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Direct excitation of spinal GABAergic interneurons by noradrenaline

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ABSTRACT

Endogenous pain control is, in part, mediated by descending inhibition of spinal nociception via spinal release of noradrenaline. Antinociception by activation of descending noradrenergic fibres has partially been attributed to the direct inhibition of nociceptive spinal neurons. Here, we tested the alternative hypothesis: the direct excitation of inhibitory spinal interneurons by noradrenaline. Transverse lumbar spinal cord slices were obtained from adult mice expressing enhanced green fluorescent protein (EGFP) in GABAergic neurons under control of the GAD67 promoter. Recordings were made from a total of 113 EGFP-expressing neurons and non-EGFP-expressing neurons in spinal laminae II and III with the perforated patch–clamp technique. In lamina II, where mainly nociceptive afferents terminate, noradrenaline (20 μM) depolarised significantly more EGFP-labelled (41%) than non-EGFP-labelled GABAergic neurons (4%). In contrast, noradrenaline hyperpolarised significantly more non-EGFP-labelled (46%) than EGFP-labelled GABAergic neurons (20%). In lamina III, where low threshold afferents terminate, EGFP-labelled neurons were never depolarised but either hyperpolarised (25%) or not affected (75%) by noradrenaline. Depolarisations of EGFP-labelled lamina II neurons were mimicked by the α_1-adrenoceptor agonist phenylephrine (10–20 μM) and abolished by the α_1-adrenoceptor antagonist prazosin (2 μM). Hyperpolarisations of EGFP- and non-EGFP-labelled neurons were abolished by the α_2-adrenoceptor antagonist yohimbine (2 μM). These results show that noradrenaline directly excites inhibitory (GABAergic) lamina II interneurons in addition to its inhibitory effect on (putatively excitatory) interneurons in superficial spinal dorsal horn. Both effects of noradrenaline constitute a synergism in descending inhibition of nociceptive information in the spinal dorsal horn.

1. Introduction

The dorsal horn of the spinal cord is a major site for the modulation of nociceptive information. Supraspinal neurons with long descending axons may inhibit or facilitate spinal nociception either directly or indirectly via interneurons [4,31,34,44]. Descending noradrenergic neurons seem to have mainly antinociceptive effects as electrical stimulation within the noradrenergic brainstem areas A5 and A6 (locus ceruleus) or A7 (subceruleus) [38], or spinal application of noradrenaline reduce C-fibre evoked activity in dorsal horn neurons [40]. Behavioural studies have shown that the activation of spinal adrenoceptors leads to antinociception [1,16,48]. Supraspinal descending pathways are the only source of noradrenaline in the spinal dorsal horn [22]. The highest density of noradrenergic terminals is found in spinal dorsal horn laminae I and II [11,46]. Part of the antinociceptive effect of noradrenaline in the dorsal horn seems to be mediated by the activation of the inhibitory α_2-adrenoceptor on lamina II interneurons [10,28,37]. However, the α_1-adrenoceptor is also present on some spinal dorsal horn neurons [27] and an excitatory effect of noradrenaline on a small but unidentified subgroup of dorsal horn neurons has been described [19,24,28]. Noradrenaline enhances GABAergic synaptic transmission in the superficial laminae of the dorsal horn [10] through the activation of the α_1-adrenoceptor [2,3]. Thus, an additional intriguing but so far unproven mechanism for an antinociceptive action of noradrenaline would be an α_1-adrenoceptor-mediated excitation of inhibitory GABAergic interneurons in the spinal dorsal horn [22,49].

Up to now, the effect of noradrenaline on dorsal horn GABAergic neurons has not been assessed directly, as it is technically quite demanding to record from identified spinal inhibitory neurons using purely electrophysiological methods [18]. Here, we took advantage of transgenic GIN (GFP-expressing inhibitory neurons) mice [29], in which about one-third of superficial spinal dorsal horn GABAergic neurons are labelled by EGFP [14]. Adrenoceptors are metabotropic, thus likely acting through signal transduction pathways which involve diffusible second messengers. To prevent dialysis of these messengers from the postsynaptic neuron, we recorded dorsal horn neurons using the perforated patch–clamp

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technique. We recorded from EGFP-labelled and non-EGFP-labelled neurons in laminae II and III of GIN mouse spinal cord slices to test the hypothesis that noradrenaline directly depolarises spinal GABAergic interneurons via α_1-adrenoceptors.

2. Methods

2.1. Animals

All procedures were in accordance with European Communities Council directives (86/609/EEC) and were approved by the Austrian Federal Ministry for Education, Science and Culture. Homozygotic transgenic mice that express EGFP under the control of the promoter for GAD67 [29] were obtained from The Jackson Laboratory [Bar Harbour, ME, USA; strain name: FVB-TgN(GadGFP)45704Swn] and interbred at a local facility (Fig. 1A). Although FVB mice show early retinal degeneration and various behavioural defects [25,41], they do not show abnormalities in pain behaviour and development of hyperalgesia, allodynia and chronic pain [35].

