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The activation of nicotinic acetylcholine receptors enhances the inhibitory synaptic transmission in the deep dorsal horn neurons of the adult rat spinal cord

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Abstract

Somatosensory information can be modulated by nicotinic acetylcholine receptors (nAChRs) in the superficial dorsal horn of the spinal cord. Nonetheless, the functional significance of nAChRs in the deep dorsal horn of adult animals remains unclear. Using whole-cell patch-clamp recordings from lamina V neurons in the adult rat spinal cord, we investigated whether the activation of nAChRs could modulate the inhibitory synaptic transmission in the deep dorsal horn. In the presence of CNQX and APV to block excitatory glutamatergic synaptic transmission, bath applications of nicotine (100 μ M) significantly increased the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in almost all neurons tested. The effect of nicotine was mimicked by N-methyl-4-(3-pyridinyl)-3-butene-1-amine (RJR-2403, 100 μ M), an α 4 β 2-nAChR agonist, and was also mimicked by choline (10 mM), an α 7-nAChR agonist. The effect of nicotine was completely blocked by the nAChR antagonist mecamylamine (5 μ M). In the presence of tetrodotoxin (0.5 μ M), nicotine (100 μ M) significantly increased the miniature IPSC frequency. On the other hand, RJR-2403 (100 μ M) or choline (10 mM) did not affect miniature IPSCs. The application of nicotine (100 μ M) also evoked a large inward current in all lamina V neurons tested when cells were held at -60 mV. Similarly, RJR-2403 (100 μ M) induced inward currents in the majority of lamina V neurons examined. On the other hand, choline (10 mM) did not elicit any detectable whole-cell currents. These results suggest that several nAChR subtypes are expressed on the presynaptic terminals, preterminals, and neuronal cell bodies within lamina V and that these nAChRs are involved in the modulation of inhibitory synaptic activity in the deep dorsal horn of the spinal cord.

Background

Neuronal nAChRs are a larger family of ligand-gated ion channels widely expressed in both the central and the

peripheral nervous system. At least 12 different subunits of nAChRs, including α 2- α 10, β 2- β 4, have been identified so far and these subunits form many different sub-

types of nAChRs with pentameric structures consisting of homomers or heteromers [1]. Homomeric nAChRs are made up of $\alpha 7$, $\alpha 8$ or $\alpha 9$ subunits, while heteromeric nAChRs comprise various combinations of $\alpha 2$ – $\alpha 6$ with $\beta 2$ – $\beta 4$ subunits, $\alpha 9$ with $\alpha 10$ subunits [2,3]. These subtypes of nAChRs have different pharmacological and biophysical properties [1]. It has been shown that nAChRs are involved in a variety of physiological functions including learning, reinforcement, development, aging and nociception [4].

Although Davis et al. (1932) first reported that nicotine has analgesic effects [5], high dosages of nicotine were required to produce antinociception and its effect was relatively modest with a short duration [6-8]. Epibatidine, a potent nAChR agonist isolated from the skin of an Ecuadorian frog, was about 100-fold more potent than morphine in rodents [9-12]. Unfortunately, the dosage of epibatidine to produce antinociception was near that to cause seizure, death, and other side effects [12]. The intolerable toxic effects of epibatidine were due to its actions on a broad range of nAChR subtypes. Therefore, the key to the development of safe and effective nicotinic agonists as analgesics is to first understand which nAChR subtypes are involved in modulating nociceptive transmission.

The spinal dorsal horn is the first site in the central nervous system where somatosensory information is processed and integrated. Multiple subtypes of nAChRs are expressed in the spinal dorsal horn and these receptors have been indicated to modulate sensory inputs from the periphery. Genzen and McGehee (2003) have demonstrated that the activation of $\alpha 7$ nAChRs located at the central terminals of primary afferents enhances the glutamatergic excitatory transmission in the spinal dorsal horn [13]. Several subtypes of nAChRs have been shown to exert tonic or phasic control on the descending inhibitory serotonergic transmission [14]. Multiple subtypes of nAChRs are found to be expressed on both inhibitory and excitatory interneurons in the spinal dorsal horn [15]. The activation of presynaptic nAChRs facilitates GABAergic and glycinergic inhibitory synaptic transmission in the superficial dorsal horn [16-19]. Although the roles of presynaptic nAChRs were extensively studied in the superficial dorsal horn, it is unclear whether nAChRs also mediate sensory modulation in the deep dorsal horn of the spinal cord in adult animals. A variety of sensory inputs, including nociceptive and non-nociceptive inputs, are transmitted into deep dorsal horn [20]. Deep dorsal horn neurons, especially those in the lamina V region, can generate long-lasting afterdischarges in response to nociceptive inputs and this hyperactivity has important implications in pathological pain states [21]. Inhibitory modulation in this region is critical in preventing the central hyperactivity and hyperalgesia. The aim of this study

was to evaluate the effects of nAChR activation on the inhibitory synaptic transmission in deep dorsal horn neurons.

