Dexmedetomidine inhibits Tetrodotoxin-resistant Na\textsubscript{v}1.8 sodium channel activity through G\textsubscript{i/o}-dependent pathway in rat dorsal root ganglion neurons

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Abstract

Background: Systemically administered dexmedetomidine (DEX), a selective α2 adrenergic receptor (α2-AR) agonist, produces analgesia and sedation. Peripherally restricted α2-AR antagonist could block the analgesic effect of systemic DEX on neuropathic pain, with no effect on sedation, indicating peripheral analgesic effect of DEX. Tetrodotoxin-resistant (TTX-R) sodium channel Na\textsubscript{v}1.8 play important roles in the conduction of nociceptive sensation. Both α2-AR and Nav1.8 are found in small nociceptive DRG neurons. We, therefore, investigated the effects of DEX on the Na\textsubscript{v}1.8 currents in acutely dissociated small-diameter DRG neurons.

Results: Whole-cell patch-clamp recordings demonstrated that DEX concentration-dependently suppressed TTX-R Na\textsubscript{v}1.8 currents in small-diameter lumbar DRG neurons. DEX also shifted the steady-state inactivation curves of Na\textsubscript{v}1.8 in a hyperpolarizing direction and increased the threshold of action potential and decrease electrical and chemical stimuli-evoked firings in small-diameter DRG neurons. The α2-AR antagonist yohimbine or α2\textsubscript{A}-AR antagonist BRL44408 but not α2\textsubscript{B}-AR antagonist imiloxan blocked the inhibition of Na\textsubscript{v}1.8 currents by DEX. Immunohistochemistry results showed that Na\textsubscript{v}1.8 was predominantly expressed in peripherin-positive small-diameter DRG neurons, and some of them were α2\textsubscript{A}-AR-positive ones. Our electrophysiological recordings also demonstrated that DEX-induced inhibition of Na\textsubscript{v}1.8 currents was prevented by intracellular application of G-protein inhibitor GDP\textsubscript{β}-s or G\textsubscript{i/o} proteins inhibitor pertussis toxin (PTX), and bath application of adenylate cyclase (AC) activator forskolin or membrane-permeable cAMP analogue B-Bromo-cAMP (8-Br-cAMP). PKA inhibitor Rp-cAMP could mimic DEX-induced inhibition of Na\textsubscript{v}1.8 currents.

Conclusions: We established a functional link between α2-AR and Na\textsubscript{v}1.8 in primary sensory neurons utilizing the G\textsubscript{i/o}/AC/cAMP/PKA pathway, which probably mediating peripheral analgesia of DEX.

Keywords: α2-adrenoceptor, Dexmedetomidine, Dorsal root ganglion, Pain, Tetrodotoxin-resistant (TTX-R) sodium channel Na\textsubscript{v}1.8, Whole-cell recording
Background

Dexmedetomidine (DEX), a potent and highly selective agonist of the alpha 2 adrenergic receptors (α2-ARs) with more favorable pharmacokinetic properties than clonidine (another commonly used α2-AR agonist) is approved for the adult intensive care unit use as sedative infusion by the US Food and Drug Administration in 1999. Three α2-ARs (α2A, α2B, and α2C-ARs) have been cloned, and all of which are coupled to inhibitory G proteins [1] and play an important role in the control of pain. The α2-ARs have a diffuse distribution in the nervous system, including in primary afferents, spinal dorsal horn and brain stem [2-5]. Systemically administered α2-AR agonists produce anti-nociceptive effects in humans and animals, suggesting that the α2-AR may be involved in anti-nociception at the supraspinal, spinal and peripheral levels [6-9]. Our previous study showed that intrathecal DEX significantly suppressed monoarthritis-induced thermal hyperalgesia and glial activation in spinal level [10]. However, intrathecal or intracerebroventricular administration of DEX produces dose-dependent sedation [11]. Peripherally restricted α2-AR antagonist could block the analgesic effect of systemic DEX on neuropathic pain, with no effect on sedation, indicating peripheral analgesic effect of DEX [7].