2.2. Preparation of spinal cord slices

Adult (>12-weeks-old) GIN mice were deeply anaesthetized with ether and killed by decapitation. A laminectomy was performed and the lumbar spinal cord was removed into ice cold incubation solution consisting of (in mM): NaCl, 95; KCl, 1.8; KH_2PO_4, 1.2; $CaCl_2$, 0.5; $MgSO_4$, 7; $NaHCO_3$, 26; glucose, 15; sucrose, 50; oxygenated with 95% O_2, 5% CO_2; pH 7.4, measured osmolarity 310–320 mosmol l^{-1}. The dura mater and the ventral and dorsal roots were removed. Transverse slices were cut at 400–600 μm thickness using a vibrating microslicer (DTK-1000, Dosaka EM, Kyoto, Japan) and kept in incubation solution at 33 °C for at least 30 min. After this, the slices were stored in the same solution at room temperature (20–24 °C).

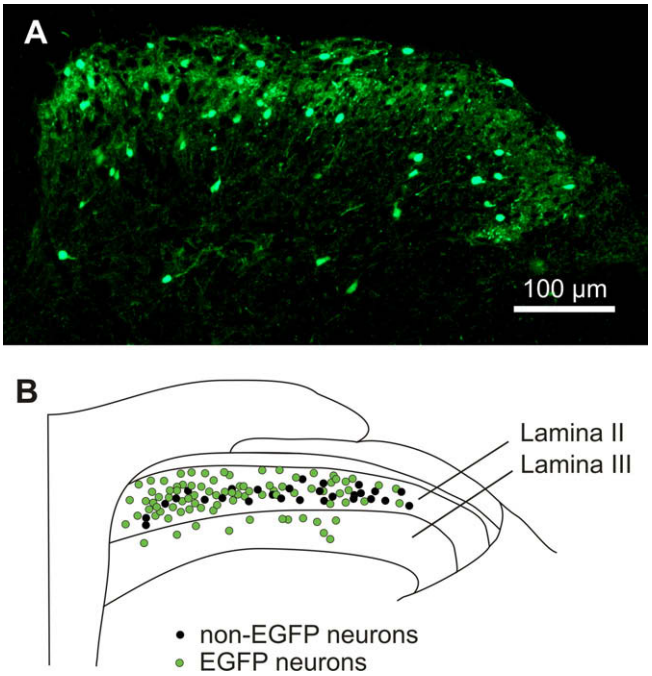

Fig. 1. EGFP expression pattern and recording sites in a transverse section of an adult mouse spinal cord. (A) Photomicrograph of the L4 segment (region of the right dorsal horn) showing the laminar distribution of EGFP-expressing neurons. (B) Recording sites of EGFP- and non-EGFP-labelled neurons in spinal cord slices. Recording sites were located through a microscope at 100× magnification and documented on a standard histological section through the L4 segment. Borders of laminae II and III were as described in methods. ●, EGFP-labelled neurons; ●, non-EGFP-labelled neurons.

2.3. Recording technique

A single slice was then transferred to a recording chamber (volume 3 ml), where it was continuously superfused at a rate of 3–4 ml/min with recording solution, which was identical to the incubation solution except for (in mM): NaCl, 127; $CaCl_2$, 2.4; $MgSO_4$, 1.3; sucrose, 0. Bicuculline (10 μM), strychnine (1 μM), D-AP5 (50 μM) and CNQX (10 μM) were routinely included in the recording solution. In some experiments, TTX (1 μM) was additionally included in the recording solution. Recordings were made at room temperature.

Dorsal horn neurons were visualized with an Olympus BX 51WI (Olympus Optical, Tokyo, Japan) microscope equipped with Dodt-infrared optics [7]. Lamina II was identified as a translucent band across the dorsal horn. EGFP-expressing neurons and non-EGFP-expressing neurons in laminae II and III were detected by epifluorescence as described before [14].

The perforated patch–clamp technique [15] was used to record passive membrane properties and membrane potential from selected neurons. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfels, Germany) on a horizontal puller (P-97, Sutter Instruments, Novato, CA, USA) and had resistances of 3–6 MΩ, when filled with internal solution (in mM: potassium gluconate, 120; KCl, 20; $MgCl_2$, 2; Na_2ATP, 2; NaGTP, 0.5; Hepes, 20; EGTA, 0.5 and lucifer yellow 1, pH 7.28 adjusted with KOH, measured osmolarity 300–310 mosmol l^{-1}). A thin quartz capillary (npi, Tamm, Germany) filled with internal solution including the antibiotic nystatin (0.1 mM) and connected to a Hamilton syringe (25 μl, Hamilton Bonaduz, Bonaduz, Switzerland) was placed inside the patch pipette, with its opening near the pipette tip. After the formation of a gigaseal, 1–3 μl of the nystatin solution was injected into the pipette tip. Usually, series resistance started to decrease 10–25 min after the application of nystatin. After reaching a stable series resistance of 20–70 MΩ, the experiment was started. Epifluorescence was used to check for the presence of lucifer yellow in the pipette tip and/or the recorded neuron at regular intervals. Leakage of lucifer yellow to the neuron indicated rupture of the perforated patch and led to rejection of the recording.

