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***In vivo* activation of the SK channel in the spinal cord reduces the NMDA receptor antagonist dose needed to produce antinociception in an inflammatory pain model**

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Abstract

N-methyl-D-aspartate receptor (NMDAR) antagonists have been shown to reduce mechanical hypersensitivity in animal models of inflammatory pain. However, their clinical use is associated with significant dose-limiting side effects. Small conductance Ca^{2+} -activated K^{+} channels (SK) have been shown to modulate NMDAR activity in the brain. Here, we demonstrate that *in vivo* activation of SK channels in the spinal cord can alleviate mechanical hypersensitivity in a rat model of inflammatory pain. Intrathecal (i.t.) administration of the SK channel activator, 6,7-Dichloro-1H-indole-2,3-dione 3-oxime (NS309), attenuates complete Freund adjuvant (CFA)-induced mechanical hypersensitivity in a dose-dependent manner. Postsynaptic expression of the SK channel subunit, SK3, as well as apamin-sensitive SK channel-mediated currents recorded from superficial laminae, are significantly reduced in the dorsal horn (DH) after CFA. CFA-induced decrease in SK mediated currents can be reversed *in vitro* by bath application of NS309. In addition, immunostaining for SK3 subunit indicates that SK3-containing channels within DH neurons can have both somatic and dendritic localization. Double immunostaining shows co-expression of SK3 and NMDAR subunit, NR1, compatible with functional interaction. Moreover, we demonstrate that i.t. co-administration of NS309 with an NMDAR antagonist reduces the dose of NMDAR antagonist, DL-2-Amino-5-phosphonopentanoic acid (DL-AP5), required to produce antinociceptive effects in the CFA model. This reduction could attenuate the unwanted side effects associated with NMDAR antagonists, giving this combination potential clinical implications.

Keywords

SK channel; NMDA receptor; NMDA receptor antagonists; inflammatory pain; mechanical hypersensitivity; spinal cord; CFA

Introduction

One property of pronociceptive neurons in the dorsal horn (DH) is increased excitability when in a sensitized state [7; 56]. One of the key regulators of neuronal excitability is a small conductance Ca^{2+} -activated K^{+} channel (SK). SK channels can regulate cell excitability in three different ways: 1) by generating afterhyperpolarization (AHP) that controls action potential firing frequency [31; 45], 2) by reducing action potential-induced Ca^{2+} transients [52], or 3) by reducing NMDA receptor- (NMDAR-) mediated excitatory synaptic transmission and plasticity [9; 18; 19; 39; 49]. Three SK channel subunits have been described, SK1, SK2 and SK3 [1], all of which are expressed both in the dorsal root ganglia (DRG) neurons [37] and in DH neurons in the spinal cord [16; 37; 46]. There is an indication that SK channels in the spinal cord are involved in nociception, in particular, it has been shown that dorsal root-evoked ventral root potentials recorded *in vitro* in neonatal rats are inhibited when SK channel activity is increased with the SK channel opener, 1-EBIO [6]. Furthermore, *in vivo* extracellular recordings from DH neurons in anesthetized rats showed increased responses to naturally evoked nociceptive stimuli after application of the selective apamin-sensitive SK2/SK3 channel blocker UCL 1848. In addition, an intraplantar injection of the selective SK channel blocker, apamin, has been shown to induce mechanical allodynia and heat hyperalgesia in naive rats [40].

In an animal model of nociception, AHP is down-regulated in DRG cells and reticulospinal neurons after nerve injury [27; 30; 34; 48]. An early study reported reduction in the AHP in primary afferent nociceptive neurons after inflammation [17]. However, whether SK channel function can alter inflammation-induced pain sensitivity is unclear, and the role of the DH SK channels in inflammatory pain is poorly understood.

NMDAR antagonists are known to reduce mechanical hypersensitivity associated with tissue inflammation [24; 44]. Although a number of NMDAR antagonists are antinociceptive in animal models of inflammatory pain, they are associated with significant dose-limiting side effects in the clinic, including sedation, nausea, dissociative reactions etc [14; 25; 55]. Given the modulatory action of SK channels on NMDAR-mediated synaptic transmission and plasticity [9; 18; 19; 39; 49], we hypothesized that activation of SK channels could modulate antinociceptive effects of NMDAR antagonists.