Evidence has emerged that the effects on ion channels may be an important mechanism underlying DEX-induced peripheral anti-nociception [12,13]. Previous studies have revealed that changes in function of voltage-gate sodium channels in nociceptive primary sensory neurons participate in the development of peripheral hyperexcitability that occurs in neuropathic and inflammatory pain conditions [14,15]. Among them, the tetrodotoxin-resistant (TTX-R) sodium channel Nav1.8 primarily expressed by small- and medium-sized dorsal root ganglion (DRG) neurons [16,17], substantially contributes to the upstroke of action potential in these neurons [18]. Na1.8-null mice displayed a pronounced increase in threshold to noxious mechanical stimuli and a slight decrease in noxious thermoreception as well as delayed development of inflammatory hyperalgesia [19]. Likewise, functional knockdown of Na1.8 in rats reduces hyperalgesia and allodynia in neuropathic pain and inflammatory pain models [14,20,21]. Several G-protein-coupled receptors (GPCRs)-mediated second-messenger cascades including PKA, PKC and MAPKs have been shown to regulate Na1.8 sodium channels [14,22]. In the present study, we investigated whether the peripheral DEX-induced analgesia might in part arise from the suppressed activation of TTX-R sodium channel Nav1.8 currents via binding to its GPCR α2-ARs in small-diameter DRG neurons.

Results

Recording of Nav1.8 currents in DRG neurons

Double immunofluorescence revealed that Na1.8 was predominantly expressed in peripherin-positive small-diameter DRG neurons (Figure 1A). In the present study, all recordings were performed in small-diameter (<25 μm) DRG neurons. With existence of TTX (500 nM) in external solution, TTX-resistant (TTX-R) sodium currents were recorded in most (170 out of 223) of the small-diameter DRG neurons. As our previous reported, the membrane potential was held at −60 mV to inhibit Na1.9 currents, leaving the Na1.8 currents intact [22]. The family of Na1.8 currents was generated with a voltage-clamp protocol (holding at −60 mV, depolarizing steps from −55 mV to +40 mV, 50 ms, 5 mV increment, Figure 1B). According to the current–voltage relationship (Figure 1C), we selected −15 mV to elicit Na1.8 currents in most of the recordings (Figure 1D). The peak amplitude of Na1.8 currents was stable during the recordings.

Effects of DEX on Na1.8 currents in small DRG neurons

Application of DEX in different doses (0.03, 0.1, 0.3, 1, 3 and 30 μM) dose-dependently reduced the peak amplitude of Na1.8 currents in small DRG neurons within 1 min and washed out within 5 min (Figure 2A and B). One-way ANOVA analysis revealed a significant effect of DEX treatment (F(5,66) = 23.885, p < 0.001). The ED50 was calculated to be 0.92 μM (95% CI: 0.77–1.68). The maximal inhibitory effect (36.51 ± 5.39%) was induced by 3 μM DEX. A higher concentration of DEX (30 μM) failed to induce more powerful inhibition (34.56 ± 2.7%), indicating a “ceiling effect” at a concentration of 3 μM (Figure 2C).

The effects of DEX on the activation and inactivation properties of Na1.8 currents were studied using the appropriate voltage protocols. As described above, a voltage-clamp protocol consisted of 50 ms depolarizing steps from −55 mV to +40 mV with 5 mV increment was used to determine the activation of Na1.8 channels. No shift in the voltage-dependent activation curve was observed in DEX-treated group compared with control one (Figure 3A). The half-maximal activation potential (V1/2 activation) was −27.30 ± 2.13 mV (n = 7) and −28.17 mV ± 0.73 mV (n = 7) in the absence and presence of 3 μM DEX, respectively. Steady-state inactivation of Na1.8 channel was determined at a series of membrane potentials from −60 mV to −20 mV with 5 mV increment for 500 ms and a following test potential of −15 mV. DEX caused a left shift toward the hyperpolarizing potential of the steady-state inactivation curve (Figure 3B). The V1/2 inactivation was −40.49 ± 2.49 mV (n = 9) of the control and −45.39 ± 2.65 mV (n = 9) of DEX treatment, respectively.