2.4. Experimental protocol and data analysis

Voltage- and current–clamp recordings were made using the Axopatch 700B amplifier and the pCLAMP 9 acquisition software (Molecular Devices, Union City, CA, USA). Signals were low-pass filtered at 4–10 kHz, amplified fivefold, sampled at 5–100 kHz and analysed offline using pCLAMP 9. The resting membrane potential was measured after establishing a stable series resistance. Only neurons that had a resting membrane potential more negative than −50 mV were studied further.

The effects of noradrenaline (20 μM) and phenylephrine (10–20 μM) on the membrane potential were studied. Concentrations were chosen according to the literature [3,9,21,28]. Noradrenaline and phenylephrine were dissolved in recording solution supplemented with sodium metabisulfite (20 μM) to prevent oxidation [5]. A gravity-fed tube system operated by a manual switch that had its outlet directly onto the surface of the dorsal horn was used to apply drugs for 10–20 s during recording of the membrane potential. Baseline and standard deviation of the membrane potential were measured in the 50 s time segment before drug application. Strict criteria on baseline stability and response timing and morphology were used to assure that spontaneous fluctuations of the baseline could not be misinterpreted as responses to noradrenaline

or phenylephrine. Only traces with a pre-drug-application standard deviation (used as a measure of the membrane potential noise level) ⩽0.8 mV (0.55 ± 0.02 mV) and without spontaneous fluctuations with a time course of one to several seconds were included into analysis (see Fig. 2, for examples). Noradrenaline or phenylephrine was considered to have a modulatory action on the membrane potential if the effect exceeded ±3 mV, appeared within 4 s (for depolarisations) or within 8 s (for hyperpolarisations) of the drug application, and could be reproduced upon a second application. Small depolarisations (3–8 mV amplitude) and hyperpolarisations of all sizes were always completely reversible. Some of the larger depolarisations (8–58 mV amplitude) rendered the membrane potential instable with incomplete return to baseline. In some experiments, slices were preincubated with the α_1-adrenoceptor antagonist prazosin (2 μM) for ⩾10 min. In some other experiments, the α_2-adrenoceptor antagonist yohimbine (2–3 μM) was added to the recording solution after the second noradrenaline application and washed in ⩾10 min before a third noradrenaline application was performed to test if the effect of noradrenaline on the membrane potential was mediated by α_2-adrenoceptors.

At the end of the recording, the distance of the neuron to the white matter overlying the dorsal horn was measured. Neurons lying within 20 μm ⩽ x < 100 μm from the white matter border were classified as lamina II neurons [14] while those lying within 100 μm < y ⩽ 250 μm were assigned to lamina III [17]. Neurons lying outside these borders were discarded.

2.5. Statistics

All values are given as mean ± SEM. Fisher's exact test, linear regression, the non-parametric Mann–Whitney rank sum test and one-way ANOVA were used for statistical comparison (SigmaStat 3.1, Systat Software GmbH, Erkrath, Germany). $P < 0.05$ was considered significant.

2.6. Drugs

All drugs were added to the superfusion solution at known concentrations. Drugs and their sources were as follows: strychnine (1 μM), (−)-bicuculline methiodide (bicuculline; 10 μM), yohimbine hydrochloride (yohimbine; 2–3 μM), (−)-norepinephrine bitartrate (noradrenaline; 20 μM), (*R*)-(−)-phenylephrine hydrochloride (phenylephrine; 10–20 μM), dimethyl sulfoxide (DMSO), sodium metabisulfite ($Na_2S_2O_5$; 20 μM), lucifer yellow (1 mM), nystatin (0.1 mM) and prazosin hydrochloride (2 μM) were obtained from Sigma, Deisenhofen, Germany. D-2-amino-5-phosphovaleric acid (D-AP5; 50 μM) and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX; 10 μM) were obtained from Alexis, Grünstadt, Germany. Tetrodotoxin citrate (TTX, 1 μM) was obtained from Ascent Scientific LLC, Princeton, USA.

Stock solutions were prepared by dissolving D-AP5, TTX, bicuculline, yohimbine, noradrenaline, phenylephrine, prazosin and $Na_2S_2O_5$ in distilled water and strychnine and CNQX in dimethyl sulfoxide (DMSO) and were stored in aliquots at −20 °C. A stock of the antibiotic nystatin was prepared by dissolving it in DMSO at 25 mM and was stored at −20 °C for a maximum of 1 week. To prevent the oxidation of noradrenaline and phenylephrine, the antioxidant sodium metabisulfite ($Na_2S_2O_5$; 20 μM) was included upon their dissolution in recording solution.