Here, we show that *in vivo* activation of SK channels can alleviate mechanical hypersensitivity induced by the administration of complete Freund adjuvant (CFA) in the hind paw of the rat, a well-established model of inflammatory pain [23; 54]. Furthermore, we demonstrate that co-administration of an SK channel activator with an NMDAR antagonist reduces the dose of NMDAR antagonist required to produce antinociceptive effects. Therefore, our data highlights DH SK channels as potential therapeutic targets for the treatment of inflammatory pain.

Materials and methods

Subjects

Three to four week old male Sprague Dawley rats (Harlan) were housed in groups of 3 per cage in a temperature-controlled vivarium on a 12/12h dark/light cycle (lights on at 7:00 A.M.) with *ad libitum* access to food and water. This age was chosen because the postnatal development of DH sensory processing is mostly complete, and it is still possible to record from visualized spinal cord neurons, which becomes increasingly difficult with heavier laminar myelination at older postnatal ages [22; 53]. Rats were acclimated to the vivarium for at least 2 days before any manipulation. All procedures were approved by the Columbia University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

CFA model

Rats were first habituated to the testing environment and then evaluated until they showed stable baseline thresholds (3–5 days). Pre-CFA data reflects the last baseline measurement taken immediately before CFA administration. Rats were injected with 100 µl (s.c.) CFA (Calbiochem) or saline in the plantar hind paw under brief isoflurane anesthesia (3%; 1 l/min) as described [8]. Forty-eight hours later animals were either used for behavioral evaluation or were sacrificed for subsequent biochemical or electrophysiological analyses.

Drugs

The following drugs were used: the potent, selective NMDAR antagonist DL-2-Amino-5-phosphonopentanoic acid (DL-AP5; Tocris); the specific SK channel blocker apamin (Sigma); and the potent SK activator 6,7-Dichloro-1H-indole-2,3-dione 3-oxime (NS309; Tocris).

Behavior

One dose of NS309 [35] or its vehicle (1% DMSO in sterile saline) was administered intrathecally (i.t.) under brief isoflurane anesthesia. A 10 µl volume [20] was injected between the L5 and L6 vertebrae in each animal, using a 25 µl Hamilton syringe and 27G needle. For the dose-response studies, CFA-treated rats were randomly assigned to four groups: 1) vehicle (DMSO), 2) NS309 25 µM, 3) NS309 50 µM, 4) NS309 100 µM. For the co-administration experiments CFA-treated rats were randomly assigned to four groups: 1) vehicle, 2) NS309 alone, 3) DL-AP5 alone, 4) DL-AP5 i.t. + NS309. Mechanical thresholds were evaluated using von Frey filament stimulation and the up-down method [12]. Animals were placed in plastic boxes on a wire mesh grid through which the filaments were applied (bending forces ranged from 0.07 to 15 g; Bioseb). Clear paw withdrawal with associated shaking, or licking in response to von Frey stimulation were considered nociceptive-like responses.

A locomotor activity test was performed as previously described [21] using chambers (41.5 × 41.5 × 30 cm) equipped with photobeams (AccuScan Instruments). All sessions began with a habituation period in which rats were allowed to explore the locomotor activity chambers for 30 min. Following habituation, rats were randomly assigned to two groups and

received an i.t. injection of 100 μ M NS309 or its vehicle. The rats were then placed in the locomotor activity chambers and activity was measured for 30 min.

Electrophysiology

Transverse lumbar spinal cord slices (300 μ m thick) from CFA- and saline-treated rats were prepared as previously described [10; 50]. Krebs solution comprised (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 26 NH_4CO_3 , 25 glucose, 1 MgCl_2 , 2 CaCl_2 , pH 7.4. 95% O_2 /5% CO_2 was bubbled continuously during dissection, incubation, and recording. Visualized whole cell patch-clamp recordings were made from laminae I-II neurons with recording pipettes containing: 135 mM potassium methylsulphate (KMeSO_4), 10 mM Hepes, 8 mM NaCl, 2 mM Mg_2ATP , and 0.3 mM Na_4GTP , 0.3 EGTA. Apamin-sensitive outward tail currents (AHP currents [I_{AHP}]) were recorded in neurons voltage clamped at holding potentials from -55 mV following 80 ms voltage steps to -10 mV applied every 20 s as described [36]. Data acquisition and analysis was performed using Clampex and Clampfit 10 (Axon Instruments).