DEX reduced Na1.8 currents via α2-AR

DEX was a selective alpha 2 adrenergic receptor (α2-AR) agonist. To address whether the attenuation of Na1.8 currents induced by DEX application was mediated by α2-ARs, the effect of yohimbine, an α2-ARs antagonist, on inhibitory effects of DEX on Na1.8 currents was
examined. Like previous report [12], yohimbine (30 μM) per se inhibited Na$_v$1.8 currents (Figure 4A). Pretreatment of DRG neurons with 3 μM yohimbine, a concentration to antagonize DEX-induced membrane hyperpolarization mediated by α2-ARs in rat hypothalamic neurons [23], DEX-induced suppression of the Na$_v$1.8 currents was significantly blocked (Figure 4B and C). The peak densities of Na$_v$1.8 currents in yohimbine (3 μM) plus DEX (3 μM)-treated group was significantly greater than that in DEX-treated group (One-way ANOVA, $F_{3, 26} = 5.451$, $p < 0.01$). Moreover, we examined the effect of BRL44408 (a preferential α2-AR antagonist) on DEX-induced inhibition of Na$_v$1.8 currents. Pre-incubation of BRL44408 (1 μM) alone did not affect the peak densities of Na$_v$1.8 currents, but significantly blocked DEX-induced suppression of Na$_v$1.8 currents (Figure 4D and F). Given that BRL44408 may also be able to block α2β-AR at a higher dose, we further examined the effect of α2β-AR antagonist imiloxan on DEX-induced inhibition of Na$_v$1.8 currents. Neither basal Na$_v$1.8 currents nor DEX-induced suppression was influenced by incubation of imiloxan (3 μM) (Figure 4E and F). These data indicated that DEX

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**Figure 1** Isolation of TTX-resistant Na$_v$1.8 currents in small-diameter DRG neurons. (A) Double immunofluorescence reveals the expression of Na$_v$1.8 in peripherin-positive small-diameter DRG neurons. (B) Representative I-V curve family of currents recorded in the presence of 500 nM TTX. Cells were depolarized to a variety of potentials (−55 mV to +40 mV) from a holding potential of −60 mV, to elicit Na$_v$1.8 currents. (C) I-V curve of Na$_v$1.8 currents shown in (B). (D) Representative traces of Na$_v$1.8 currents elicited by a single pulse of −15 mV.

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**Figure 2** DEX dose-dependently inhibits Na$_v$1.8 currents. (A, B) Typical traces illustrating the Na$_v$1.8 currents in small-diameter DRG neurons recorded pre- (control) and post- (DEX) perfusion of DEX and wash out. (C) Dose-effect curve of DEX-induced inhibition of Na$_v$1.8 currents. The currents were measured after a 1-min application of different concentrations of DEX.
modulated Na\textsubscript{v}1.8 currents mainly through α2\textsubscript{AR}. Also, the colocalization of Na\textsubscript{v}1.8 with α2\textsubscript{AR} in DRG small-diameter neurons provided a cellular basis for the involvement of α2\textsubscript{AR} in the DEX modulating Na\textsubscript{v}1.8 currents (Figure 4G).

**G\textsubscript{i/o}-proteins participate in DEX-induced Na\textsubscript{v}1.8 currents inhibition**

Given α2-ARs act through G-proteins, we examined the effect of GDP\textsubscript{β}-s, a G protein inhibitor, on DEX-induced rapid suppression of Na\textsubscript{v}1.8 currents in small DRG neurons. Inclusion in pipette solution of GDP\textsubscript{β}-s (1 mM) did not impair Na\textsubscript{v}1.8 activation. On the other hand, the inhibition of Na\textsubscript{v}1.8 currents by DEX was completely abolished (Figure 5A and C) (One-way ANOVA, F\textsubscript{3,38} = 12.757, p < 0.01).