Results

Effects of nicotine and nAChR agonists on spontaneous IPSCs in the lamina V neurons

Whole-cell patch-clamp recordings were performed from lamina V neurons of spinal cord slices prepared from adult rats. Stable recordings could be obtained from slices maintained *in vitro* for more than 12 hours. Glutamatergic excitatory postsynaptic transmission was blocked by CNQX (20 μ M) and APV (50 μ M). All lamina V neurons tested exhibited spontaneous inhibitory postsynaptic currents (sIPSCs) when cells were held at -10 mV. In the presence of bicuculline (20 μ M) and strychnine (2 μ M), sIPSCs were completely abolished in all lamina V neurons tested ($n = 3$; data not shown), indicating these sIPSCs were mediated by GABA and/or glycine receptors. Perfusion of nicotine (100 μ M) for 1 min resulted in a rapid and significant increase in sIPSC frequency in all neurons tested (Fig. 1A–C). The average sIPSC frequency in controls was 2.1 ± 0.6 Hz (0.4 – 5.1 Hz, $n = 9$) and the frequency increased to 15.8 ± 2.3 Hz (4.2 – 28.1 Hz, $n = 9$, $P < 0.05$) following the application of 100 μ M nicotine (Fig. 1C); the sIPSC frequency increased to $1330 \pm 310\%$ of the control ($n = 9$, $P < 0.05$). The nicotine-induced increase in sIPSC frequency was completely blocked in the presence of nAChR antagonist mecamylamine (5 μ M) administered 5 min prior to the application of nicotine ($n = 3$; Fig. 1D, E). After the washout of mecamylamine, a second application of nicotine (100 μ M) increased the sIPSC frequency in all neurons tested (Fig. 1D, E).

We tested RJR-2403, a selective $\alpha 4\beta 2$ nAChR agonist, to see if it also increased sIPSC frequency. Similar to nicotine, application of 100 μ M RJR-2403 for 1 min markedly increased sIPSC frequency in 13 out of 14 neurons recorded (Fig. 2Aa–c). The average sIPSC frequency in the control and following the application of RJR-2403 was 5.8 ± 1.0 Hz (0.5 – 11.6 Hz, $n = 14$) and 15.7 ± 1.9 Hz (3.7 – 25.2 Hz, $n = 14$), respectively (Fig. 2Ac). The sIPSC frequency following the applications of RJR-2403 significantly increased to $573 \pm 189\%$ of the control ($n = 14$, $P < 0.05$; Fig. 2C). While RJR-2403 alone produced a significant increase in sIPSC frequency, the effects of RJR-2403 was completely blocked in the presence of dihydro-beta-erythroidine (Dh β E, 1 μ M), an $\alpha 4\beta 2$ nAChR antagonist ($97 \pm 2\%$ of control, $n = 3$). Perfusion of choline (10 mM), a selective $\alpha 7$ nAChR agonist, for 1 min also increased the sIPSC frequency in 11 out of the 13 neurons examined (Fig. 2Ba–c). The average sIPSC frequency in the control and following the application of choline was 7.4 ± 1.2 Hz (2.9 – 16.9 Hz, $n = 13$) and 15.2 ± 2.3 Hz (5.2 – 27.6 Hz, $n = 13$), respectively (Fig. 2Bc). The sIPSC frequency fol-

