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RESEARCH PAPER

Dexmedetomidine and ST-91 analgesia in the formalin model is mediated by α_{2A}-adrenoceptors: a mechanism of action distinct from morphine

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Background and purpose: Intrathecal administration of α_2-adrenoceptor agonists produces potent analgesia. This study addressed the subtype of spinal α_2-adrenoceptor responsible for the analgesic effects of i.t. dexmedetomidine and ST-91 in the formalin behavioural model and their effects on primary afferent substance P (SP) release and spinal Fos activation.
Experimental approach: The analgesic effects of i.t. dexmedetomidine and ST-91 (α_2 agonists) were tested on the formalin behavioural model. To determine the subtype of α_2-adrenoceptor involved in the analgesia, i.t. BRL44408 (α_{2A} antagonist) or ARC239 ($\alpha_{2B/C}$ antagonist) were given before dexmedetomidine or ST-91. Moreover, the ability of dexmedetomidine and ST-91 to inhibit formalin-induced release of SP from primary afferent terminals was measured by the internalization of neurokinin$_1$ (NK_1) receptors. Finally, the effects of dexmedetomidine on formalin-induced Fos expression were assessed in the dorsal horn.
Key results: Intrathecal administration of dexmedetomidine or ST-91 dose-dependently reduced the formalin-induced paw-flinching behaviour in rats. BRL44408 dose-dependently blocked, whereas ARC239 had no effect on the analgesic actions of dexmedetomidine and ST-91. Dexmedetomidine and ST-91 had no effect on the formalin-induced NK_1 receptor internalization, while morphine significantly reduced the NK_1 receptor internalization. On the other hand, both dexmedetomidine and morphine diminished the formalin-induced Fos activation. The effect of dexmedetomidine on formalin-induced Fos activation was reversed by BRL44408, but not ARC239.
Conclusion and implications: These findings suggest that α_{2A}-adrenoceptors mediate dexmedetomidine and ST-91 analgesia. This effect could be through a mechanism postsynaptic to primary afferent terminals, distinct from that of morphine.
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Keywords: Dexmedetomidine; ST-91; α_2-adrenoceptors; substance P; neurokinin 1 receptor; primary afferent; nociception; morphine; BRL44408; ARC239

Abbreviations: PBS, sodium phosphate buffer; SP, substance P

Introduction

Activation of spinal α_2-adrenoceptors by noradrenaline or synthetic agonists produces potent analgesia in animals and humans (Eisenach *et al*., 1996; Pertovaara, 2006). There are three different subtypes of α_2-adrenoceptors (α_{2A}, α_{2B} and α_{2C}), which have similar homology and signal transduction mechanisms (Bylund *et al*., 1994; Kable *et al*., 2000). Histological evidence indicates that the predominant subtypes of α_2-adrenoceptors in the spinal cord are the α_{2A}- and α_{2C}-adrenoceptors. α_{2A}-Adrenoceptors are largely located on substance P (SP)-containing C-fibre primary afferent terminals; although a smaller population also exists on other sites within the superficial dorsal horn (Stone *et al*., 1998; Chen *et al*., 2007). On the other hand, α_{2C}-adrenoceptors are found on inhibitory interneurons and axons of projections neurons (Olave and Maxwell, 2002). The spinal cord contains negligible levels of α_{2B}-adrenoceptors (Zeng and Lynch, 1991; Nicholas *et al*., 1993). On the basis of the distribution of binding and findings from transgenic animals, spinally administered α_2-adrenoceptor agonists are considered to produce their analgesic actions through activation of α_{2A}-adrenoceptors. That is, disabling α_{2A}-adrenoceptor function by a point mutation in the α_{2A} gene resulted in the inability of clonidine and dexmedetomidine to be analgesic in acute thermal nociception and SP-elicited pain behaviour in mice (Lakhlani *et al*., 1997; Stone *et al*., 1997).

The distribution of α_{2A}-adrenoceptors raises the possibility that spinal α_2-adrenoceptor agonists produce their analgesic effects by a presynaptic mechanism. Indeed, previous

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findings have shown that dexmedetomidine, clonidine and ST-91 reduced the evoked release of SP from spinal cord preparations (Pang and Vasko, 1986; Ono *et al*., 1991; Takano *et al*., 1993). Work from our laboratory and others (Honore *et al*., 1999; Nazarian *et al*., 2008) demonstrated that intraplantar formalin evokes the release of SP, as measured by the internalization of neurokinin$_1$ (NK_1) receptors (NK1r). Since α_{2A}-adrenoceptors are involved in the analgesia produced by α_2-adrenoceptor agonists, it would be reasonable to predict that α_{2A}-adrenoceptors are also involved in the presynaptic inhibition of SP release. Thus, in this study, it was determined whether α_{2A}-adrenoceptors mediate the analgesic effects of dexmedetomidine and ST-91 in the formalin-induced pain behaviour model, as well as regulating formalin-induced primary afferent SP release, as measured by NK1r internalization. Finally, the effects of dexmedetomidine on formalin-evoked Fos expression in the dorsal horn were also measured. The present findings indicate that dexmedetomidine and ST-91 produce their analgesic actions through α_{2A}-adrenoceptors in the formalin behaviour test; however, this effect appears to be mediated postsynaptically in the spinal dorsal horn.

Materials and methods

Animals

Male Holtzman Sprague–Dawley rats (250–350 g; Harlan, Indianapolis, IN, USA) were individually housed in standard cages and maintained on a 12-h light/dark cycle (lights on at 07 h). Testing occurred during the light cycle. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23, Bethesda, MD, USA) and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Food and water were available *ad libitum*.

Intrathecal catheter implantation

Rats were implanted with a single i.t. catheter for drug delivery, as described previously (Malkmus and Yaksh, 2004). In brief, rats were anaesthetized by induction with 4% isoflurane in a room air/oxygen mixture (1:1), and the anaesthesia was maintained with 2% isoflurane delivery by mask. The animal was placed in a stereotaxic headholder with the head flexed forward. A midline incision was made on the back of the occipital bone and the neck to expose the cisternal membrane. The membrane was carefully opened with a stab blade, and a single lumen polyethylene-5 (outer diameter 0.36 mm) catheter (8.5 cm) was inserted and passed into the i.t. space surrounding L3–L4 spinal segments. The other end of the catheter was jointed to a polyethylene-10, which was tunnelled s.c. to exit through the top of the head. Catheters were flushed with 10 μl of saline and then plugged. The rats were given 5 mL of lactated ringer's solution s.c. and allowed to recover under a heat lamp; those showing motor weakness or signs of paresis on recovery from anaesthesia were killed immediately. The rats were allowed to recover for 5–7 days before the experiment.