3. Results

Perforated patch–clamp recordings were obtained from a total of 113 neurons in spinal laminae II and III (73 lamina II EGFP-expressing neurons, 28 lamina II non-EGFP-expressing neurons and 12 lamina III EGFP-expressing neurons). The three groups of neurons had similar resting membrane potentials (−63 ± 0.9, −60 ± 1.3 and −63 ± 1.8 mV, respectively, $p > 0.05$, one-way ANOVA). Recording sites are shown in Fig. 1B.

3.1. Effects of noradrenaline on the membrane potential of superficial dorsal horn neurons

The application of noradrenaline (20 μM, 10–20 s) had differential effects on the membrane potential of laminae II and III dorsal horn neurons (Fig. 2). Some neurons responded with a depolarisation that could range from 3 to 58 mV ($n = 22$). Most depolarisations were of rather small amplitude and did not evoke action potential firing, but larger depolarisations elicited action potentials (Fig. 2A). Depolarisations were also observed in the presence of

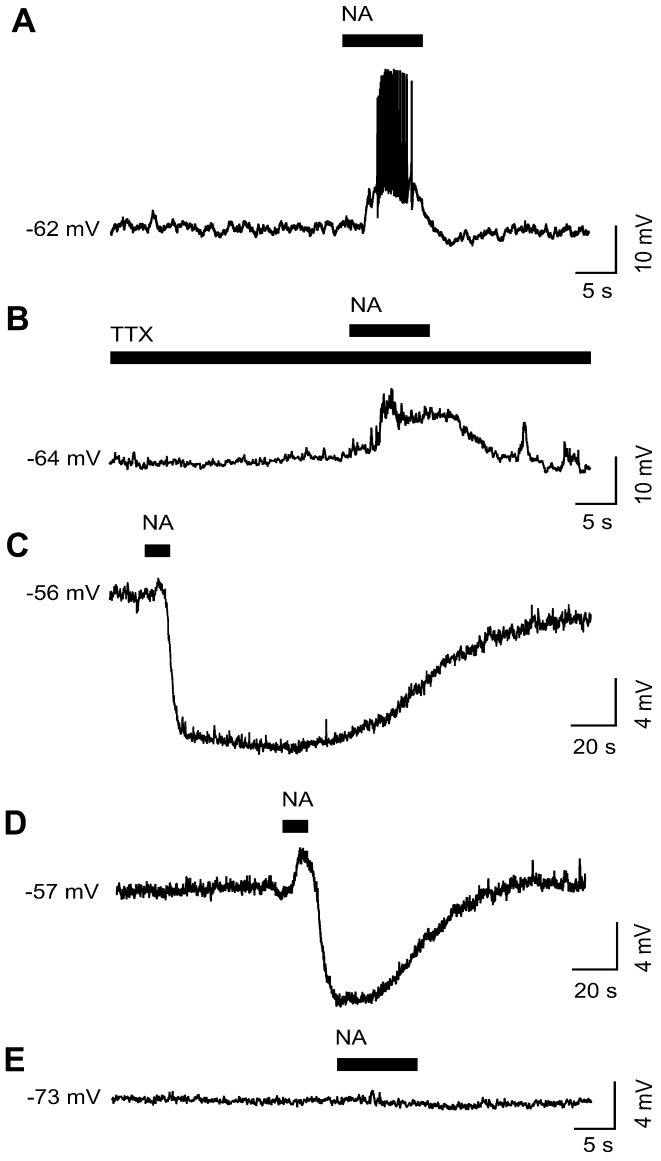

Fig. 2. Effects of noradrenaline on the membrane potential of spinal laminae II and III neurons. Examples of original membrane potential recordings are shown. In some neurons, the application of noradrenaline (NA, 20 μM, 10 s, as indicated by the bar) caused depolarisations ranging from 3 to 58 mV without (A) and in the presence of TTX (1 μM) (B). Hyperpolarisations (C), depolarisations followed by hyperpolarisations (D) or no change (E) were other possible responses of spinal dorsal horn neurons to noradrenaline.

TTX (1 μM) (Fig. 2B). A number of dorsal horn neurons were hyperpolarised by the application of noradrenaline (Fig. 2C, $n = 26$). Eight cells responded with a depolarisation that was followed by a hyperpolarisation (Fig. 2D). In a subgroup of neurons, the membrane potential was unaffected by noradrenaline (Fig. 2E, $n = 35$). As the responses to noradrenaline were steep once they started, the delay of 2–4 s between the application of noradrenaline and the onset of the response (Fig. 2) was most probably caused by the time noradrenaline needed to reach the neuron within the slice rather than by a slow pharmacological response to the drug.

To test if the differential responses to noradrenaline were caused by the same conductance activated at different resting membrane potentials, a linear regression between resting membrane potential and amplitude of the response to noradrenaline was performed in those neurons showing a unidirectional response or no response to noradrenaline. No dependence between the two variables was detected (data not shown, $R = 0.197$, $p = 0.066$, $n = 88$ neurons).