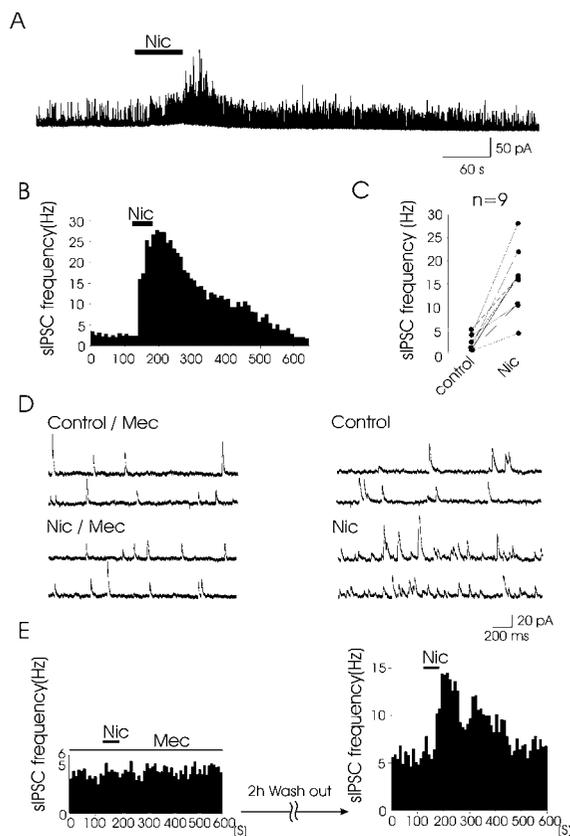


Figure 1
Effects of nicotine on spontaneous IPSCs in lamina V neurons. **A**, A continuous recording of sIPSCs in the control and following the application of nicotine (Nic, 100 μ M). **B**, A histogram shows the time course of the changes in sIPSC frequency following the application of nicotine; time bin is 10 s. **C**, The graph shows the individual result from 9 lamina V neurons. **D**, Effects of mecamlamine (Mec) on nicotine-induced increase in sIPSC frequency. The consecutive traces on the left are sIPSCs in the control (upper panel) and following the application of nicotine (lower panel) in the presence of mecamlamine (5 μ M). The consecutive traces on the right are sIPSCs in the control (upper panel) and following the application of nicotine (lower panel) after the washout of mecamlamine. Note that the bath application of nicotine did not affect the sIPSCs in the presence of mecamlamine, but it markedly increased sIPSC frequency after the washout of mecamlamine. **E**, Two histograms show time courses of changes in sIPSC frequency following the application of nicotine in the presence of mecamlamine (left) and after the washout of mecamlamine (right); time bin is 10 s.

lowing the applications of choline significantly increased to $221 \pm 22\%$ of control ($n = 13$, $P < 0.05$; Fig. 2C). While choline alone produced a significant increase in sIPSC frequency, choline did not produce any significant increase in sIPSC frequency in the presence of methyllycaconitine (MLA, 50 nM), an $\alpha 7$ nAChR antagonist ($98 \pm 2\%$ of control, $n = 4$).

Effects of nicotine and nAChR agonists on mIPSCs in lamina V neurons

We examined the effects of nicotine or nAChR agonists on mIPSC frequency in the presence of tetrodotoxin (TTX, 0.5 μ M) to determine whether nAChRs might be localized at the presynaptic terminals of GABAergic and/or glycinergic inhibitory interneurons. Application of 0.5 μ M TTX itself blocked the action potential-driven synaptic transmission and decreased the amplitude of IPSCs from 149.9 ± 125.6 pA to 30.0 ± 18.4 pA ($n = 4$). Under this condition, bath application of nicotine (100 μ M) largely increased mIPSC frequency in all neurons recorded, but there was no effect on mIPSC amplitude ($n = 10$; Fig. 3A). The average mIPSC frequency in the control and following the applications of nicotine was 1.7 ± 0.4 Hz (0.4 – 5.3 Hz, $n = 10$) and 15.0 ± 2.3 Hz (1.4 – 25.2 Hz, $n = 10$), respectively. The mIPSC frequency following the applications of nicotine significantly increased to $1043 \pm 153\%$ of control ($n = 10$, $P < 0.05$, Fig. 3D). On the other hand, perfusion of 100 μ M RJR-2403 ($n = 6$) or 10 mM choline ($n = 7$) did not affect mIPSC frequency and amplitude (Fig. 3B, 3C). The average mIPSC frequency following the application of RJR-2403 and choline was $95 \pm 3\%$ of control ($n = 6$) and $98 \pm 2\%$ of control ($n = 7$), respectively (Fig. 3D).

Whole-cell currents directly evoked by nicotine or nAChR agonists in lamina V neurons

We determined whether nicotine, RJR-2403 and choline could evoke whole-cell currents in lamina V neurons. In this set of experiments, cells were held at -60 mV and recordings were conducted in the presence of 20 μ M CNQX, 50 μ M APV, 20 μ M bicuculline and 10 μ M PMBA (3-[2'-Phosphonomethyl][1,1'-biphenyl]-3-yl]alanine). Under this condition, both excitatory and inhibitory postsynaptic currents were completely disappeared. The bath application of nicotine (100 μ M) for 1 min evoked an inward current in all neurons tested (Fig. 4A). The average peak amplitude of the inward currents evoked by nicotine was 95 ± 19 pA ($n = 8$; Fig. 4D). The bath application of RJR-2403 (100 μ M) for 1 min also evoked large inward currents in 6 out of 8 neurons examined (Fig. 4B). The average peak amplitude of the inward currents induced by RJR-2403 was 119 ± 42 pA ($n = 6$; Fig. 4D). In contrast to nicotine and RJR-2403, choline (10 mM) did not elicit any detectable currents ($n = 6$; Fig. 4C).