α2\textsubscript{AR}s are generally known to coupled to the inhibitory G\textsubscript{i} proteins [24] through which they inhibit adenylate cyclase (AC) activity. Therefore, we examined whether DEX-induced inhibition of Na\textsubscript{v}1.8 currents occurs via G\textsubscript{i} proteins. As shown in Figures 5B and C, pertussis toxin (PTX, 1 μg/ml), an irreversible inhibitor of G\textsubscript{i/o}-proteins, significantly prevented the Na\textsubscript{v}1.8 currents amplitude change induced by DEX (One-way ANOVA, F\textsubscript{3,30} = 15.765, p < 0.01). Inclusion in pipette solution of PTX (1 μg/ml) did not change Na\textsubscript{v}1.8 currents amplitude (Figure 5B and C).

Because G\textsubscript{i} proteins inhibit the catalytic activity of AC, which catalyzes cAMP production, the G\textsubscript{i}-mediated suppression of Na\textsubscript{v}1.8 currents can be the consequence of decreased levels of intracellular cAMP and a concomitant reduction in PKA-dependent phosphorylation of Na\textsubscript{v}1.8. Here, we used Rp-cAMP, 8-Br-cAMP and forskolin to examine the effects of AC-cAMP-PKA pathway on the Na\textsubscript{v}1.8 currents. Incubate with PKA inhibitor Rp-cAMP (50 μM) for 5 min, Na\textsubscript{v}1.8 currents were significantly suppressed. Co-application of 3 μM DEX did not cause a further reduction of the currents amplitudes (Figure 5D and E). Moreover, 5-min pretreatment of DRG neurons with 8-Br-cAMP (500 μM) a membrane-permeable cAMP
anallogues, or forskolin (0.1 μM), a AC activator, caused slight increase in Na\textsubscript{v}1.8 currents amplitude (Figure 5 F-I). Although the increase did not reach statistical significance, it totally removed the inhibitory effect of DEX on Na\textsubscript{v}1.8 currents (Figure 5G and I).

**Effect of dexmedetomidine on excitability of DRG neurons**
Na\textsubscript{v}1.8 is the main contributor to the upstroke of action potentials in small-diameter DRG neurons [18]. Thus, modulation of this channel by DEX should influence the excitability of DRG neurons. We applied 10 ms depolarizing currents pulse to evoke action potentials. In 13 of 23 neurons tested, DEX 3 μM significantly increase the injected currents threshold to evoke action potentials from 37.5 ± 4.97 pA to 61.88 ± 5.78 pA respectively (paired \(t\)-test, \(p < 0.01\)) (Figure 6A and B). Moreover, by injection of maximum currents pulse (500 ms, 200 pA), action potential firing
frequencies of DRG neurons were significantly decreased by DEX treatment (Figure 6C and E). DEX-induced inhibitory effect on action potential firing can be mimicked by selective $\alpha_2$-AR agonist guanfacine ($30 \mu M$) (Figure 6D and E). Similarly, $0.5 \mu M$ capsaicin-induced action potentials were also significantly blocked by DEX (Figure 6F and G).

**Discussion**

In this study, we demonstrated that selective $\alpha_2$-AR agonist dexmedetomidine (DEX) reduced Na$_{v}$1.8 currents in small-diameter acutely dissociated DRG neurons. We also showed that DEX decreased excitability of small sensory neurons by increasing the activation threshold and decreasing the action potential firing. This inhibition of Na$_{v}$1.8 currents was completely blocked by the selective $\alpha_2$-AR antagonist, suggesting that $\alpha_2$-AR might be directly involved in DEX-induced changes in Na$_{v}$1.8 activity. We also found that the PTX-sensitive G$_{i/o}$ proteins/AC/cAMP/PKA signaling cascade is primarily responsible for the activation of Na$_{v}$1.8 currents in response to DEX.