3.2. Noradrenaline has differential effects on EGFP-labelled and non-EGFP-labelled neurons in laminae II and III

The different responses to noradrenaline were not evenly distributed among the three groups of neurons tested (Fig. 3A). Depolarisation was the most frequent effect of noradrenaline on EGFP-expressing, i.e. GABAergic lamina II neurons (41%, 21 of 51 neurons). In contrast, depolarisations were scarce in non-EGFP-expressing lamina II neurons (4%, 1 of 28 neurons, $p < 0.001$ compared to EGFP-expressing lamina II neurons, Fisher's exact test followed by Bonferroni adjustment). Conversely, significantly less EGFP-expressing GABAergic than non-EGFP-expressing neurons in lamina II responded with a hyperpolarisation to noradrenaline (10 of 51 and 13 of 28 neurons, respectively, $p < 0.05$). The remainder of the lamina II neurons showed a mixed response or no change in membrane potential.

Interestingly, in lamina III, EGFP-expressing neurons were never depolarised by noradrenaline. Most neurons were unaffected by noradrenaline (9 of 12) and some were hyperpolarised (3 of 12). EGFP-expressing neurons in lamina III were significantly more often unaffected by noradrenaline than EGFP-expressing neurons in lamina II ($p < 0.05$). The incidence of hyperpolarisation was similar.

The amplitudes of noradrenaline-evoked depolarisations in lamina II neurons are illustrated in Fig 3B. For each neuron, the average of the responses evoked by the first and second application of noradrenaline is shown. The mean amplitude of depolarisation was 14.7 ± 3.0 mV ($n = 31$) in lamina II EGFP-expressing neurons and 8.3 ± 2.3 mV ($n = 4$) in lamina II non-EGFP-expressing neurons ($p = 0.97$, Mann–Whitney rank sum test). In three of the neurons which showed strong depolarisations, the membrane potential did not fully return to the baseline level after the end of the noradrenaline application. In these cases, noradrenaline was applied only once. Most experiments were performed in the presence of blockers of fast synaptic transmission (CNQX, D-AP5, bicuculline and strychnine) but in the absence of TTX to allow detection of noradrenaline-evoked action potentials that were seen in 9 of 41 EGFP-expressing lamina II neurons but only in 1 of 28 non-EGFP-expressing lamina II neurons ($p < 0.05$). To further corroborate that the effects of noradrenaline were caused by direct action on the recorded neuron and not by synaptic transmission, a subgroup of EGFP-expressing lamina II neurons ($n = 10$) was tested in the additional presence of TTX (1 μM). Membrane potential responses evoked in lamina II EGFP-expressing neurons by noradrenaline in the presence of TTX were not significantly different from those obtained in the absence of TTX (all $p > 0.2$, Fisher's exact test; with TTX: 40% depolarisations, $n = 10$; without TTX: 41.5% depolarisations, $n = 41$).