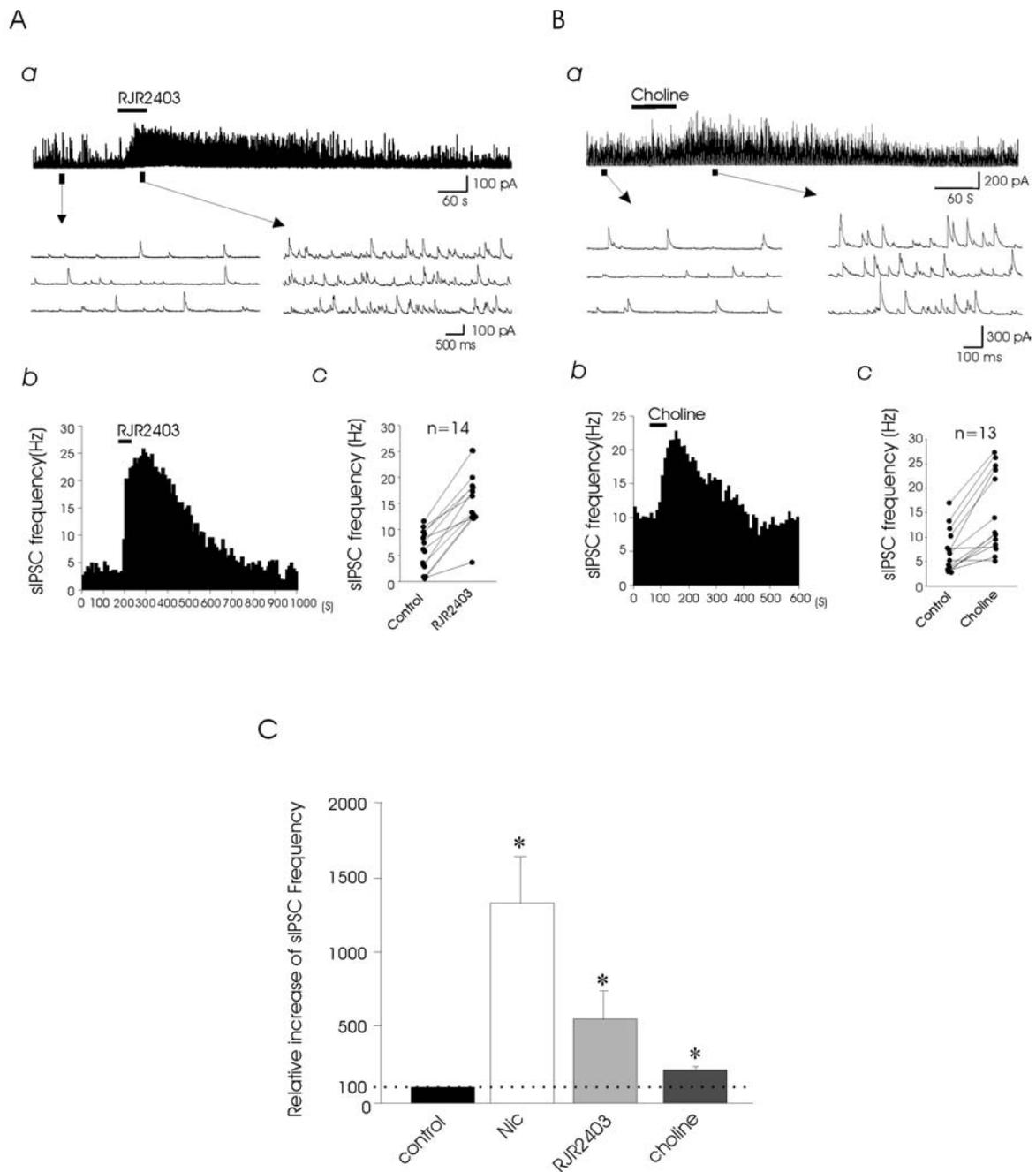
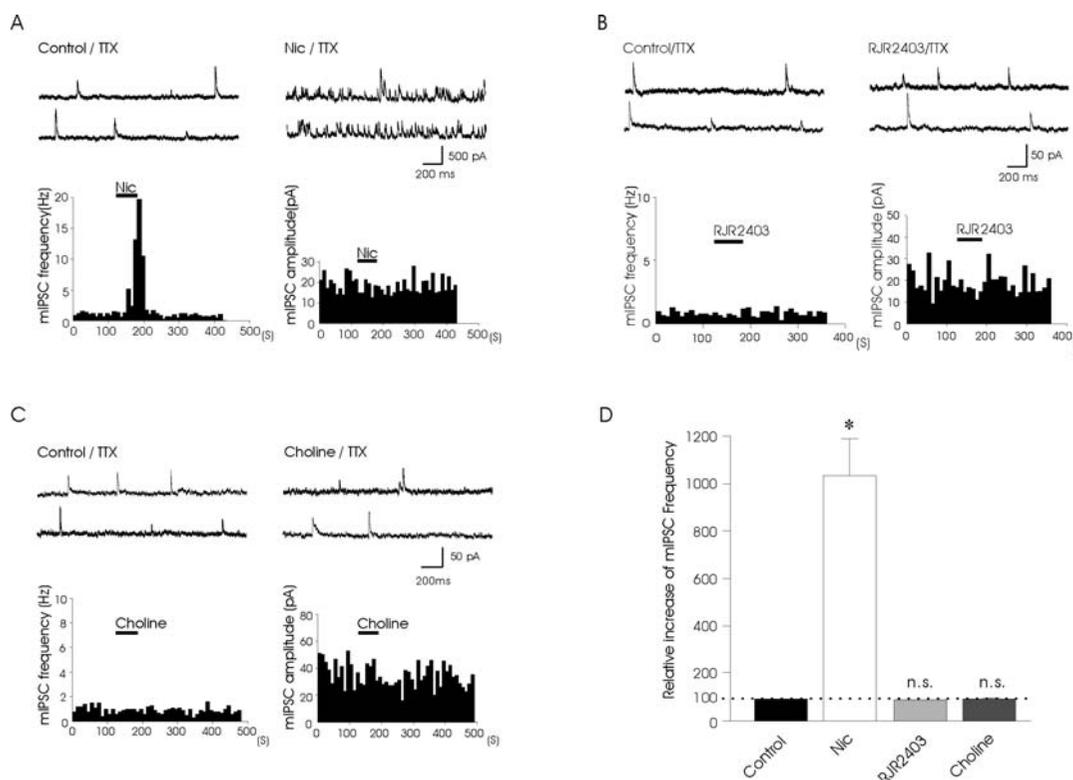