Immunohistochemistry

Rats were first perfused with 30 mL ice cold phosphate buffered saline (PBS) followed by 50 mL ice cold 4% paraformaldehyde in PBS, then spinal cords were removed and post-fixed for 24 h in 4% paraformaldehyde in PBS followed by submersion in 30% sucrose until tissue was infiltrated. Lumbar L4-L5 spinal cord sections were cut at 40 μ m on a cryostat (MicromTM HM 525). For NR1 and SK3 co-immunostaining, free-floating sections were first pretreated with PBS with 0.2% Triton X-100 at 37 °C for 30 min followed by 3–4 min in 0.2 M HCl with 1 mg/ml pepsin [10]. After washing with PBS and blocking with 30% goat serum in PBS with 0.5% Triton X-100, sections were incubated overnight with SK3 (1:1000, Alomone-labs) antibody in PBS with 0.5% Triton X-100 at 4 °C or SK3 and NR1 (1:100, Invitrogen) antibodies in PBS Triton X-100 0.2% or SK3 and NeuN (1:5000, Millipore) antibodies in PBS Triton X-100 0.2%. Following a rinse in PBS, the sections were incubated for 2 h at room temperature with anti-rabbit IgG conjugated with Alexa 488 (1:1000, Invitrogen) in PBS with 0.5% Triton X-100 or with anti-rabbit IgG conjugated with Alexa 488 and anti-mouse IgG conjugated with Alexa 594 (1:500, Invitrogen) in PBS Triton X-100 0.2%. After rinsing in PBS they were mounted on slides and coverslipped with either DAPI Fluoromount-G mounting media (SouthernBiotech) or ProLong Gold Antifade Reagent (Invitrogen). Control immunostaining included processing tissue as described above in the absence of primary antibody. Specific binding of the SK3 antibody was previously described [28]. Color digitized images of sections were captured using the 10x and 60x objectives using an Eclipse Ti confocal microscope (Nikon).

Subcellular Fractionation

Several cohorts of rats on independent days were treated with intraplantar CFA or saline injections. Ipsilateral dorsal spinal cords were harvested 48 hours later, immediately frozen and stored at -80°C until subcellular fractionation was performed. Total homogenate (TH) and postsynaptic density (PSD) fractions were prepared as previously described [10; 21]. In order to obtain an adequate amount of tissue for PSD fraction isolation three ipsilateral lumbar dorsal spinal cords from either CFA-treated or saline-treated rats were pooled to

obtain each individual sample. Experiments were conducted with and n equal to 6–7 samples per treatment. The spinal cords were homogenized in 0.32 M sucrose/0.1 mM CaCl_2 solution containing protease and phosphatase inhibitors (Sigma-Aldrich). A sucrose gradient, generated by diluting the homogenates to 1.25 M sucrose and overlaying with 1.0 M sucrose, was centrifuged at 100,000 g (3 hr) and the synaptosomal fraction was collected at the interface. This fraction was incubated in 20 mM Tris pH6 and centrifuged at 40,000 g for 30 min to obtain synaptic junctions which were then incubated/homogenized in 20 mM Tris pH8. After centrifugation at 40,000 g (30 min) PSD fractions were solubilized in 1% SDS.

Western blotting

The TH and PSD fractions were loaded equally (10 μg) and separated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in TBS containing 0.1% Tween-20, membranes were incubated with SK1 (1:500, Alomone-labs), SK2 (1:500, Sigma-Aldrich) or SK3 (1:500, Alomone-labs) antibody overnight at 4 °C. The specific binding of these antibodies have been previously described [3; 28; 33]. Then membranes were probed with anti-rabbit-HRP 1:5000 followed by Amersham ECL Prime detection. The intensity of each sample's SK1, SK2 or SK3 band and its respective β -actin was measured using ImageJ software. For the analysis, intensity of each sample's SK1, SK2 and SK3 band was normalized to its respective β -actin and represented as a percent of the average intensity of saline bands.