α_2-Adrenoceptor agonists and antagonists on formalin-induced flinching

Formalin-induced flinching was measured using an automated detection system (Yaksh *et al*., 2001). A soft metal band (10 mm wide and 27 mm long, shaped into a C, and weighing ~0.5 g) was placed around the left hindpaw of the animal being tested. Animals were allowed to acclimate in individual Plexiglas chambers for 30 min before experimental manipulations. For dexmedetomidine and ST-91 dose–response studies, rats were administered with dexmedetomidine (0.01, 0.1, 0.3, 1 or 2 μg), ST-91 (0.3, 0.9, 3, 10 or 30 μg) or saline 10 min before a s.c. injection of formalin (5%, 50 μL) into the dorsal side of the banded paw. Immediately after the formalin injection, rats were placed into the test chamber and nociceptive behaviour was quantified by automatic counting of spontaneous flinching and shaking of the injected paw. Flinches were counted in 1-min intervals for 60 min. The data are expressed as total number of flinches observed during phase 1 (0–9 min) and phase 2 (10–60 min). In studies with the antagonist drugs, the α_{2A}-adrenoceptor antagonist, BRL44408 (Young *et al*., 1989) or the $\alpha_{2B/C}$-adrenoceptor antagonist, ARC239 (Bylund *et al*., 1992) were administered 10 min before the administration of dexmedetomidine or ST-91. All drugs were injected i.t. in a volume of 10 μL followed by a 10 μL saline flush.

α_2-Adrenoceptor agonists on formalin- and paw compression-induced NK_1 receptor internalization

After recovery from i.t. catheter implantation, rats were administered i.t. with dexmedetomidine (0.3–10 μg), ST-91 (30 μg), morphine (20 μg) or saline. Five minutes after i.t. drug administration, rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.). For formalin-induced NK1r internalization, intraplantar formalin injection (5%, 50 μL) to the left hindpaw occurred 5 min after the anaesthesia. Rats were killed and transcardially perfused with fixative 8 min after the formalin injection. For paw compression-induced NK1r internalization, dexmedetomidine (1 or 10 μg), ST-91 (30 μg), morphine (20 μg) or saline was administered i.t. Five minutes after i.t. drug administration, rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) and 5 min after anaesthesia, the hindpaw was positioned perpendicularly across the jaws of a 6-inch mosquito forceps with non-serrated jaws. The jaws were closed to the third click of the hemostat ratchet. Compression was applied for 60 s. Application of the forceps produces an evident compression of the soft tissue resulting in approximately a 30% reduction in paw thickness during the compression. This stimulus has been previously used to evoke NK1r internalization (Ghilardi *et al*., 2004; Kondo *et al*., 2005; Nazarian *et al*., 2008). Rats were transcardially perfused 5 min after the paw compression (see Immunocytochemistry).

Dexmedetomidine and α_2-adrenoceptor antagonists on formalin-induced Fos expression

After the rats had recovered from i.t. catheter implantation, they were administered i.t. with BRL44408 (100 μg), ARC239 (100 μg) or saline. Ten minutes after antagonist administration,

rats were administered with dexmedetomidine (1 μg), morphine (20 μg) or saline. Intraplantar formalin (5%, 50 μL) was injected 10 min after agonist administration. Rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.), 1 h 55 min after formalin administration. Five minutes after induction of anaesthesia rats were transcardially perfused.

Tissue preparation and immunocytochemistry

Anaesthetized rats were transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4. The lumbar spinal cord was removed and postfixed overnight. After cryoprotection in 20% sucrose, coronal sections were made using a sliding microtome (30 μm for NK1r staining and 50 μm for Fos staining). Immunofluorescent staining was performed to examine NK1r expression in the spinal dorsal horn. In brief, sections were incubated in a rabbit anti-NK1r polyclonal antibody overnight at room temperature. The antibody was diluted to a concentration of 1:3000 in 0.01 M PBS containing 10% normal goat serum and 0.3% Triton X-100. After rinses in PBS, sections were then incubated for 90 min at room temperature in a goat anti-rabbit secondary antibody conjugated with Alexa 488 diluted at 1:1000 in 0.01 M PBS containing 10% normal goat serum and 0.3% Triton X-100. All sections were finally rinsed and mounted on glass slides and coverslipped with ProLong mounting medium.

Immunocytochemical staining for Fos consisted of blocking endogenous peroxidate activity by incubating the spinal cord sections in 0.3% H_2O_2 for 30 min. Non-specific binding sites were blocked in 0.5% Triton X-100 and 5% normal goat serum in PBS. For primary antibody incubation, tissues were incubated in rabbit anti-Fos polyclonal antibody (1:10 000) for 72 h at 4 °C. The binding sites were visualized by using an ABC kit. The signal was developed in diaminobenzidine–tetrahydrochloride solution containing H_2O_2 in PBS. Upon the presence of a light background, the reaction was stopped by two washes in distilled water. Sections were mounted on slides, air-dried, dehydrated through graded ethanol solution followed by citrisolve and then coverslipped with Permount mounting medium.

Quantification of NK_1 receptor internalization and Fos expression

Neurokinin$_1$ receptor internalization was counted using an Olympus fluorescence microscope at ×60 magnification and followed the standard of previous reports (Mantyh *et al*., 1995; Abbadie *et al*., 1997). The total number of NK1r-immunoreactive neurons in lamina I, with or without NK1r internalization, was counted and taken as a ratio of cells showing NK1r internalization vs all NK1r-positive cells and then converted into a percentage of NK1r-immunoreactive cells. Neuronal profiles that had 10 or more endosomes in their soma and the contiguous proximal dendrites were considered to have internalized NK1rs. NK1r-positive neurons in both sides of the dorsal horn were counted. The person counting the neurons was blinded to the experimental treatments. Mean counts from three to five sections per segment of the lumbar spinal cord were used as representative counts for a given animal. Three to five animals per drug treatment group were used for statistical analysis ($n = 3$–5).

Fos immunoreactivity was quantified by counting Fos-positive cells in lamina I of the lumbar L3–4 segments of the spinal cord ipsilateral and contralateral to the formalin-treated paw. The mean counts from five sections were used as representative counts for a given animal. Four animals per drug treatment group were used for the statistical analysis ($n = 4$).

Confocal microscopy and image processing

Confocal images of representative NK1r cells were acquired by a Leica TCS SP2 confocal system equipped with AOBS with a ×63 objective (1.4 numerical aperture) and an argon 488 nm laser line with a pinhole of 1 airy unit. Images were acquired at a digital size of 1024 × 1024 pixels. Five to ten adjacent optical sections (~0.5-μm separation) along the z-axis were projected together for demonstration. Images were processed with Adobe Photoshop software (version 8.0) by using the 'curves' option to adjust image contrast.