Figure 2

Effects of nicotinic receptor agonists on sIPSCs in lamina V neurons. **A, (a)** A continuous recording of sIPSCs in the control and following the application of the selective $\alpha 4\beta 2$ nAChR agonist RJR-2403 (100 μ M). **(b)** A histogram shows the time course of changes in sIPSC frequency following the application of RJR-2403; time bin is 10 s. **(c)** The graph shows the individual result from 14 lamina V neurons. **B,** The experiment was similar to that shown in **(A)** except that the selective $\alpha 7$ nAChR agonist choline (10 mM) was tested. Similar results were obtained in 11 out of 13 neurons. **C,** A histogram shows relative sIPSC frequency following the application of nicotine ($n = 9$), RJR-2403 ($n = 14$), or choline ($n = 13$). sIPSC frequency before the applications of testing drugs is used as control and is scaled at 100%. Data represent Mean \pm SEM; * $P < 0.05$.

**Figure 3**

Effects of nicotinic receptor agonists on mIPSCs in lamina V neurons. **A**, The consecutive traces of mIPSCs are in the control (left) and following the application of nicotine (100 μ M, right). Two histograms on the bottom panel show the time course of changes in mIPSC frequency (left) and amplitude (right) during the application of nicotine; time bin is 10 s. **B**, **C**, Experiments were similar to that shown in (**A**) except that 100 μ M RJR-2403 or 10 mM choline was tested. **D**, A histogram shows relative mIPSC frequency following the applications of nicotine ($n = 11$), RJR-2403 ($n = 6$), or choline ($n = 7$). mIPSC frequency before the applications of testing drugs is used as control and is scaled at 100%. Data represent Mean \pm SEM; * $P < 0.05$; n.s., not significant.

Discussion

The present study demonstrated in lamina V neurons of the adult rat spinal cord that nicotine increased sIPSC frequency when glutamatergic excitatory transmission was blocked in the presence of CNQX and APV and that nicotine also increased mIPSC frequency when action potential-driven synaptic transmission was not permitted in the presence of TTX. Interestingly, however, neither the $\alpha 4\beta 2$ nor $\alpha 7$ nAChR agonists increased mIPSC frequency although both of them increased sIPSC frequency in lamina V neurons. Together with the findings of our previous study conducted on superficial laminae of the spinal cord of adult rats [18], we have provided electrophysiological evidence showing that inhibitory synaptic activity in both superficial and deep laminae of the spinal cord dorsal horn are modulated by different nAChR subtypes.

Nicotinic receptors are abundant in different CNS regions, where they are shown to regulate the release of various

neurotransmitters, including serotonin, norepinephrine, glutamate, GABA and glycine [22-24]. In the present study, the activation of nAChRs enhanced the GABA and/or glycine release onto lamina V neurons. A similar enhancement of the inhibitory synaptic transmission by the activation of nAChRs has been reported in the superficial layers of the spinal dorsal horn [16,18,19]. In neonatal rats, $\alpha 4\beta 2$ nAChR subtype has been suggested to be expressed at presynaptic terminals and these receptors mediate significantly increases in the glycinergic [16] and GABAergic inhibitory synaptic transmission in the superficial lamina of the spinal cord dorsal horn [19]. Interestingly, the expression of nAChR subunits in the spinal cord changes during development [25]. Consistent with the development changes of nAChR subunits, our previous study demonstrated that a non- $\alpha 4\beta 2$ and non- $\alpha 7$ subtype nAChR mediated an enhancement of both the GABAergic and glycinergic mIPSC frequency in the superficial laminae of the adult rat spinal dorsal horn [18]. In the deeper

