \$p<0.05 vs. contralateral.