Drugs, antibodies and materials

Dexmedetomidine (Precedex, Hospira Inc., Lake Forest, IL, USA) was purchased from the pharmacy of the University of California, San Diego Medical Center. Dexmedetomidine in powder form was kindly provided by Dr Donna L Hammond from the University of Iowa. ST-91 (N-(2,6-diethylphenyl)-4,5-dihydro-1H-imidazol-2-amine hydrochloride) was provided by Boehringer Ingelheim (Ridgefield, CT, USA), Formalin solution was purchased from Sigma Chemicals (St Louise, MO, USA). BRL44408 (2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole maleate) and ARC239 (2-[2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2 H,4 H)-isoquinolindione dihydrochloride) were purchased from Tocris Biosciences (Ellisville, MO, USA). Dexmedetomidine, ST-91 and BRL44408 were dissolved in saline, whereas ARC239 was dissolved in 10% 2-hydroxypropyl-β-cyclodextrin. All drugs were prepared on the day of testing. The rabbit anti-NK1r polyclonal antibody was purchased from the Advanced Targeting Systems (San Diego, CA, USA) and the rabbit anti-Fos polyclonal antibody was purchased from Calbiochem-EMD Biosciences (La Jolla, CA, USA). Secondary Alexa 488-conjugated antibody was purchased from Invitrogen (Eugene, OR, USA). ProLong mounting medium was obtained from Invitrogen, ABC kit from Vector Laboratories (Burlingame, CA, USA) and Permount mounting medium was from Fisher Scientific (Pittsburgh, PA, USA).

Nomenclature for drugs and receptors conform with the guide to receptors and channels of the *British Journal of Pharmacology* (Alexander *et al*., 2008).

Statistical analysis

Changes in formalin-induced paw-flinching behaviour were analysed using separate one-way ANOVA for phases 1 and 2. Upon detection of a significant ANOVA, Tukey's *post hoc* tests were performed for pair-wise comparisons of drug-treated

groups with their relative control within phase 1 or 2. The analyses for NK1r internalization data consisted of one-way or two-way ANOVAs. To detect the differences in the presence of a significant one-way ANOVA, Tukey's *post hoc* analysis was conducted. In the presence of a significant two-way ANOVA, Bonferroni *post hoc* tests were applied. The analysis of the Fos data consisted of a paired *t*-test and a one-way ANOVA. Tukey's *post hoc* test was used for pair-wise comparisons. In all analysis, probability to detect the differences was set at the 5% level ($P<0.05$).

Results

Effects of dexmedetomidine and ST-91 on formalin-induced paw-flinching behaviour

The effects of dexmedetomidine and ST-91 on formalin-induced paw-flinching behaviour are shown in Figures 1a and b, respectively. Dexmedetomidine dose-dependently reduced the formalin-induced paw flinches in phase 1: $F(5,21)=6.42$, $P<0.001$; with doses of 1 and 2 μg producing a significant reduction in flinching. In phase 2, dexmedetomidine reduced the formalin flinches at doses of 0.3, 1 and 2 μg: $F(5,21)=8.05$, $P<0.001$. In this experiment, the 2 μg dose of dexmedetomidine produced mild sedation, but the 1 μg dose of dexmedetomidine did not appear to have sedative effects; similarly, i.t. dexmedetomidine (1 μg) was not found to alter the rotarod performance in rats (Idänpään-Heikkilä *et al*., 1994). Accordingly, the 1 μg dose of dexmedetomidine was typically employed in subsequent experiments, unless otherwise noted. ST-91 dose-dependently reduced the formalin-induced paw flinches in phase 1: $F(5,31)=5.74$, $P<0.001$, with 10 and 30 μg doses producing a significant reduction in flinching. In phase 2, ST-91 at doses of 3, 10 and 30 μg reduced the formalin flinching: $F(5,31)=11.36$, $P<0.001$, with no effect upon arousal noted at the highest dose.

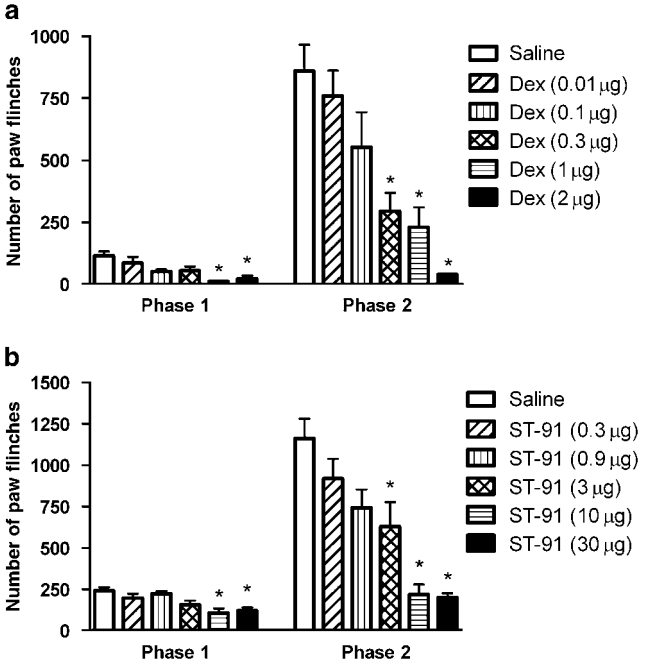

Figure 1 Analgesic effects of dexmedetomidine and ST-91 on formalin-induced paw-flinching behaviour. An injection of formalin (5%) into the hindpaw of rats produced a biphasic (phases 1 and 2) paw-flinching behaviour. (**a**) Intrathecal dexmedetomidine and (**b**) i.t. ST-91 dose-dependently reduced the formalin-induced paw flinches. Data are presented as mean number of paw flinches with vertical bars showing s.e.mean. *Represents a significant difference between saline-treated and drug-treated animal within phase 1 or 2, $P<0.001$. In panel a, $n=4$–8 rats per group; in panel b, $n=5$–9 rats per group.

α_2-Adrenoceptor antagonists on dexmedetomidine and ST-91 analgesia

The effects of the α_{2A}-adrenoceptor antagonist, BRL44408 on dexmedetomidine and ST-91 analgesia are presented in Figures 2a and b. In phase 1, dexmedetomidine reduced the formalin-flinching behaviour: $F(4,22)=13.14$, $P<0.001$, Whereas the i.t. pretreatment with BRL44408 (10 and 100 μg) blocked the dexmedetomidine-induced reduction in formalin flinching. Equally, in phase 2, dexmedetomidine reduced the formalin-flinching behaviour; whereas pretreatment with BRL44408 (10 and 100 μg) blocked the dexmedetomidine-induced reduction in formalin-flinching behaviour: $F(4,22)=17.72$, $P<0.001$. ST-91 did not significantly alter phase 1 formalin-flinching behaviour and BRL44408 pretreatment had no effect on ST-91-treated rats during this phase (Figure 2b). In phase 2, however, ST-91 reduced the formalin-flinching behaviour: $F(4,20)=13.40$, $P<0.001$. Intrathecal pretreatment with BRL44408 (10 and 100 μg) blocked the ST-91-induced reduction of formalin-flinching behaviour.