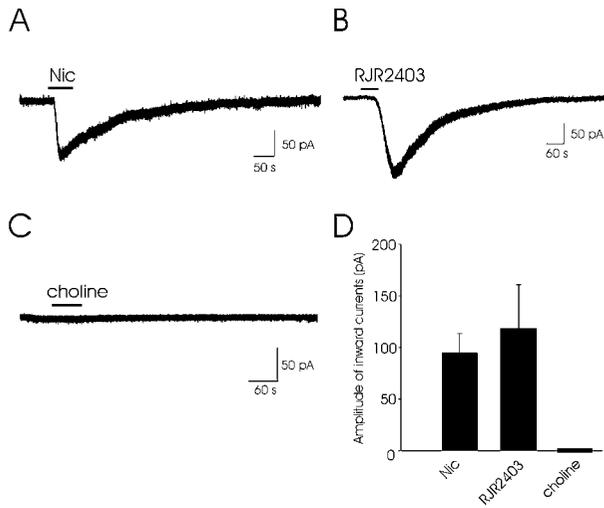


Figure 4
Whole-cell currents evoked by nicotine and nAChR agonists in lamina V neurons. **A**, Whole-cell currents evoked by bath application of nicotine (100 μ M) in a lamina V neuron. **B**, Whole-cell currents evoked by RJR-2403 (100 μ M) in a different lamina V neuron. **C**, Choline (10 mM) did not evoke any membrane current in a neuron. **D**, The histogram shows the average amplitude of the whole-cell inward currents induced by nicotine ($n = 8$), RJR-2403 ($n = 6$), and choline ($n = 6$).

laminae (lamina III–V) of neonatal rats, a previous study showed that presynaptic $\alpha 4\beta 2$ nAChRs mediate the facilitation of GABA release [19], a result similar to those shown in superficial laminae of neonatal rats [16,19]. However, the present study revealed that the nAChR-mediated modulation of inhibitory synaptic transmission in the adult stage is more complicated than that in the neonatal stage. We showed that nicotine largely increased both sIPSC frequency and mIPSC frequency in the lamina V neurons in adult rats. On the other hand, RJR-2403, a potent activator of $\alpha 4\beta 2$ nAChR, and choline, a selective agonist for $\alpha 7$ nAChR, significantly increased the sIPSC frequency, but did not change mIPSC frequency. It has been demonstrated that nAChRs are expressed at two cellular locations in the central nervous system [26]. One is presynaptic sites or synaptic boutons where nAChR activation modulates transmitter release in a TTX-insensitive manner. The other is preterminals at terminal axon branches where nAChR activation affects transmitter release by depolarizing axonal membranes to fire action potentials. A recent immunohistochemical study revealed the immunoreactivity of nAChRs in lamina V neurons of the spinal dorsal horn at both presynaptic and preterminal sites [27]. At the preterminal sites, nAChR-mediated regulation of transmitter releases is TTX-sensitive and can be blocked in the presence of TTX. The block by TTX of the

RJR-2403- and choline-induced increase in sIPSC frequency in our results suggests that $\alpha 4\beta 2$ and $\alpha 7$ nAChRs are not expressed at presynaptic terminals. These receptors are likely to be expressed at the preterminals or other parts of GABAergic and/or glycinergic neurons whose axons innervate lamina V neurons. Because nicotine could still increase IPSC frequency in the presence of TTX, it suggests that a non- $\alpha 4\beta 2$, non- $\alpha 7$ subtype of nAChR is located at the presynaptic terminals of GABAergic and/or glycinergic neurons that innervate lamina V neurons (Fig. 5). The

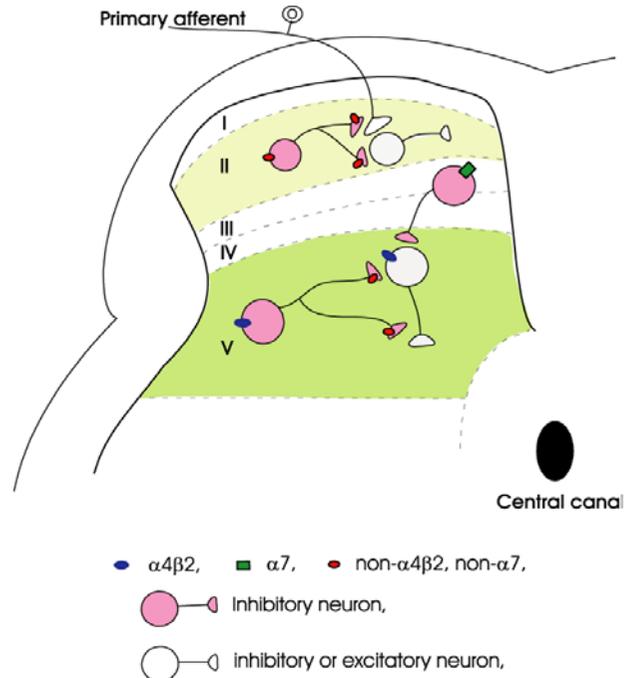


Figure 5
Schematic diagram of nAChR-mediated modulation of sensory synaptic transmission in the dorsal horn of adult rats. In adult rats, $\alpha 4\beta 2$ nAChRs are expressed on the soma of inhibitory interneurons located in the lamina V region. These nAChRs may be also expressed on preterminals but not at presynaptic sites of the lamina V inhibitory neurons. Lamina V neurons are also synapsed by inhibitory neurons expressing $\alpha 7$ nAChRs at their preterminals and/or on their somas and these inhibitory neurons are likely to be located in other lamina regions. The modulation of inhibitory activity in lamina V by both $\alpha 4\beta 2$ nAChRs and $\alpha 7$ nAChRs depends on membrane depolarization and action potentials. There is a non- $\alpha 4\beta 2$ /non- $\alpha 7$ subtype of nAChRs that are expressed at the presynaptic terminals of inhibitory neurons in lamina V region. The modulation of inhibitory transmission in lamina V by non- $\alpha 4\beta 2$ /non- $\alpha 7$ subtype of nAChRs is independent of membrane depolarization and action potentials. The distribution of nAChR subtypes in the spinal cord lamina II region of adult rats [18] is also presented in this diagram for a comparison.