Statistical analyses

In the dose response experiments with NS309 a One-way ANOVA with repeated measures followed by a Bonferroni multiple comparison test was used to compare differences within or between the groups. For co-administration experiments a Two-way ANOVA with repeated measures followed by Bonferroni multiple comparison test was used to compare differences within and between the groups. For biochemistry and electrophysiology experiments unpaired t-tests were used to compare differences between saline and CFA groups. Paired t-test was used for electrophysiological recordings before and after apamin application. Data are expressed as mean $\pm$ SEM, and statistical analyses were performed using Prism (GraphPad) or SPSS (ver.13.0; IBM) software. Significance level was always set at $p < 0.05$.

Results

In vivo activation of SK channels reverses CFA-induced mechanical hypersensitivity

NS309 potently and selectively activates apamin-sensitive SK channels (SK2/SK3) by increasing their sensitivity to Ca^{2+} , leading to a decrease in both apamin-sensitive AHP and evoked excitatory postsynaptic potentials (EPSP) in hippocampal neurons [35; 42]. In order to test whether SK channels in the spinal cord can be activated *in vivo* and whether this activation can affect nociceptive behavior, we studied the effect of i.t. administration of NS309 on inflammation-induced mechanical hypersensitivity. Prior to NS309 injections, CFA-treated rats in all groups showed robust reduction in mechanical thresholds ($n = 8$ animals per group, $p < 0.0001$, One-way ANOVA with repeated measures followed by

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was generated by SK channel activation we used bath application of apamin (100 nM). We found that apamin abolished I_{AHP} indicating SK-channel involvement. In order to test whether *in vitro* activation of SK channels could reverse CFA-induced decrease in I_{AHP} we used bath application of NS309 (100 μ M). In 5 neurons that showed CFA-induced decrease NS309 produced an average of 174% increase (Fig. 2B; $p < 0.001$, $n = 5$ neurons per 5 rats per group), reversing the average peak amplitude of I_{AHP} to 26.7 ± 6.7 pA.

Postsynaptic expression of the SK3 subunit is reduced in the DH after CFA

Since both SK2 and SK3 subunits are NS309- and apamin-sensitive, our data indicates that either SK2 or SK3 subunits may contribute to the I_{AHP} reduction after CFA. Next, we conducted biochemical analyses to evaluate SK channel subunit expression levels in total homogenate (TH) and postsynaptic density (PSD) fractions [10; 21] from pooled ipsilateral lumbar dorsal horns of CFA- and saline-treated rats. Channel expression was analyzed using SK1, SK2, or SK3 antibody. We found a significant decrease in the postsynaptic levels of SK3 ($24.21 \pm 7.82\%$, $p = 0.01$, unpaired t-test, $n = 6-7$ samples per group, $t_{(11)} = 3.096$) in the DH of CFA-treated animals compared to saline-treated rats (Fig. 3), β -actin did not change across treatment groups. No significant changes in the SK1 or SK2 expression levels were observed in either TH or PSD fractions from CFA- compared to saline-treated controls (PSD: SK1, $p = 0.66$, $n = 6-7$ samples per group, $t_{(12)} = 0.45$; SK2, $p = 0.64$, $n = 7$ samples per group, $t_{(12)} = 0.4743$; TH: SK1, $p = 0.51$, $n = 7$ samples per group, $t_{(12)} = 0.6776$; SK2, $p = 0.53$, $n = 9-10$ per group, $t_{(17)} = 0.6483$; SK3, $p = 0.57$, $n = 7$ per group, $t_{(12)} = 0.5859$; unpaired t-test). These data suggest that postsynaptic expression of SK3 may play a role in inflammation-induced hypersensitivity. Taken together with the previously reported increase in NMDAR NR1 subunit expression in the PSD after CFA [59], our data may indicate changes in SK-NMDAR interaction at the postsynaptic level.

SK3-containing channels are localized on DH soma and dendrites, in close proximity with NMDAR

In the hippocampus, SK channels have been shown to control synaptic transmission by hyperpolarizing postsynaptic membrane and reducing activation of dendritically located NMDARs [39]. In the spinal cord, both dendritic and somatic localization of SK3 subunit was reported in motoneurons [6; 16], however, in DH neurons, dendritic SK3 expression has not been demonstrated. We used SK3 antibody to immunostain tissue sections taken from rat lumbar spinal cord. Consistent with previous publications [6; 16; 37; 46] SK3 immunostaining was found throughout the spinal cord (Fig. 4A). High magnification (60x) images showed numerous puncta outlining cell bodies and dendrites, which was consistent with the channels having a postsynaptic location (Fig. 4B, D). Double immunostaining for SK3 and a neuronal marker, NeuN, confirms neuronal localization of SK3 (Fig. 4C). In addition, co-immunostaining of spinal cord tissue with antibodies to SK3 and NMDAR subunit NR1 demonstrated co-expression of these proteins (Fig. 5), indicating that SK3-containing channels and NMDAR could be localized in close proximity to be able to interact at the synaptic level.