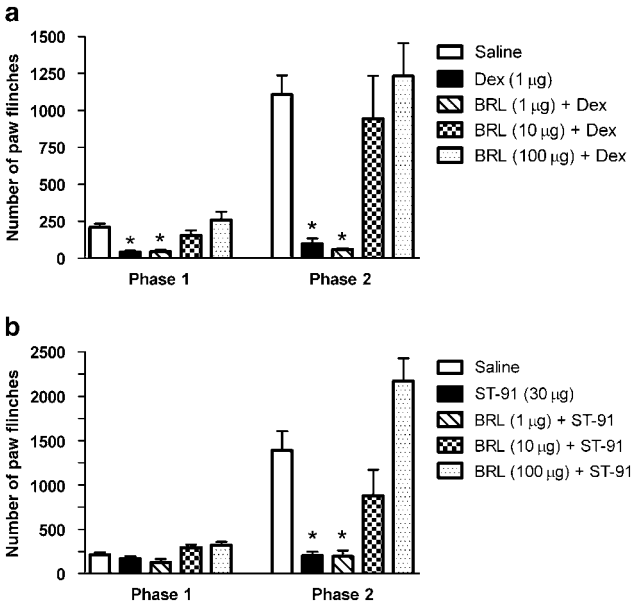

Figure 2 Effects of the α_{2A}-adrenoceptor antagonist BRL44408 on dexmedetomidine and ST-91-induced analgesia in the formalin model. Pretreatment of i.t. BRL44408 blocked the analgesic effects of i.t. (**a**) dexmedetomidine and (**b**) ST-91 in a dose-dependent manner. Data are presented as mean number of paw flinches with vertical bars showing s.e.mean. *Represents a significant difference between saline-treated and drug-treated animal within phase 1 or 2, $P<0.001$, $n=3$–6 rats per group.

The effects of the $\alpha_{2B/C}$-adrenoceptor antagonist ARC239 on dexmedetomidine and ST-91 analgesia is shown in Figures 3a and b. Dexmedetomidine reduced the formalin-flinching behaviour in phase 1: $F(3,12)=6.14$, $P<0.01$, and pretreatment with ARC239 had no effect on the dexmedetomidine-induced reduction of formalin-flinching behaviour. In phase 2, dexmedetomidine reduced the formalin-flinching behaviour: $F(3,12)=8.82$, $P<0.01$. Again, ARC239 pretreatment did not block the dexmedetomidine-induced analgesia. ST-91 reduced the formalin-flinching behaviour in phase 1: $F(3,14)=11.96$, $P<0.001$, but pretreatment with ARC239 did not alter the ST-91-induced reduction in formalin-flinching behaviour (Figure 3b). In phase 2, ST-91 reduced the formalin-flinching behaviour: $F(3,14)=7.13$, $P<0.01$. This reduction was not blocked by pretreatment with ARC239.

Dexmedetomidine and ST-91 on formalin-induced NK_1 receptor internalization

The effects of i.t. dexmedetomidine (1 μg), ST-91 (30 μg) and morphine (20 μg) on formalin-induced NK1r internalization are shown in Figures 4 and 5. Unilateral intraplantar injection of formalin (5%) produced significant ipsilateral NK1r internalization at all levels of the lumbar spinal cord: $F(2,24)=14.12$, $P<0.001$ (Figures 4a and 5a). NK1r internalization in the spinal cord was not observed on the side contralateral to the formalin-injected paw (Figures 4a and 5d). Intrathecal dexmedetomidine or ST-91 did not reduce the NK1r internalization in any of the spinal segments measured (Figures 4b and 5b). Intrathecal morphine, serving as a positive control, did significantly attenuate the formalin-induced NK1r internalization in spinal segments L3–4 and L5–6: $F(6,36)=2.52$, $P<0.05$ (Figures 4b and 5c).

To determine whether dexmedetomidine at doses other than 1 μg is able to reduce the formalin-induced NK1r internalization, varying doses of dexmedetomidine (0.3–10 μg) were administered i.t. before an injection of formalin (Figure 4c). Once again, none of the dexmedetomidine doses were able to diminish the formalin-induced NK1r internalization at any level of the lumbar spinal cord: $F(8,26)=1.87$, $P>0.05$, in spite of the 10 μg dose producing evident sedation in rats.

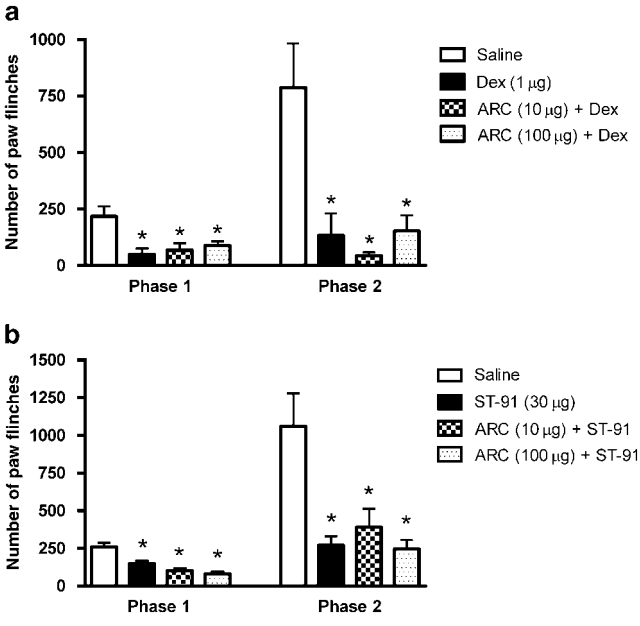

Figure 3 Effects of the $\alpha_{2B/C}$-adrenoceptor antagonist ARC239 on dexmedetomidine and ST-91-induced analgesia in the formalin model. Pretreatment of i.t. ARC239 did not reduce the analgesic effects of i.t. (**a**) dexmedetomidine and (**b**) ST-91. Data are presented as mean number of paw flinches with vertical bars showing s.e.mean. *Represent a significant difference between saline-treated and drug-treated animal within phase 1 or 2, $P<0.01$. In panel a, $n=4$ rats per group; in panel b, $n=3$–6 rats per group.