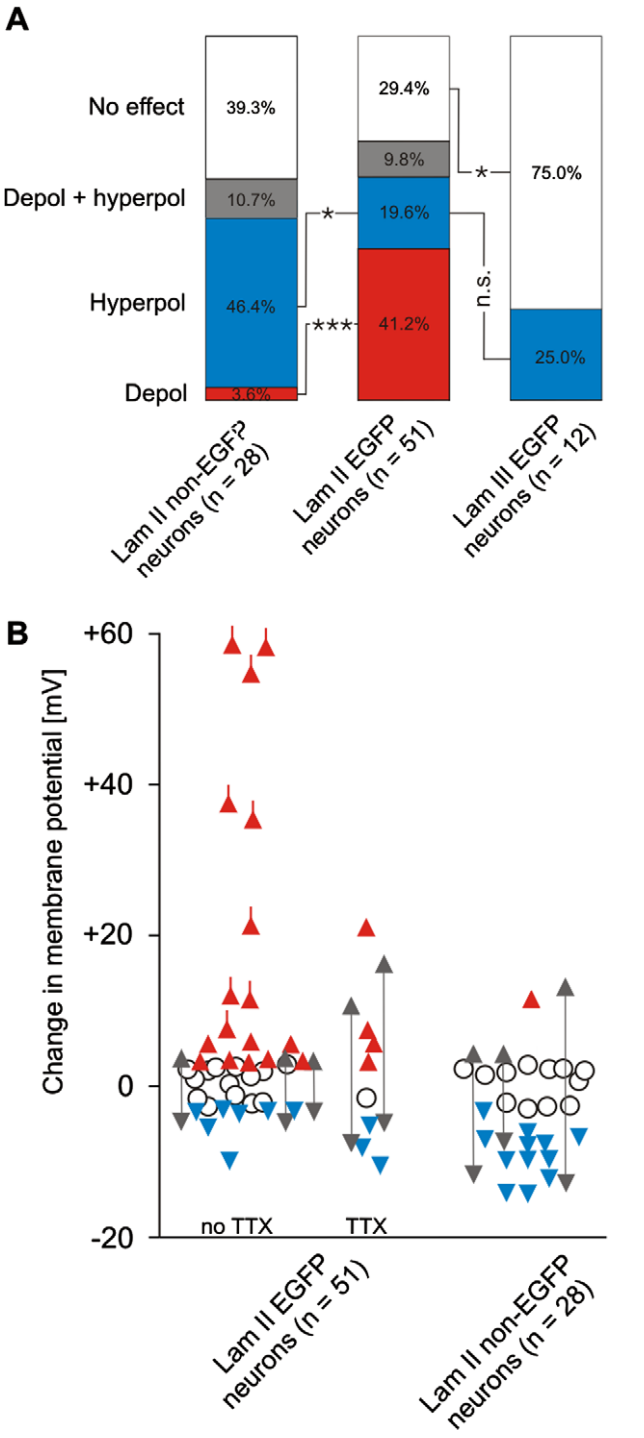

Fig. 3. Distribution of the different responses to noradrenaline among superficial dorsal horn neurons. (A) Summary of the effects of noradrenaline (20 μM) on the membrane potential of EGFP-labelled and non-EGFP-labelled neurons in spinal laminae II and III. $^{***}p < 0.001$; $^{*}p < 0.05$ in Fisher's exact test followed by Bonferroni adjustment. n.s., not significant. (B) illustrates the amplitudes of noradrenaline-induced changes in membrane potential in lamina II EGFP- vs. non-EGFP-labelled neurons. Each symbol represents the average magnitude of the changes evoked by the first two noradrenaline applications in one neuron. ▲ and ▼, pure depolarisation or hyperpolarisation, ▲ and ▼ connected by a line, depolarising/hyperpolarising portion of mixed depolarising–hyperpolarising responses, ▲ depolarisation that evoked action potentials, ○, no effect. Lamina II EGFP neurons recorded in the absence ($n = 41$) or presence ($n = 10$) of TTX are shown separately.

Application of recording solution containing the antioxidant $Na_2S_2O_5$ without noradrenaline did not affect the membrane potential ($n = 5$, lamina II EGFP-expressing neurons, data not shown).

3.3. Adrenergic receptor types involved

We next identified the adrenergic receptor subtypes responsible for the noradrenaline-induced depolarisations and hyperpolarisations. We hypothesised that depolarisations were due to the activation of α_1-adrenoceptors and hyperpolarisations were due to the activation of α_2-adrenoceptors [28,32]. Eleven EGFP-expressing lamina II neurons were exposed to the selective α_1-adrenoceptor agonist phenylephrine (10–20 μM, 10 s). After two applications of phenylephrine, noradrenaline (20 μM, 10 s) was applied. Phenylephrine depolarised five of the eleven EGFP-expressing neurons in lamina II by 4.1 ± 0.8 mV (Fig. 4A) and this depolarisation was in all cases reproduced by subsequent application of noradrenaline. The six neurons unaffected by phenylephrine showed either a hyperpolarisation ($n = 2$) or no reaction ($n = 4$) but never a depolarisation to subsequent superfusion with noradrenaline. These results suggested that the α_1-adrenoceptor was responsible for the noradrenaline-induced depolarisations. To further confirm this conclusion, we used the α_1-adrenoceptor selective antagonist prazosin. As depolarisations, especially large ones, in many cases rendered the neurons too unstable to support the full wash-in procedure required for the subsequent application of prazosin (⩾10 min), we used a different approach. Slices were preincubated (⩾10 min) with prazosin (2 μM) and the selective α_1-adrenoceptor agonist phenylephrine (20 μM, 10–20 s) was applied two times (Fig. 4B). None of the 13 EGFP-expressing lamina II neurons tested showed a response to phenylephrine. This is significantly different from the results obtained in the absence of prazosin (where 5 of 11 neurons reacted to phenylephrine with a depolarisation, $p = 0.01$; and 21 of 51 neurons reacted to noradrenaline with a depolarisation, $p < 0.01$, Fisher's exact test).

In contrast to depolarisations, hyperpolarisations were well supported by neurons so that it was possible to wash in an α_2-adrenoceptor antagonist after completion of the two initial noradrenaline applications. Hyperpolarisations in response to noradrenaline were completely blocked by the α_2-adrenoceptor antagonist yohimbine (2–3 μM, ⩾10 min wash in) in every case ($n = 6$ lamina II EGFP- and non-EGFP-expressing neurons, Fig. 4C), showing that the hyperpolarisations were α_2-adrenoceptor dependent.