presence of a non- $\alpha 4\beta 2$, non- $\alpha 7$ subtype of nAChR in spinal cord dorsal horns are supported by previous studies using $\alpha 4\beta 2$ nAChR knock-out mouse [28], *in situ* hybridization [29] and the combination of patch-clamp recordings with single-cell RT-PCR [15]. All these previous studies pointed to the potential presence of other functional nAChR subtypes in addition to $\alpha 4\beta 2$ and $\alpha 7$ receptors. However, the subunit compositions of the non- $\alpha 4\beta 2$ and non- $\alpha 7$ subtype of nAChRs remain to be identified.

We have shown that both nicotine and the $\alpha 4\beta 2$ nAChR agonist RJR-2403 directly evoked whole-cell inward currents in the majority of lamina V neurons. On the other hand, the $\alpha 7$ nAChR agonist choline did not evoke any detectable inward currents in lamina V neurons. Since strychnine has been noted to also be an effective antagonist at $\alpha 7$ nAChRs [30,31], the effect of choline was examined in the presence of PMBA, a glycine receptor antagonist that has no effect on $\alpha 7$ nAChRs [32,33]. The lack of choline-evoked whole-cell currents raise a possibility that $\alpha 7$ nAChRs are not expressed on lamina V neurons. If this is the case, then choline-induced increases of sIPSC frequency might be mediated by $\alpha 7$ nAChRs that are expressed on the inhibitory interneurons whose cell bodies are located in other lamina regions in the spinal cord. In contrast to $\alpha 7$ nAChRs, our results suggest that $\alpha 4\beta 2$ nAChR expressing inhibitory interneurons are located in lamina V. Consistently, several reports have indicated the presence of $\alpha 4\beta 2$ nAChR in the deep dorsal horn and $\alpha 7$ nAChRs in other lamina of the spinal cord [15,33-35]. Bradia et al. (2002) has reported that α -bungarotoxin-sensitive $\alpha 7$ nAChRs are located in the parasympathetic preganglionic neurons surrounding the central canal of the spinal cord (lamina X) [33]. A low level of $\alpha 7$ transcripts were also detected by *in situ* hybridization in the area around the central canal [35]. Moreover, the single-cell RT-PCR study revealed a more widespread expression of $\alpha 7$ nAChR subunits in mouse spinal dorsal horn neurons [15]. These findings support the idea that $\alpha 7$ nAChR-expressing inhibitory interneurons innervate lamina V neurons from other lamina regions in the spinal cord.

The role of nAChRs in modulating pain transmission has been reported by a number of studies. Using $\alpha 4\beta 2$ knock-out mice, Marubio et al. (1999) showed a reduced antinociceptive effect in a behavior study [28]. In a neuropathic mouse model, epibatidine, a potent agonist of nAChRs showed strong analgesic effects. However, the effects of epibatidine were not completely prevented by the $\alpha 4\beta 2$ nAChR antagonist dihydro- β -erythroidine [36]. These studies suggested that in addition to $\alpha 4\beta 2$ nAChR, other nAChRs were involved in nAChR-mediated analgesic effects. Consistent with this idea, the intrathecal injection of choline, an $\alpha 7$ nAChR agonist, has been reported to

have an antinociceptive effect [37]. Several mechanisms have been proposed to contribute to nAChR-mediated analgesic effects, including the desensitization of nAChRs on nociceptive primary afferent fibers, the increase of noradrenaline and serotonin release within the spinal cord, the activation of the descending inhibitory pathways [14,38], and the increases of GABA and glycine release from inhibitory interneurons in the superficial spinal cord dorsal horn [39]. Our study suggest that $\alpha 4\beta 2$, $\alpha 7$ nAChRs, and another undefined subtype of nAChRs are involved in regulating GABA and/or glycine release in the deep lamina of the spinal cord dorsal horn in adult animals.

In this study, the application of nicotine or nicotinic agonists significantly facilitated GABAergic and/or glycinergic inhibitory synaptic transmission in the deep dorsal horn of the spinal cord. This raises a possibility that acetylcholine released endogenously may modulate inhibitory synaptic transmission in a similar fashion. Recently, Rashid et al. (2006) suggested that endogenous acetylcholine tonically stimulated the GABA and glycine release via $\alpha 4\beta 2$ subtype of nAChRs in the superficial dorsal horn in mice [40]. Acetylcholine may be released from the interneurons in the dorsal horn since the cell bodies of cholinergic interneurons have been found in lamina III-V [41]. It appears that in the deep dorsal horn there are no descending cholinergic systems in the rat [20,42]. Thus, cholinergic interneurons in the dorsal horn [43,44] may play an important role in modulating inhibitory synaptic transmission.