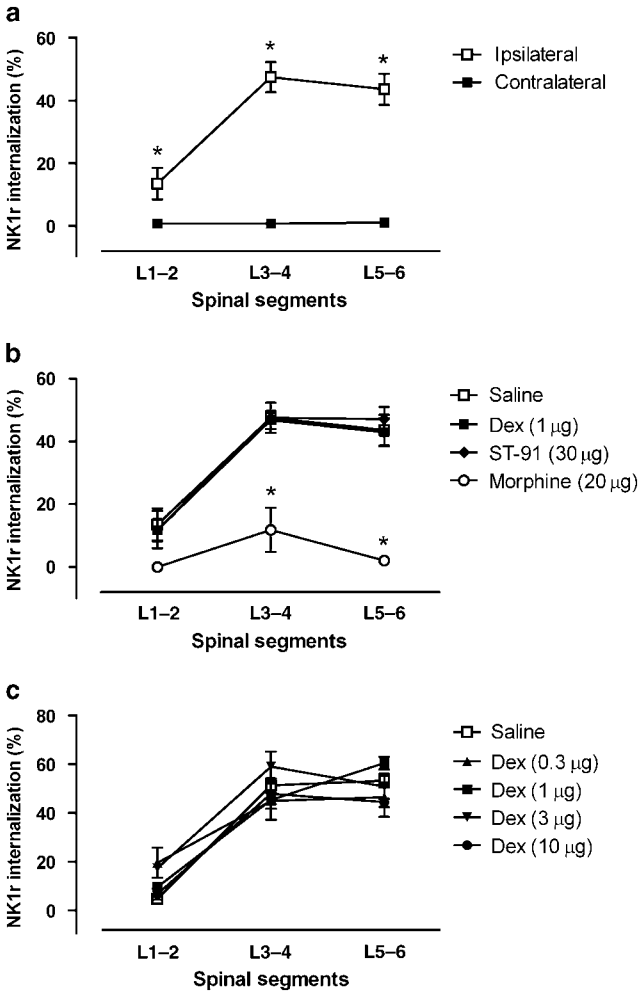

Figure 4 α_2-Adrenoceptor agonists on formalin-induced NK_1 receptor (NK1r) internalization. (**a**) Unilateral injection of formalin (5%) into the hindpaw produced a robust ipsilateral NK1r internalization at all levels of the lumbar lamina I compared with the contralateral side. (**b**) Intrathecal dexmedetomidine (1 μg) or ST-91 (30 μg) did not reduce the formalin-evoked NK1r internalization. Intrathecal morphine significantly reduced the NK1r internalization in spinal segments L3–4 and L5–6 compared with saline. (**c**) Intrathecal dexmedetomidine (0.3–10 μg) failed to reduce the formalin-evoked NK1r internalization. Data are presented as mean percentage of NK1r internalization with vertical bars showing s.e.mean. *Represent a significant difference between ipsilateral and contralateral sides of the spinal cord or in NK1r internalization between saline-treated and drug-treated animal, $P<0.05$. In panel a, $n=5$ per group; in panel b, $n=3$–5 per group; in panel c, $n=3$ per group.

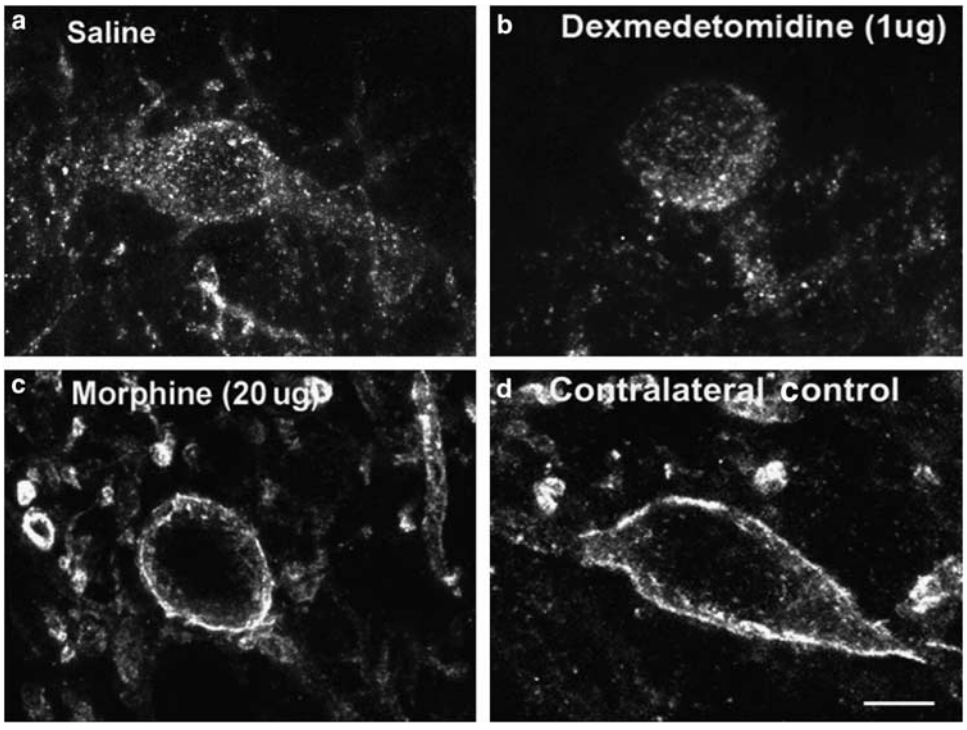

Figure 5 Representative confocal images of NK_1 receptor (NK1r) internalization induced by a unilateral injection of formalin (5%) into the hindpaw. Images in (**a**–**c**) are from the ipsilateral spinal cord. (**a**) An image of formalin-evoked NK1r internalization from a rat administered i.t. saline. Notice the lack of a homogeneous cell membrane and the presence of NK_1-containing endosomes internalizing into the cytoplasm. (**b**) Intrathecal dexmedetomidine (1 μg) did not reduce the formalin-evoked NK1r internalization. (**c**) Intrathecal morphine (20 μg) blocked the formalin-evoked NK1r internalization. Notice the presence of a homogeneous cell membrane and the lack of NK_1-containing endosomes internalizing into the cytoplasm. (**d**) Sample image of a cell from the contralateral superficial dorsal horn of a saline-treated rat demonstrating the lack of NK1r internalization. Confocal images were taken using a $\times 63$ objective with 5–10 optical sections along the z-axis separated by approximately 0.5 μm. Scale bar is 10 μm.

Dexmedetomidine and ST-91 on paw compression-induced NK_1 receptor internalization

To confirm the lack of effects of i.t. dexmedetomidine and ST-91 on noxious stimuli-induced NK1r internalization, we examined the effects of dexmedetomidine and ST-91 on NK1r internalization evoked by hindpaw compression (Figure 6), a model of robust spinal NK1r internalization in response to stimulation (Kondo *et al*., 2005). Thus, unilateral hindpaw compression produced significant ipsilateral NK1r internalization at L3–4 and L5–6 segments of the spinal cord compared with the contralateral side: $F(2,12) = 42.55$, $P < 0.001$ (Figure 6a). Intrathecal dexmedetomidine (1 and 10 μg) or ST91 (30 μg) had no effect on the paw compression-induced NK1r internalization (Figure 6b). However, i.t. morphine (20 μg), once again, did reduce the paw compression-induced NK1r internalization: $F(4,40) = 5.82$, $P < 0.001$.