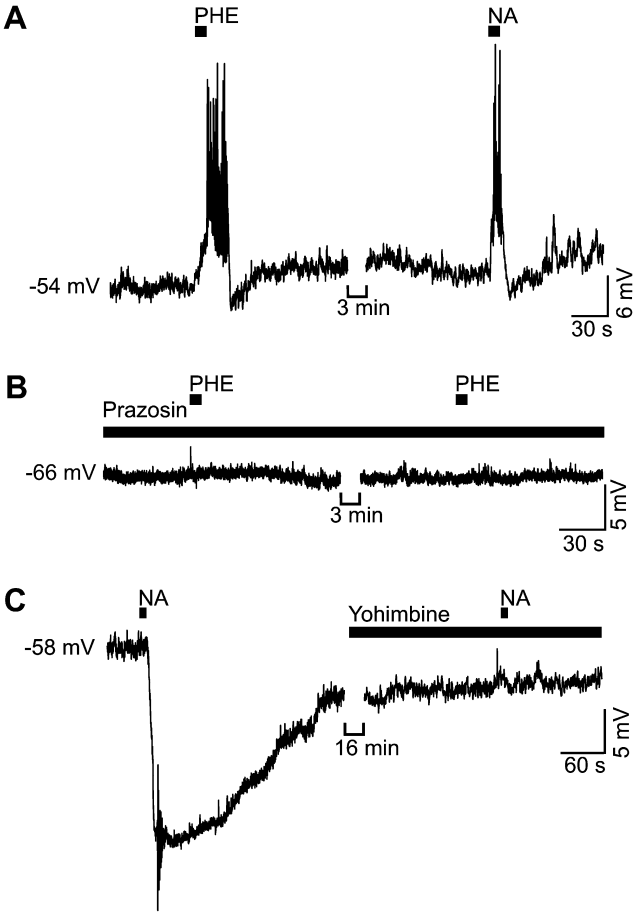

Fig. 4. Identification of the α-adrenoceptor types involved in depolarising and hyperpolarising responses to noradrenaline (NA, 20 μM). Original membrane potential traces are shown. (A) Example of a lamina II EGFP-labelled neuron that was depolarised both by the α_1-selective adrenoceptor agonist phenylephrine (PHE, 20 μM) and by NA. Depolarisation reached the threshold for action potential generation. (B) Example of a lamina II EGFP-labelled neuron recorded in a slice preincubated with prazosin (2 μM). The selective α_1-adrenoceptor agonist phenylephrine (20 μM) did not evoke a depolarisation. (C) Example of a lamina II non-EGFP-labelled neuron where the noradrenaline-induced hyperpolarisation was abolished by the α_2-adrenoceptor antagonist yohimbine (2–3 μM).

4. Discussion

The main finding of the present study is that noradrenaline directly excites GABAergic spinal lamina II but not lamina III neurons via the activation of α_1-adrenoceptors. Excitation of GABAergic neurons and subsequent induction or facilitation of GABA release in the dorsal horn may therefore be a mechanism of noradrenergic descending inhibition in addition to its direct inhibitory action on excitatory spinal dorsal horn neurons.

4.1. Effects of noradrenaline on the membrane potential of superficial dorsal horn neurons

In the present study, both α_2-adrenoceptor-mediated hyperpolarisations and α_1-adrenoceptor-mediated depolarisations were observed in lamina II neurons in response to noradrenaline. α_2-Adrenoceptors are concentrated in the laminae of the superficial dorsal horn [27,39]. Consistently, noradrenaline has been shown to induce α_2-adrenoceptor-mediated membrane hyperpolarisations and/or outward currents in unidentified neurons of the superficial dorsal horn [2,3,10,28,37]. α_2-Adrenoceptor antagonists depress the analgesia which is induced by spinal noradrenaline application [28]. Thus, hyperpolarisation of excitatory superficial dorsal horn interneurons has been deemed a major mechanism of descending pain control [22]. Consistently, we found that an α_2-adrenoceptor-mediated hyperpolarisation was the most frequent reaction to noradrenaline in non-EGFP-labelled neurons. As only about 35% of lamina II GABAergic neurons are labelled by EGFP in the transgenic GIN mice used here [14], non-EGFP-labelled lamina II neurons can be excitatory or inhibitory. However, knowing this figure and the fact that two thirds of superficial dorsal horn neurons are excitatory and one-third is inhibitory (GABAergic, sometimes with coexpression of glycine) [42], it can be estimated that about 75% of the non-EGFP-labelled neurons in lamina II are excitatory.

In addition to inhibitory α_2-adrenoceptors, α_1-adrenoceptors have also been detected in the spinal dorsal horn by in situ hybridisation and binding assays [6,27,45]. Using conventional whole-cell patch–clamp recordings, only hyperpolarisations were observed after the application of noradrenaline [10,37]. In contrast, a small group of superficial dorsal horn neurons shows α_1-adrenoceptor dependent depolarisations when sharp electrodes were used [28]. Recently, a subpopulation of lamina II islet neurons which shows an inward current to noradrenaline was identified [21]. Depolarisations were constantly detected in the present study using the perforated patch–clamp technique for recording, at least