*Figure 5 Involvement of G$_{i/o}$ proteins/AC/cAMP/PKA pathway in DEX-induced inhibition of Na$_{v}$1.8 currents. (A-C) Loading neurons with 1 mM GDP$\beta$s or PTX completely abolished the inhibitory effect of DEX on Na$_{v}$1.8 currents. *p < 0.05. (D, E) Pre-incubation PKA inhibitor Rp-cAMP (50 $\mu M$) inhibited Na$_{v}$1.8 currents. Co-application of DEX did not cause a further reduction of the current amplitudes. *p < 0.05. (F, G) Pre-incubation cAMP analogues 8-Br-cAMP ($500 \mu M$) prevented DEX-induced inhibition of Na$_{v}$1.8 currents. (H, I) Pre-incubation AC activator forskolin (0.1 $\mu M$) prevented DEX-induced inhibition of Na$_{v}$1.8 currents.*
Figure 6 Effect of DEX on the action potential threshold and firing rate of DRG neurons. (A) In current clamp model, depolarizing current pulse required to evoke an action potential in a DRG neuron before and after application of DEX (a = 25 pA, b = 30 pA, c = 40 pA, d = 45 pA). (B) DEX (3 μM) reduced the amount of currents required to evoke action potential. (C, D) Firing response of DRG neurons to a 200 pA depolarizing current pulse (500 ms) before and after application of DEX (C) and selective α2A-AR agonist guanfacine (D). (E) Summary data indicate the inhibitory effects of DEX and guanfacine on firing rate in DRG neurons. (F, G) Current clamp recording showing suppression of capsaicin-induced action potential firing by DEX (3 μM). *p < 0.05; **p < 0.01.
in DRG neurons. These results suggest a peripheral mechanism of DEX analgesia.

TTX-R sodium channel primarily expresses in DRG nociceptors [25,26]. In the two distinct TTX-R sodium channel isoforms Na\textsubscript{1.8} and Na\textsubscript{1.9}, Na\textsubscript{1.8} likely mediates the majority of TTX-R currents. Accumulating evidence points up that TTX-R sodium channel plays an important role in peripheral pain processing [27]. Nociceptive signals evoke a dynamic change of TTX-R sodium channel, for example, chronic compression (CCD) of the DRG [15,28] or local inflammation of the DRG by the application of zymosan [29] and subcutaneous injection of carrageenan [30] or complete Freund’s adjuvant (CFA) [31] produces an increase in TTX-R sodium currents in small DRG neurons. Either the physiological or pathological pain was alleviated in the Na\textsubscript{1.8}-null mice or Na\textsubscript{1.8} knock-down rats [14,27,32,33]. Peripheral inflammation or nerve injury has been shown to upregulate Na\textsubscript{1.8} expression in nociceptive DRG neurons [34,35]. Blockade of Na\textsubscript{1.8} sodium channel by A-803467, a potent and selective Na\textsubscript{1.8} sodium channel blocker, could inhibit nerve injury-induced mechanical allodynia and inflammation-induced thermal hyperalgesia [36].

Given that both α\textsubscript{2}-AR and Nav1.8 are found in small nociceptive DRG neurons [4,5,37], and α\textsubscript{2A}-AR and Na\textsubscript{1.8} co-localized in the same small DRG neurons, we propose that stimulation of α\textsubscript{2}-AR in sensory neurons may lead to an attenuation of the painful symptoms of hypersensitivity via the inhibition of Na\textsubscript{1.8} channel activity. Consistently, application of DEX concentration-dependently decreased the current density of Na\textsubscript{1.8} in small DRG neurons and shifted the voltage-dependence of steady-state inactivation curve for Na\textsubscript{1.8} in the hyperpolarizing direction, which could result in a lower threshold for Na\textsuperscript{+} channel inactivation. DEX also increased the threshold of action potential and decreased firing rate in small DRG neurons. Despite of the previous reports that yohimbine did not alter DEX-induced inhibition of TTX-R Na\textsuperscript{+} currents in small DRG neurons [12] and voltage-gated Na\textsuperscript{+} currents in NG108-15 cells [13], the present study showed that 3 μM yohimbine, a concentration to antagonize DEX-induced membrane hyperpolarization mediated by α\textsubscript{2}-ARs in rat hypothalamic neurons [23], completed blocked DEX-induced suppression of the Na\textsubscript{1.8} currents, suggesting an involvement of α\textsubscript{2}-ARs in DEX effect. Considering the affinity of yohimbine for α\textsubscript{1}-ARs, serotonin and dopamine receptors [38], inhibition of high dose yohimbine per se on Na\textsubscript{1.8} currents may relate to the interaction of yohimbine with these receptors.