Dexmedetomidine and α_2-adrenoceptor antagonists on formalin-induced Fos expression

This experiment also tested the ability of i.t. delivered α_2-adrenoceptor antagonists and agonist to reduce formalin-induced Fos expression in the dorsal horn. Accordingly, i.t. BRL44408 (100 μg), ARC239 (100 μg) or saline were administered 10 min before the administration of dexmedetomidine (1 μg), morphine (20 μg) or saline. Ten minutes after agonist administration, a unilateral intraplantar injection of formalin (5%) was given. This injection of formalin produced a significant enhancement of Fos expression on the ipsilateral, but not contralateral L3–4 segment of the dorsal horn, $t(3) = 8.18$, $P < 0.01$ (Figures 7 and 8). Intrathecal administration of either dexmedetomidine or morphine reduced the formalin-evoked Fos expression on the ipsilateral side: $F(4,21) = 7.01$; $P < 0.01$. BRL44408 blocked the ability of dexmedetomidine to reduce formalin-evoked Fos expression, whereas ARC239 did not alter the effects of dexmedetomidine.

Discussion

The aim of this study was to elucidate the spinal α_2-adrenoceptor subtype responsible for the analgesic properties of dexmedetomidine and ST-91 in the formalin test. In addition, the effects of dexmedetomidine and ST-91 on formalin-induced NK1r internalization and Fos expression were assessed to further demonstrate their mechanism of action in the spinal cord.

Spinal α_{2A}-adrenoceptors and nociception

Intrathecal dexmedetomidine and ST-91 produced a dose-dependent reduction in the formalin-flinching behaviour in both phases 1 and 2, indicating the potent analgesic actions

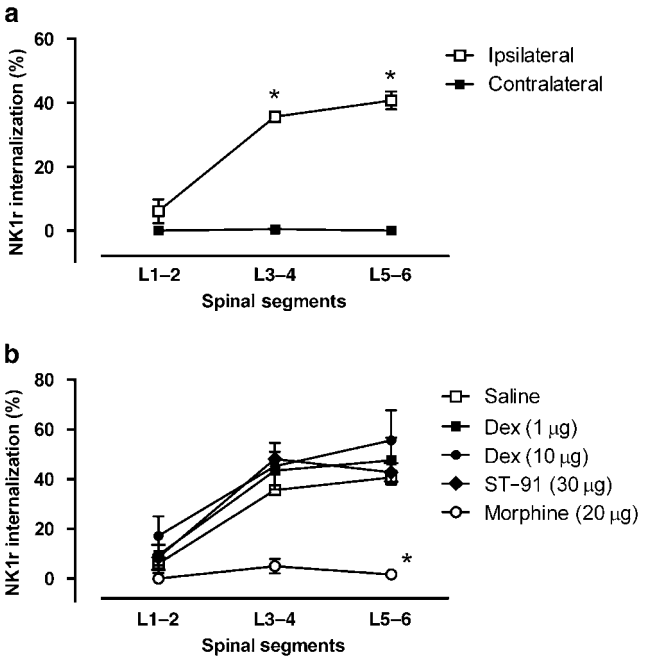

Figure 6 α_2-Adrenoceptor agonists on hindpaw compression-induced NK_1 receptor (NK1r) internalization. (**a**) Unilateral paw compression of the hindpaw produced significant ipsilateral NK1r internalization at L3–4 and L5–6 spinal segments. (**b**) Intrathecal dexmedetomidine (1 or 10 μg) or ST-91 (30 μg) did not reduce the paw compression-induced NK1r internalization, whereas i.t. morphine markedly reduced it. Data are presented as mean percentage of NK1r internalization with vertical bars showing s.e.mean. In panel a, asterisk represents a significant difference in NK1r internalization between ipsilateral and contralateral sides of the spinal cord, $n=3$. In panel b asterisk represents the difference in NK1r internalization between morphine-treated and saline-treated rats, $n=3$.

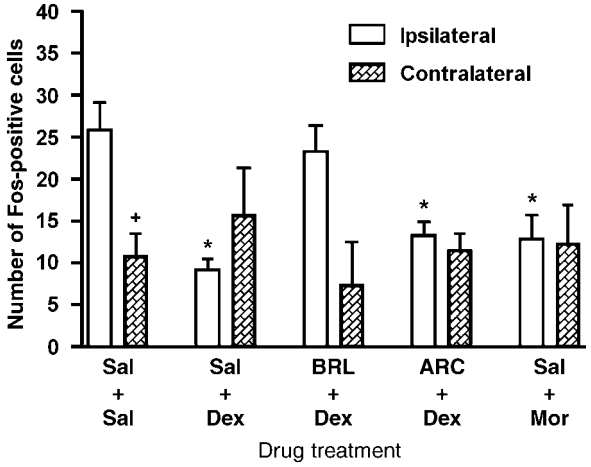

Figure 7 Effects of dexmedetomidine and morphine on formalin-induced Fos expression. Unilateral injection of formalin (5%) into the hindpaw enhanced the expression of Fos on the ipsilateral side compared with the contralateral side of i.t. saline-treated rats. Intrathecal dexmedetomidine (1 μg) and morphine (20 μg) reduced the formalin-evoked Fos expression on the ipsilateral side compared with the saline control. Intrathecal pretreatment of BRL44408 (100 μg) significantly blocked the ability of dexmedetomidine to decrease the formalin-induced Fos expression. However, i.t. pretreatment of ARC239 (100 μg) did not alter the effects of dexmedetomidine. Data are presented as mean number of Fos-positive cells with vertical bars showing s.e.mean. $^{+}$Represents a difference in formalin-induced Fos expression between ipsilateral and contralateral to the injected paw, $P<0.01$. *Represents a difference in formalin-induced Fos expression on the ipsilateral side between saline control and i.t. dexmedetomidine, dexmedetomidine with ARC239 and morphine-treated rats, $P<0.01$, $n=4$ per group.