Intrathecal co-administration of low doses of NS309 and DL-AP5 reduces CFA-induced mechanical hypersensitivity

The NMDAR antagonist, DL-AP5, dose-dependently reduces CFA-induced mechanical hypersensitivity in rats [24; 44]. In order to test whether *in vivo* activation of SK-channels could reduce the dose of DL-AP5 required to produce the antinociceptive effect, we used 10 nmol DL-AP5, a dose shown to have less than 10% reversal effect on CFA-induced mechanical hypersensitivity [44]. When co-administered i.t. with a subeffective dose of NS309 (25 μ M), this dose of DL-AP5, reversed CFA-induced mechanical hypersensitivity to $48.4 \pm 4.8\%$, $38.4 \pm 2.9\%$ and $26.2 \pm 4.2\%$ of the baseline values at 30 min, 60 min and 120 min after i.t. administration, respectively (Fig. 6; $n = 6$ animals per group; $p_{30 \text{ min}} < 0.0001$, $p_{60 \text{ min}} < 0.0001$ and $p_{120 \text{ min}} < 0.00845$; Two-way ANOVA followed by Bonferroni multiple comparisons). Indeed, the Two-way ANOVA with repeated measures found statistical differences in the interaction time $\times$ group ($n = 6$ animals per group, $p < 0.0001$, $F_{9,60} = 12.490$) and also within the group ($p = 0.004$, $F_{3,20} = 6.202$). Further posthoc analysis found that the 10 nmol DL-AP5 + 25 μ M NS309 group was significantly different from DL-AP5 alone ($p_{30 \text{ min}} < 0.0001$, $p_{60 \text{ min}} = 0.006$), NS309 alone ($p_{30 \text{ min}} < 0.0001$, $p_{60 \text{ min}} < 0.0001$) and from vehicle groups ($p_{30 \text{ min}} < 0.0001$, $p_{60 \text{ min}} = 0.001$). These data demonstrate that administration of an SK channel activator can reduce the dose of NMDAR antagonist needed to produce antinociceptive effects.

Discussion

The SK channel subunits (particularly SK3) are abundantly expressed both in the DH neurons and in the DRGs [16; 37; 46]. Previous reports suggested a role for SK channels in nociception [6; 40], but these studies were conducted in naive rats. Data from nociceptive animal models remained scarce, however, AHP was shown to be down-regulated in primary afferents after nerve injury [27; 30; 34; 48] and after CFA [17]. Here we demonstrate that activation of the SK channels in the spinal cord *in vivo* can alleviate CFA-induced mechanical hypersensitivity. Our data highlights for the first time a role for the DH SK channels in inflammatory pain. Whole-cell patch-clamp recordings allowed measurement of SK-mediated AHP currents in laminae I-II neurons directly, indicating I_{AHP} decrease 48 h after CFA. *In vitro* application of NS309 reversed CFA-induced I_{AHP} in the affected neurons. Moreover, the I_{AHPs} were apamin-sensitive suggesting the involvement of SK2- or SK3-containing channels. Western blot analyses of subcellular fractions revealed a significant decrease in SK3 protein expression at the PSD 48 h after CFA, confirming the role of postsynaptic SK3 channels in the DH in the inflammation-induced hypersensitivity. This is in agreement with the previous finding that apamin can increase amplitude of the mEPSCs in the superficial DH indicating the effect of postsynaptic SK channels on synaptic transmission in this area [40]. The role of SK2-containing channels in nociception cannot be excluded since apamin and NS309 can affect both SK2- and SK3-containing channels, however our western blot analyses showed no change in the PSD levels of SK2 after CFA.