Although three subtypes of the α\textsubscript{2}-ARs mRNAs were expressed in the rat DRGs [5,39,40], α\textsubscript{2A}-AR mRNA was only found in small numbers of neuron profiles [5,41], and following peripheral nerve injury, α\textsubscript{2A}-AR and α\textsubscript{2C}-AR mRNA levels increased and decreased, respectively [5,40]. Also, the immunohistochemical analysis showed that α\textsubscript{2A}-AR and α\textsubscript{2C}-AR proteins in DRG neurons was respectively increased and decreased after chronic constriction injury of sciatic nerve, whereas no α\textsubscript{2B}-AR neurons were detected in either normal or nerve injury DRG [42]. Moreover, α\textsubscript{2A}-AR rather than α\textsubscript{2C}-AR in the superficial layers of spinal dorsal horn was observed in the terminals of capsaicin-sensitive and substance P-containing primary afferent fibers [2]. Degeneration of TRPV1 afferent terminals, the level of α\textsubscript{2A}-AR, but not α\textsubscript{2C}-AR, was largely reduced in primary afferent terminals [43]. Our present study further showed co-localization of α\textsubscript{2A}-AR and Na\textsubscript{1.8}-like immunoreactivity in small DRG neurons. DEX-induced inhibition of Na\textsubscript{1.8} currents was prevented by pretreatment of BRL44408, a preferential α\textsubscript{2A}-AR antagonist, but not of imiloxan, a α\textsubscript{2B}-AR antagonist. In addition to Na\textsubscript{1.8}, other cation channels, for example, TRPM8 may also participate in α\textsubscript{2A}-AR-mediated nociceptive inhibition. Stimulation of α\textsubscript{2A}-AR inhibited TRPM8 in DRG neurons [44]. Taken together, these findings suggest that the α\textsubscript{2A}-AR subtype represents the most likely candidate in DRG neurons to be involved in the modulation of nociceptive information.

Our data strongly suggest that DEX inhibits Na\textsubscript{1.8} currents in a G\textsubscript{i/o} proteins/AC/cAMP/PKA signaling-dependent manner in small DRG neurons. Specifically, we showed that preventing G\textsubscript{i/o} recruitment by PTX treatment blocked DEX-induced inhibition of Na\textsubscript{1.8} current density, and PKA inhibitor mimicked the effect of DEX through the receptor. In support of this, it has been also reported that blockade of PKA activity inhibited the baseline Na\textsubscript{1.8} currents in small-diameter node ganglion neurons [45]. Consistent with the general notion that stimulation of α\textsubscript{2A}-AR by DEX brings about G\textsubscript{i/o}-mediated inhibition of AC and reduction of intracellular CAMP levels, AC activator forskolin and cAMP analogues 8Br-cAMP completely reversed DEX-induced inhibition of Na\textsubscript{1.8} currents. These findings suggest that the classical AC/cAMP/PKA signaling pathway resulting from the α\textsubscript{2}-ARs-mediated activation of PTX-sensitive G\textsubscript{i/o} proteins is involved in the regulation of Na\textsubscript{1.8} by DEX.

**Conclusions**

DEX attenuated TTX-R sodium channel Na\textsubscript{1.8} currents in small-diameter DRG neurons via α\textsubscript{2A}-AR/G\textsubscript{i/o}/AC/cAMP/PKA cascade, which probably constitutes a mechanism of peripheral DEX analgesia.