of spinal α_2-adrenoceptor agonists. To determine the subtype of α_2-adrenoceptor responsible for dexmedetomidine- and ST-91-induced analgesia, rats were pretreated with the α_{2A}-adrenoceptor antagonist, BRL44408 or the $\alpha_{2B/C}$-adrenoceptor antagonist, ARC239. The analgesic effects of dexmedetomidine and ST-91 were dose-dependently attenuated by BRL44408, whereas ARC239 was unable to alter the analgesia produced by the two agonists. These findings support previous findings showing that α_{2A}-adrenoceptors mediate the analgesic effects of typical α_2-adrenoceptor agonists (i.e., dexmedetomidine) in acute pain (Lakhlani *et al*., 1997; Stone *et al*., 1997). Although the present findings with the α_2-adrenoceptor selective antagonists suggest a comparable pharmacology between dexmedetomidine and ST-91, previous studies have suggested that ST-91-induced analgesia is mediated by receptors other than α_{2A}. For instance, work from our group has indicated that i.t. prazosin ($\alpha_{2B/C}$ and α_1 antagonist) reduced the analgesic effect of ST-91 in an acute thermal escape model (Takano and Yaksh, 1992). Likewise, i.t. prazosin reduced ST-91 analgesia in the SP behavioural assay (Stone *et al*., 2007). Finally, i.t. ARC239 attenuated ST-91 analgesia in a postincisional pain model (Duflo *et al*., 2003). Therefore, the disparities between these findings and those of the past may be because of the use of formalin as the noxious stimulus. It is noteworthy to consider that the pharmacology of analgesia achieved in the formalin test is different from that induced in acute nociceptive and postincisional models. Preliminary data from our laboratory, as well as findings by Kanui *et al*. (1993) indicate that i.t. prazosin, alone, is analgesic in the formalin model. As differences in α_2-adrenoceptor pharmacology exist between nociceptive models, care should be taken when interpreting the mechanism of action of ST-91. According to our present behavioural data, ST-91 seems to produce its analgesic effect through α_{2A}-adrenoceptors in the formalin model.

Spinal α_2-adrenoceptors and SP release

It has been shown that dorsal horn α_{2A}-adrenoceptors are located presynaptically on primary afferent fibres and our behavioural data suggest that spinal dexmedetomidine- and ST-91-induced analgesia are mediated through α_{2A}-adrenoceptors; therefore, it was hypothesized that dexmedetomidine and ST-91 would reduce the formalin-induced NK1r internalization. However, we found that i.t. administration of equianalgesic doses of dexmedetomidine (1 μg) or ST-91 (30 μg) did not reduce the formalin-evoked NK1r internalization. In contrast, morphine, our positive control, effectively reduced the formalin-evoked NK1r internalization. To assess the possibility of non-specific or insufficient effects of the dose of dexmedetomidine used, a wide range of dexmedetomidine doses (0.3–10 μg) were tested to determine whether other doses of the drug could reduce NK1r internalization. None of the doses tested was able to decrease the formalin-

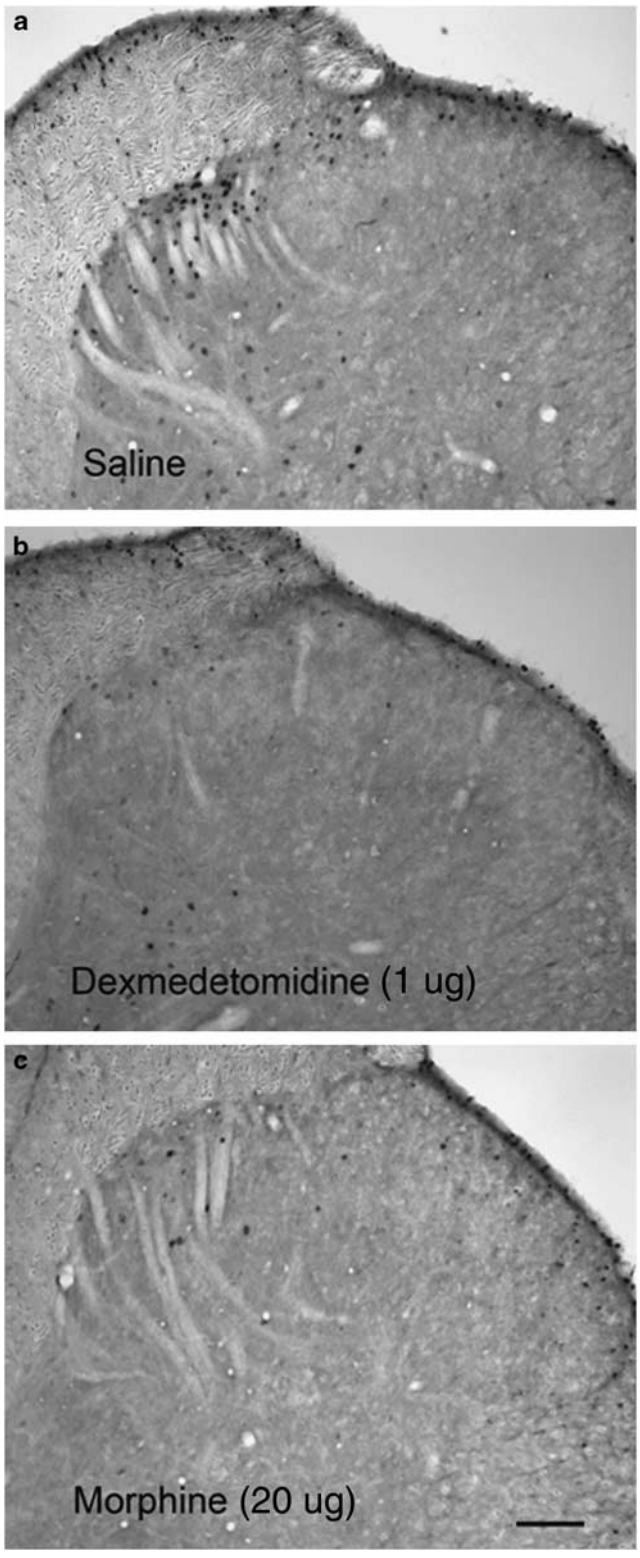

Figure 8 Representative light microscopy image of Fos expression induced by a unilateral injection of formalin (5%) into the hindpaw. (**a**) An image of formalin-evoked Fos expression from a rat administered i.t. saline. (**b** and **c**) Intrathecal dexmedetomidine (1 μg) or morphine (20 μg) significantly reduced the formalin-evoked Fos expression. Images were captured using a ×10 objective. Scale bar is 60 μm.

induced NK1r internalization. To determine whether the inability of dexmedetomidine and ST-91 to reduce NK1r internalization was unique to the use of formalin as a noxious stimulus, the effects of dexmedetomidine and ST-91 on paw compression-induced NK1r internalization were measured. Similar to the formalin data, neither dexmedetomidine nor ST-91 reduced the paw compression-induced NK1r internalization, whereas morphine, again, significantly reduced it.