The SK channels can control cell excitability by reducing the function of postsynaptically located NMDARs. This was first observed in the hippocampus [39] and amygdala [19], however, later studies demonstrated this mechanism in other structures, such as prefrontal

cortex [18]. Therefore, SK-NMDAR interactions seem to be a more generalized CNS phenomenon rather than a local mechanism restricted only to the hippocampus. Nearly all excitatory synapses in the superficial laminae of the DH contain postsynaptically localized NMDARs, as determined using an antibody recognizing all splice variants of the NR1 subunit [4]. Moreover, NR1 subunit expression in the DH has been shown to increase at the PSD after CFA [59]. This finding, taken together with our data showing a decrease in the PSD levels of SK3 after CFA, may indicate a CFA-induced decrease in SK channel control over NMDAR at the postsynaptic level. A secondary slow-rising current was observed in the NK1+ cells at the DH following NMDAR-mediated EPSCs [51]. The current has not been identified, however, it was argued in the paper that SK channel could be a possible candidate generating the current. Similar coupling of NMDAR activation and SK channel has been reported in the ventral horn [2]. These findings are in line with our data suggesting SK-NMDAR interaction at the DH. It is possible that CFA-induced decrease in SK-mediated currents observed in the DH could be a result of reduction of voltage-activated Ca^{2+} currents after CFA. The fact that I_{AHP} decrease could be reversed by NS309 that directly activates SK channel, as well as our biochemistry data showing decrease in surface expression of SK3 argue against this possibility. In addition, reduced I_{AHP} was not accompanied by reduced inward currents evoked by depolarization pulse, indicating that SK current decrease could not be explained by reduced voltage-activated Ca^{2+} currents. However, direct measurement of Ca^{2+} currents will be needed to rule out voltage-activated Ca^{2+} channel involvement.

It is important to note that the SK-NMDAR interaction shown in the brain takes place at the dendritic spines [39]. In the spinal cord, both dendritic and somatic localization of SK3 subunits was reported in motoneurons [6; 16], however, in DH neurons dendritic SK3 expression has not been demonstrated. We show SK3 immunostaining throughout the spinal cord DH, and this is consistent with previous publications [6; 16; 37; 46]. Moreover, our immunohistochemistry studies for the first time demonstrated SK3 subunit expression distribution within DH neurons. We found both somatic and dendritic expression of SK3 subunits. Dendritic localization of SK3 supports our hypothesis that SK3-containing channels can be located in close proximity with postsynaptic NMDARs to provide control over NMDAR-mediated transmission. In addition, our double-immunostaining experiments indicate that SK3 and NR1 subunits can be co-expressed on the same DH neuron. Confocal imaging does not have sufficient resolution to directly confirm co-localization of the SK channels and NMDAR at the postsynaptic level, thus future studies using electron microscopy may be needed to further evaluate compartmental localization of SK channel-NMDAR interaction.

NMDARs show an increased contribution to spinal sensory transmission in a number of chronic and acute models of pain hypersensitivity [13; 15; 32; 47; 57]. The antinociceptive effects of NMDAR antagonists in animal models support the potential use of this class of drugs for the treatment of neuropathic and inflammatory pain [11; 29; 38; 44]. A number of studies on experimental pain conditions in human subjects confirmed analgesic potential of NMDAR antagonists in the treatment of chronic pain and have helped to define the role of NMDAR activation in the development of mechanical hyperalgesia [5; 26; 41; 43]. The majority of clinical studies with NMDAR antagonists have shown efficacy in the treatment of chronic pain; however, all of them were associated with significant dose-limiting side

effects involving sedation, nausea, dissociative reactions, dizziness, visual distortions, and hallucinations [14; 25; 55]. Hence, clinical use of NMDAR antagonists as a pain treatment has been limited by a very narrow therapeutic window and the inability to adequately manage the adverse effects that can occur at therapeutic doses. We show that co-administration of a sub-effective dose of an SK activator with an NMDAR antagonist can significantly reduce the dose of NMDAR antagonist required to produce antinociceptive effects. Therefore, our study addresses a long-standing problem of limited clinical application of these potentially useful analgesics and puts treatment of chronic pain with NMDAR antagonists in a new perspective.

To summarize, our studies demonstrate for the first time that activation of the SK channels in the spinal cord *in vivo* can alleviate CFA-induced mechanical hypersensitivity, and highlight an SK channel and NMDA receptor interaction in DH neurons. Our data does not exclude a role for SK channels in the DRGs in inflammation-induced hypersensitivity, however it indicates that SK channel activation in the DH is sufficient to result in antinociception following low-dose NMDAR antagonist treatment. Furthermore, we show that co-administration of an SK activator with an NMDAR antagonist can significantly reduce the dose of NMDAR antagonist required to produce antinociceptive effects. Given the importance of NMDAR in nociceptive processing and the dose-limiting side effects overshadowing otherwise efficient anti-hyperalgesic properties of NMDAR antagonists, these findings could have important clinical implications.