**Materials and methods**

**Animals**

Male adult (100–150 g) Wistar rats were obtained from the Experimental Animal Center, Shanghai Medical College of Fudan University, China. Rats were on a 12 h
light/dark cycle with a room temperature of 23 ± 1°C and received food and water ad libitum. All experiments protocols were permitted by the Shanghai Animal Care and Use Committee and followed the policies issued by the International Association for the Study of Pain on the use of laboratory animals. All efforts were made to minimize animal suffering and reduce the numbers of animals used.

**Preparation of DRG neurons**

Animals were anesthetized with ether and rapidly decapitated. DRGs from L4-L6 lumbar segments were removed and immediately transferred onto DMEM (Gibco, Life Technologies, Grand Island, NY, USA) on ice. The ganglia were minced with fine spring scissors and treated with collagenase (2.67 mg/ml, type IA, Sigma, St. Louis, MO) and trypsin (1 mg/ml, type I, Sigma) in DMEM saturated with CO$_2$/O$_2$, mixed gas at 37°C for 35 min. After wash with standard external solution (in mM, 150 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH) three times, the ganglia were then gently triturated using fine fired-polished Pasteur pipettes. The dissociated DRG neurons were plated onto 10-mm diameter coverslips in the 3.5 cm culture dishes and incubated with standard external solution. Each culture dishes contained three or four coverslips and all the experiments were performed within 2–8 h after plating.

**Patch-clamp recordings**

Whole-cell voltage-clamp and current-clamp recordings of DRG neurons were performed at room temperature (RT, 23 ± 1°C) with an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Stimulation protocols and data acquisition were controlled by the software Pulsefit 8.5 (HEKA Elektronik). All of the recordings were performed in small-diameter (15–25 μm) DRG neurons with resting membrane potentials more negative than ~50 mV. Microelectrodes (N51A borosilicate glass, Sutter Instruments) with a resistance of 2–6 MΩ were pulled using a PP7 puller (Sutter Instruments). The pipette solution contained (in mM): 140 CsF, 1 MgCl$_2$, 1 EGTA, 2.5 Na2ATP, 10 HEPES, pH was adjusted to 7.2 with CsOH. Seals (1–10 GΩ) between the electrode and the cells were established. After the whole-cell configuration was established, the cell membrane capacitance and series resistance were compensated (>80%). Leak currents were subtracted using the online P/4 protocol. The data were sampled at 10 kHz and low-passed at 2 kHz. For Na$_{1.8}$ recordings, the external solution contained (in mM): 32 NaCl, 20 TEA-Cl, 105 choline-Cl, 1 MgCl$_2$, 1 CaCl$_2$, 0.1 CdCl$_2$, 10 HEPES, 0.0005 TTX and 10 glucose, adjusted to pH 7.4 with NaOH. For current-clamp recordings, the electrode solution was changed to: 140 KCl, 1 MgCl$_2$, 0.5 CaCl$_2$, 5 EGTA, 10 HEPES, 2.5 Na2ATP, pH was adjusted to 7.2 with KOH. The external solution was changed to: 150 NaCl, 5 KCl, 2.5 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH was adjusted to 7.4 with NaOH. DRG neurons were held at ~60 mV and Na$_{1.8}$ currents were evoked by depolarizing pulses to ~15 mV. The activation and inactivation properties of Na$_{1.8}$ currents were studied using the appropriate voltage protocols. The voltage-clamp protocol consisted of 50 ms depolarizing steps from ~55 mV to ~40 mV with 5 mV increment was used to determine the activation of Na$_{1.8}$ channels. The Boltzmann function of the form $G_{Na} / G_{Na_{max}} = 1 / [1 + exp ((V_{m1/2} - V_m) / k)]$ was used to describe the voltage dependence of activation and half activation potential was obtained. Steady-state inactivation of Na$_{1.8}$ channel was determined at a series of membrane potentials from ~60 mV to ~20 mV with 5 mV increment for 500 ms and a following test potential of ~15 mV. The steady-state inactivation curve was fitted by the Boltzmann function $I_{Na} / I_{Na_{max}} = 1 / [1 + exp ((V - V_{m1/2}) / k)]$, where $I_{Na_{max}}$ is the maximal peak current, $V$ is the prepulse membrane potential.