In the dorsal horn, GABAergic and glycinergic inhibitory synapses undergo developmental changes [45-47]. In the present study, we did not separate inhibitory activity between those of GABAergic synapses and those of glycinergic synapses. It would be interesting to further study whether nAChR subtype expression on GABAergic and glycinergic neurons is different in the spinal cord dorsal horn.

Conclusion

We have demonstrated that several nAChR subtypes are expressed on the presynaptic terminals, preterminals, and neuronal cell bodies within lamina V and that they are involved in the facilitation of inhibitory synaptic transmission. Therefore, the activation of nAChRs in the deep dorsal horn of the spinal cord may be capable of inhibiting nociceptive signaling in physiological and pathological pain sensations.

Methods

All the experimental procedures involving the use of animals were approved by the Ethics Committee on Animal Experiments, Wakayama Medical University, and were in

accordance with the UK Animals (Scientific Procedures) Act 1986 and all associated guidelines.

Spinal cord slice preparation

The method used to prepare adult rat spinal cord slices has been described previously [48]. In brief, male adult Sprague-Dawley rats (6–8 weeks of age, 200–300 g) were deeply anaesthetized with isoflurane through a nose cone inhalation, and then lumbosacral laminectomy was performed. The lumbosacral spinal cord (L1-S3) was removed and placed in pre-oxygenated Krebs solution at 1–3°C. Immediately after the removal of the spinal cord, the rats were killed by exsanguination. The pia-arachnoid membrane was removed after cutting all the ventral and dorsal roots near the root entry zone. The spinal cord was mounted on a vibratome and then a 600 µm-thick transverse slice was cut. The slice was placed on nylon mesh in the recording chamber, which had a volume of 0.5 ml, and then was perfused at a rate of 10–15 ml/min with Krebs solution saturated with 95% O₂ and 5% CO₂, and maintained at room temperature. A platinum grid was placed on the top of the slice to prevent slice movement. The Krebs solution contained (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃ and 11 glucose.

Patch-clamp recordings from lamina V neurons

Blind whole-cell patch-clamp recordings were made from lamina V neurons with patch-pipette electrodes having a resistance of 5–10 MΩ [48]. The patch-pipette solution was composed of (in mM) 110 Cs₂SO₄, 5 Tetraethylammonium (TEA), 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 5 HEPES, 5 ATP-Mg, pH 7.2. Signals were acquired with a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA, USA). The data were digitized with an A/D converter (Digidata 1200, Axon Instruments) and stored and analyzed with a personal computer using the pCLAMP data acquisition program (Version 8.2, Axon Instruments). Lamina V neurons were viable for up to 24 h in slices perfused with a pre-oxygenated Krebs solution. All the recordings described in this study were made within 12 h. Whole-cell patch-clamp recordings were stable for up to 4 h. All of the neurons had membrane potentials more negative than -50 mV. Unless otherwise noted, all the recordings in this study were performed in the presence of CNQX (20 µM) and APV (50 µM).

Drug Applications

Drugs were dissolved in Krebs solution and then were applied by perfusion via a three-way stopcock without any change in the perfusion rate or the temperature. The time necessary for the solution to flow from the stopcock to the surface of the spinal cord slice was approximately 20 s. The drugs used in this study were nicotine (Sigma-Aldrich, St. Louis, MO, USA), RJR2403 (Tocris, Ballwin, MO,

USA), choline (Sigma-Aldrich), mecamylamine (Sigma-Aldrich), 3-[2'-Phosphonomethyl[1,1'-biphenyl]-3-yl]alanine (PMBA, Sigma RBI), bicuculline (Sigma-Aldrich), strychnine (Sigma-Aldrich), 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX, Tocris), D(-)-2-Amino-5-phosphonopentanoic acid (D-APV, Tocris), and tetrodotoxin (TTX, Tocris).

Statistical analysis

All numerical data were expressed as the mean ± S.E.M. Statistical significance was determined as P < 0.05 using paired Student's t-test. For electrophysiological data, *n* refers to the number of neurons recorded.

Abbreviations

nACh, nicotinic acetylcholine receptor;

IPSC, inhibitory postsynaptic current;

RJR2-403, N-methyl-4-(3-pyridinyl)-3-butene-1-amine;

TTX, tetrodotoxin;

GABA, gamma-aminobutyric acid;

TEA, tetraethylammonium;

EGTA, ethyleneglycol bis(2-aminoethylether)tetraacetic acid;

HEPES, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid);

ATP-Mg, adenosine triphosphate-magnesium;

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dion;

PMBA, 3-[2'-Phosphonomethyl[1,1'-biphenyl]-3-yl]alanine;

D-APV, D(-)-2-Amino-5-phosphonopentanoic acid;

Competing interests

The author(s) declare that they have no competing interests.

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