in GABAergic lamina II neurons. One possible explanation for the observation that depolarisations are more easily detected with sharp electrodes and perforated patch–clamp than with whole-cell patch–clamp while hyperpolarisations are detected with either technique is that the dialysis of the postsynaptic cell leads to elution of diffusible signals required for depolarisation but not for hyperpolarisation [43]. Both α_1- and α_2-adrenoceptors are metabotropic receptors, which activate different intracellular pathways. α_1-Adrenoceptors couple to $G_{q/11}$ and activate phospholipase C which leads to an increase of intracellular calcium and protein kinase C activation [12,31,32]. α_2-Adrenoceptors couple to $G_{i/o}$, leading to adenylyl cyclase inhibition, increase of K^+-currents and decrease of Ca^{2+} influx [22,49]. α_2-Adrenoceptor-coupled $G_{i/o}$ triggered pathways in neurons are mainly membrane delimited ($G_{\beta/\gamma}$ mediated) [36] and may therefore be less likely to be lost during whole cell recording than α_1-adrenoceptor-coupled $G_{q/11}$ dependent signalling pathways.

The involvement of β-adrenoceptors in depolarising noradrenaline actions on superficial dorsal horn neurons was not tested in the present study for two reasons. First, the density of β-adrenoceptors in the superficial laminae of the dorsal horn is extremely low [22,26,27]. Second, numerous electrophysiological and behavioural studies have found no evidence for β-adrenoceptor-mediated noradrenaline actions in the dorsal horn [2,23,28,48].

Our sample contained some neurons that responded with a depolarisation followed by a hyperpolarisation to noradrenaline. This type of neurons has been described previously [10] and may possess both α_1 and the α_2-adrenoceptors.

4.2. Differential effects of noradrenaline on GABAergic neurons in laminae II and III of the spinal cord

We found that a major subgroup of EGFP-labelled (i.e. GABAergic) but very few non-EGFP-labelled (75% of them excitatory as estimated above) lamina II neurons responded to noradrenaline with an α_1-adrenoceptor-mediated depolarisation. These depolarisations often reached the threshold for action potential generation in EGFP-labelled neurons, and thus very probably induce GABA release from their terminals. This explains the findings of Baba et al. [2], who reported that noradrenaline increases the frequency of spontaneous GABAergic inhibitory postsynaptic currents in lamina II neurons. A few GABAergic lamina II neurons were hyperpolarised by noradrenaline (present study and [13]), suggesting that lamina II GABAergic neurons are not a homogenous group of neurons.

The density of noradrenergic terminals is much higher in lamina II than in lamina III [11]. In contrast to lamina II GABAergic neurons, none of the tested lamina III GABAergic neurons were depolarised but they were either unaffected (75%) or hyperpolarised (25%) by noradrenaline. This suggests that non-nociceptive information, which is mostly processed in lamina III and deeper laminae [47], is not affected by noradrenaline. Apparently, laminae II and III GABAergic neurons represent two functionally different groups of GABAergic neurons.

One of the 28 non-EGFP-labelled neurons was depolarised by noradrenaline. As 25% of the non-EGFP-labelled neurons are GABAergic this might have been an inhibitory neuron.

As many studies before [8,9,28], the present study used exogenous application of noradrenaline to study its effect on the membrane potential. It remains to be demonstrated that synaptically released noradrenaline has the same effects. However, as descending pathways are the only source of noradrenaline in the dorsal horn [46], it seems probable that the release of noradrenaline reaches the same receptors as exogenously applied noradrenaline.

4.3. The antinociceptive effect of noradrenaline

In the spinal dorsal horn, noradrenaline may act via various receptors and groups of neurons to inhibit nociceptive processing. Behavioural studies have shown that a major part of the antinociceptive effect is due to the action on α_2-adrenoceptors [1,16,33]. Noradrenaline depresses glutamate release from nociceptive, primary afferent fibres by an α_2-adrenoceptor-mediated action on their presynaptic terminals [20,30]. Postsynaptic action on somatodendritic α_2-adrenoceptors hyperpolarises spinal dorsal horn interneurons [10,28,37]. As the spinal dorsal horn contains both excitatory and inhibitory interneurons [42], it is *a priori* not clear if this action is anti- or pronociceptive. The present study shows that the activation of α_2-adrenoceptors by noradrenaline preferentially hyperpolarises non-EGFP-labelled lamina II neurons (75% of them excitatory as estimated above) over GABAergic neurons which is consistent with an antinociceptive effect of noradrenaline.

Pharmacological activation of (α_1-adrenoceptors may also induce behavioural antinociception according to one study [16], but this effect has not yet been studied in great detail. The results of the present study suggest that postsynaptic activation of α_1-adrenoceptors depolarises GABAergic dorsal horn neurons, leading to increased GABA release and probably antinociception.

In conclusion, the present study using the perforated patch–clamp technique and transgenic mice with EGFP-labelled GABAergic neurons, suggests that the antinociceptive effect of spinal noradrenaline is mediated both by the suppression of neuronal activity via the α_2-adrenoceptor and by the activation of GABAergic neurons via the α_1-adrenoceptor.

Conflict of interest

The authors declare no conflict of interest.

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