**Drugs**

All the drugs were purchased from Sigma (St. Louis, MO, USA). The drugs were dissolved in normal saline as stock solutions. All of the stock solutions were stored at ~20°C or ~80°C until use. Working concentrations of the drugs were prepared on the day of the experiment from the stock solutions. The drug dosages were selected based on previous reports and our preliminary studies. Dexmedetomidine was applied continuously for 1 min closed to cells via ALA-VM8 perfusion system (ALA Scientific Instruments, Westbury, NY). Yohimbine and BRL44408 were applied to the chamber 30 min before and during the perfusion of dexmedetomidine (DEX). Rp-cAMP, 8-Br-cAMP and forskolin were applied to chamber 5–10 min before and during the DEX perfusion. GDPβ-s and PTX were applied in the pipette internal solution.

**Immunohistochemistry**

Animals were given an overdose of urethane and were then transcardially perfused with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). DRGs (L4–L6 segments) were removed and postfixed in the same fixative for 2 h at 4°C and then immersed in a 10–30% gradient of sucrose in phosphate buffer for 24–48 h at 4°C for cryoprotection. DRGs were embedded in OCT compound, cut in a cryostat (Leica 1900, Leica) at 7 μm (to study Na$_{1.8}$ and α2A-AR coexistence) or 14 μm thickness and mounted onto gelatin coated slides. The sections were placed in a humid chamber and processed for immunohistochemistry. The sections were blocked with 10% donkey serum in 0.01 M PBS (pH 7.4) with 0.3% Triton X-100 for 1 h at RT. For Na$_{1.8}$ and peripherin (a small-diameter DRG neuronal marker) double immunofluorescence, the sections were incubated...
with a mixture of rabbit anti-Na\textsubscript{v}1.8 (1:1000; Alomone) and mouse anti-peripherin (1:2000; Millipore) overnight at 4°C, followed by a mixture of Alex Fluor 488- and Alex Fluor 546-conjugated secondary antibodies (1:200; Invitrogen) for 2 h at 4°C. For detecting Na\textsubscript{v}1.8 and α2\textsubscript{A}-AR coexistence, two adjacent sections (7 μm) was respectively incubated with rabbit anti-Na\textsubscript{v}1.8 (1:1000) and rabbit anti-α2\textsubscript{A}-AR (1:100; Alomone) primary antibodies in PBS with 1% normal donkey serum and 0.3% Triton X-100 overnight at 4°C, followed by incubation within Alex Fluor 546- and Alex Fluor 488-conjugated secondary antibodies for 2 h at 4°C, respectively. All of the slides were coverslipped with 50% glycerin in 0.1 M PBS and then examined with an Olympus FV1000 confocal laser scanning microscope (Olympus). Images were acquired using FV10-ASW software. The specificities of the immunostaining were verified by observing no immunostaining after omitting the primary antibodies, which resulted in the disappearance of the immunostaining signals. The specificities of the primary antibodies were verified by a preabsorption experiment. Sections were first incubated overnight with a mixture of Na\textsubscript{v}1.8 or α2\textsubscript{A}-AR primary antibody and the corresponding blocking peptide (5:1 blocking peptide: primary antibody), followed by incubation with a secondary antibody. The immunostaining signals were abolished after absorption.

Data analysis
The data were presented as means ± standard error of mean (SEM). Statistical comparisons were performed using Student’s t-test, paired t-test and one-way ANOVA followed by post hoc Student-Newman-Keuls test. In all cases, p < 0.05 was considered statistically significant.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GXY, LBL and P HL performed the patch clamp recording in DRG neurons. ZK performed the immunofluorescence experiments. YL, XH and ZZQ participated in the statistical analysis. GXY and ZQ performed the study, designed the experiments, and wrote the paper. All of the authors read and approved the final manuscript.

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