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Fig. 1.

Intrathecal (i.t.) injection of the SK channel activator, NS309, attenuates CFA-induced mechanical hypersensitivity. **A** NS309 reverses CFA-induced mechanical hypersensitivity of the rats ($n=8$) without affecting their locomotor activity (right inset; $n=6$). No significant effect of NS309 100 μM on mechanical thresholds was observed when rats received intraplantar injection of saline instead of CFA (left inset; $n=6$). Post-i.t. measurements shown here are taken 30 min after i.t. administration. #, + statistical significance within the group. a, b, c statistical difference between the groups: a time point with a different letter indicates significant difference with all the groups that do not show the same letter; when data points show the same letters it indicates that there is no significant difference between those groups at the data point. **B** Dose-response curve summarizing the reversal effects of three doses of NS309 (25 μM , 50 μM and 100 μM) on mechanical thresholds calculated as percent change from baseline.

Fig. 2.

Bath applied NS309 reverses CFA-induced decrease in SK-mediated currents in the superficial laminae. **A** Whole-cell patch-clamp recordings in spinal cord slices from CFA-treated rats revealed a significant decrease in average peak SK-mediated current, I_{AHP} , in half of the recorded neurons (17 out of 35; middle column). No change was observed in the rest of the neurons (18 out of 35; right column) compared to saline-treated controls (left column). Sample traces (average of 15) show I_{AHP} decrease after CFA (left), and I_{AHP} s recorded before and after bath application of 100 nM apamin (right). * statistical significance between the groups. **B** Graph shows time course of NS309 (100 μ M) bath application. NS309 increases I_{AHP} in CFA-affected neurons (inset). Full traces (average of 15) with a zoom on I_{AHP} are recorded before, during and after NS309.

Fig. 3. Postsynaptic expression of the SK channel subunit, SK3, is reduced after CFA. Average histograms and representative western blots showing protein expression levels of SK1 (lower band), SK2 and SK3 subunits in the total homogenate (TH) and postsynaptic density fractions (PSD) in the DH of the CFA- and saline-treated rats. * statistical significance between the groups.

Fig. 4.

SK3 channels show both somatic and dendritic expression. **A** SK3 (green) immunolabeling shows abundant distribution throughout DH laminae (10x). Control (blank) image shows that there was no immunoreaction product in the spinal cord sections in the absence of primary antibody. **B** Higher magnification (60x) image of lamina II neurons reveals both somatic and dendritic expression of SK3 (red arrows), nuclei are stained with DAPI (blue). The image on the right is a high magnification of the neuron in the white square (zoomed 4.3x). Schematics in **B**, **C** and **D** show image locations within DH. **C** Double immunostaining with NeuN (red) confirms neuronal localization of SK3 (green). The image on the right is a high magnification of the neurons in the white square (zoomed 2.63x). **D** Another lamina II neuron (60x). Dendritic profiles (red arrows) are seen adjacent to the labeled neuron (marked by “+”) with several SK3-labeled puncta. Another dendrite with SK3-labeled puncta is seen at the bottom left side of the image (red arrow). The image on the right is a high magnification of the neuron in the white square (zoomed 2.89x).

Fig. 5. SK3-containing SK channels can be co-expressed in close proximity with NMDA receptors. Double immunolabeling with SK3 (green) and NR1 (red) antibodies shows co-expression (yellow) of NR1 subunit of NMDAR and SK3-containing channels within the same neuron (60 x). Control (blank) image shows that there was no immunoreaction products in the spinal cord sections in the absence of primary antibodies.

Fig. 6. I.t. co-administration of NS309 and DL-AP5 reduces CFA-induced mechanical hypersensitivity. The graph shows reversal of CFA-induced mechanical hypersensitivity following i.t. co-administration of 25 μ M NS309 and 10 nmol DL-AP5, but not following vehicle, 10 nmol DL-AP5 alone, or 25 μ M NS309 alone. * statistical significance between the groups; # statistical significance within the group.