Since we found that dexmedetomidine did not attenuate peripheral noxious stimulation-induced NK1r internalization, the effect of this drug on postsynaptic dorsal horn neuronal activation produced by an intraplantar injection of formalin was studied. To that end, the Fos protein was measured as a marker of postsynaptic neuronal activation. Intraplantar formalin notably enhanced Fos expression in the ipsilateral superficial dorsal horn and i.t. application of both dexmedetomidine and morphine clearly attenuated this formalin-induced Fos expression. In agreement with our behavioural data, i.t. BRL44408 blocked the ability of dexmedetomidine to reduce the formalin-induced Fos expression, whereas ARC239 was devoid of this effect. These findings are in line with previous results indicating that noxious stimulation-induced Fos expression was reduced by systemic administration of α_2-adrenoceptor agonists (Pertovaara *et al*., 1993; Sawamura *et al*., 1999; Sanders *et al*., 2005), as well as systemic and spinal administration of morphine (Presley *et al*., 1990; Labuz *et al*., 2003). In addition, blockade of the effects of dexmedetomidine on formalin-induced Fos expression by BRL44408 further implicates the role of postsynaptic α_{2A}-adrenoceptors in the analgesic properties of dexmedetomidine in the formalin model.

The effects of α_2-adrenoceptor agonists on SP release are controversial. Whereas studies measuring SP release using *in vitro* spinal cord preparations often show an attenuation of evoked SP release by the activation of spinal α_2-adrenoceptors (Pang and Vasko, 1986; Ono *et al*., 1991; Takano *et al*., 1993), *in vivo* studies have demonstrated either a reduction in peripheral noxious stimulation-induced SP release (Kuraishi *et al*., 1985) or no effect of α_2-adrenoceptor agonists on SP release induced by electrical stimulation of the tibial nerve (Lang *et al*., 1994; Zhao *et al*., 2004). Regardless of the *in vitro* or *in vivo* experimental approach, it is interesting that in studies where SP was measured from cerebrospinal fluid sample collections, a reduction of SP release by α_2-adrenoceptor agonists was obtained (Kuraishi *et al*., 1985; Pang and Vasko, 1986; Ono *et al*., 1991; Takano *et al*., 1993), whereas, similar to the present findings, in studies where SP release was measured in the superficial dorsal horn, little or no effect of α_2-adrenoceptor agonists on evoked SP release was found (Lang *et al*., 1994; Zhao *et al*., 2004). The reason for the differences in these findings is unclear. On the basis of our present data, we are inclined to consider spinal α_2-adrenoceptors as having little effect on primary afferent SP release.

Pre- vs postsynaptic α_2-adrenoceptor sites of spinal action

The potent effects of i.t. α_2-adrenoceptor agonists acting through α_{2A}-adrenoceptors on pain behaviour occurred at

doses, which had no effect upon SP release, as measured by NK1r internalization. These results are particularly unexpected, given the association of this receptor protein with peptidergic afferents (Stone *et al*., 1998; Chen *et al*., 2007). On the other hand, at the doses tested, dexmedetomidine produced a potent effect on spinal transmission, as evidenced by the effects on formalin-induced Fos expression. At least two possibilities are proposed to explain these findings. First, it is possible that the analgesic effects of dexmedetomidine and ST-91 work through presynaptic α_{2A}-adrenoceptors by reducing the release of neurotransmitters other than SP, such as glutamate. For instance, previous work has demonstrated inhibitory effects of α_2-adrenoceptor activation on capsaicin-evoked glutamate release from spinal cord synaptosomes and dorsal horn slices (Ueda *et al*., 1995; Li and Eisenach, 2001). Moreover, *in vivo* and *in vitro* electrophysiological recordings show that α_2-adrenoceptor activation decreased excitatory postsynaptic currents evoked by dorsal root stimulation, indicating presynaptic inhibition (Pan *et al*., 2002; Kawasaki *et al*., 2003; Sonohata *et al*., 2004). Although a presynaptic mechanism of α_2-adrenoceptors cannot be ruled out, it has been demonstrated that glutamate and SP are colocalized in the same afferent terminals (De Biasi and Rustioni, 1988) and are thought to be coreleased upon noxious stimulation. We are not aware of any data indicating that α_2-adrenoceptor activation leads to a selective inhibition of glutamate, but not SP release. Alternatively, it is possible that dexmedetomidine and ST-91 act through postsynaptic α_{2A}-adrenoceptors. A small population of α_{2A}-adrenoceptors in the superficial dorsal horn is present after the sensory fibres containing the transient receptor potential vanilloid 1 receptors have been abolished or after dorsal rhizotomy (Stone *et al*., 1998; Chen *et al*., 2007), suggesting that these receptors are indeed located postsynaptically on dorsal horn neurons. Activation of these receptors could lead to hyperpolarization of postsynaptic neurons and a reduction in formalin-induced Fos expression, while conserving formalin-induced NK1r internalization.

The possibility of a postsynaptic mechanism for the analgesic effects of α_2-adrenoceptor agonists is further supported by the findings indicating that the analgesic effects of clonidine were significantly reduced in mice lacking the G-protein-coupled inwardly rectifying potassium channel subunit 11 (Mitrovic *et al*., 2003), an effector channel likely to be selectively involved in postsynaptic inhibition. Similarly, using *in vivo* spinal cord patch clamp recordings, Sonohata *et al*. (2004) indicated that noradrenaline and clonidine produced postsynaptic inhibition by inducing an outward potassium current by α_2-adrenoceptor activation. Also, the destruction of transient receptor potential vanilloid 1-containing afferents by resiniferatoxin was found not to attenuate the analgesic effects of clonidine on noxious mechanical stimuli (Chen *et al*., 2007). Finally, non-peptidergic primary afferents do not appear to have a function in presynaptic inhibition by α_2-adrenoceptor agonists, as the destruction of isolectin B4-positive afferents by intrasciatic nerve injections of saporin-conjugated isolectin B4 did not reduce the analgesic effects of clonidine on thermal and mechanical stimuli (Eisenach JC, personal communication).

The current consensus on the mechanism of action of spinally administered α_2-adrenoceptor agonists and morphine has been the presynaptic inhibition of transmitter release from the primary afferent terminals. However, the present findings suggest a different mechanism of action for spinal α_2-adrenoceptors and morphine. Whereas morphine works presynaptically by reducing noxious stimulation-induced primary afferent release of SP, dexmedetomidine and ST-91 appear to have a minor presynaptic effect. Though we cannot conclusively exclude the possibility of an inhibitory action of α_{2A}-adrenoceptors on the release of excitatory neurotransmitters other than SP, it is likely that postsynaptic inhibition is the primary mechanism for α_{2A}-adrenoceptor-mediated antinociception. Different sites of spinal action for morphine and α_2-adrenoceptor agonists may provide a mechanism for the significant potentiating effects of α_2-adrenoceptor agonists on morphine analgesia (Monasky *et al*., 1990; Ossipov *et al*., 1990; Sullivan *et al*., 1992).

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Conflict of interest

The authors state no conflict of interest.

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