Tonic nicotinic modulation of serotoninergic transmission in the spinal cord

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The spinal serotoninergic projection from the raphe magnus has been shown to modulate nociceptive inputs, and activation of this projection mediates nicotine-elicited analgesia. Here, we investigate the interactions between cholinergic and serotoninergic systems in the spinal cord, by conducting serotonin [5-hydroxytryptamine (5-HT)] efflux experiments on mouse spinal slices. At least three spinal populations of nicotinic receptors are distinguished that affect 5-HT release. The first could be directly located on serotoninergic terminals, is insensitive to nanomolar concentrations of methyllicaconitine (MLA), and may be subjected to a basal (not maximal) cholinergic tone. The second is tonically and maximally activated by endogenous acetylcholine, insensitive to nanomolar concentrations of MLA, and present on inhibitory neurons. The last is also present on inhibitory neurons but is sensitive to nanomolar concentrations of MLA and not tonically activated by acetylcholine. Multiple nicotinic acetylcholine receptor populations thus differentially exert tonic or not tonic control on 5-HT transmission in the spinal cord. These receptors may be major targets for nicotine effects on antinociception. In addition, the presence of a tonic nicotinic modulation of 5-HT release indicates that endogenous acetylcholine plays a role in the physiological regulation of descending 5-HT pathways to the spinal cord.

Cholinomimetic drugs have long been shown to produce antinociception in animal models (1). Although early studies suggested an action via muscarinic receptors, it is now clear that nicotinic agonists exert strong antinociceptive actions. Epibatidine, a nicotinic agonist extracted from the skin of an Ecuadorian frog, has been shown to be 200 times more potent than morphine at blocking pain in animals (2), and some of its analogs are presently under clinical trial. Yet, the precise mechanism by which nicotinic agonists produce analgesia is far from completely understood.

Nicotine binds to nicotinic acetylcholine receptors (nAChRs), thus provoking the opening of their nonspecific cationic channel. nAChRs are transmembrane proteins formed of five subunits surrounding a central channel, with two (or five) ligand-binding sites (for hetero- or homopentamers, respectively) situated at the interface between subunits (3). Fifteen mammalian subunits (α1–7, α9–10, β1–4, γ, and δ) have been cloned to date (4), and several combinations of these subunits may associate to produce functional receptors *in vitro* (5).

In situ hybridization studies (6, 7) reveal that almost all nAChR subunits are expressed along the ascending nociceptive pathway (dorsal root ganglia, spinal cord, thalamus, and cortex), as well as in its descending modulatory correlates (rostro-ventral medulla). Moreover, binding experiments (8) clearly show a labeling of these regions by several nicotinic agonists and antagonists, suggesting that the expressed subunits indeed form receptors with intact binding sites in these regions. No preferential site for the analgesic effects of nicotine can therefore be deduced from such experiments.

In contrast, local injections of nicotinic drugs have shown that the serotoninergic descending control issuing from the raphe magnus may be of primary importance for nicotine-elicited analgesia (9, 10). Among the seven strains of knockout (KO) mice constructed for one of the nAChR subunits (11), only the α4- and the β2-KO mice have been analyzed to date in an analgesia paradigm (12). In both strains not only were functional nAChRs eliminated from the raphe magnus, but the analgesic effect of nicotine in the "hot plate" test also was suppressed. However, in α4 (and to a lesser extent in β2) KO mice, nicotine still had antinociceptive effects in the "tail flick" test, although much reduced in comparison to wild-type mice (12). These experiments suggest that there is a spinal (non-α4β2)-nAChR component of nicotinic analgesia. Indeed in the same study, electrophysiological experiments unraveled presynaptic (non-α4β2)-nAChRs in the dorsal horn of the spinal cord (12).

In the present investigation, we continued the exploration of spinal presynaptic nAChRs by analyzing the effects of nicotinic agonists and antagonists on tritiated neurotransmitter efflux. As the descending serotoninergic projection from the raphe magnus appears relevant to the understanding of nicotine-induced analgesia, we examined interactions between cholinergic and serotoninergic systems in the spinal cord. In spinal slices, we show not only that nicotine elicits the release of 5-hydroxytryptamine (5-HT), but also that endogenous ACh modulates serotoninergic transmission in the spinal cord through nicotinic receptors.

Materials and Methods

Animals. C57 Black6 female mice (3 mo old) were purchased from Iffa Credo. Female α4 +/+ and α4−/− mice, killed between 3 and 5 mo of age, were obtained from crossing of F_3 homozygotes (the KO and control animals were therefore cousins). Male β2 +/+ and β2−/− mice, killed at 3 mo of age, were F_7.

Drugs and Chemicals. [^{3}H]5-HT was purchased from Amersham Pharmacia (specific activity: 6.5–11.5 Ci/mmol, depending on the batches). Agonists and antagonists were purchased from Sigma/Research Biochemicals. The acetylcholine esterase (AChE) used was also purchased from Sigma and extracted from electric eel (activity: 1,070 units/mg protein).

Preparation of Spinal Slices. Mice were decapitated and the spinal cords were rapidly dissected on ice. The thoracic spinal cords were then transversely cut (300 μm) in a DSK-1000 slicer (Dosaka, Japan). The slices were collected and maintained for 30 min at room temperature in a Krebs buffer containing 120 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 10 mM glucose, gassed with 95% to 5% O_2/CO_2 mixture. This buffer is hereafter called superfusion buffer or SB. The slices were then incubated in the presence of 430 nM [^{3}H]5-HT and 10 mM pargyline in SB for 30

Abbreviations: 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; nAChR, nicotinic acetylcholine receptor; AChE, acetylcholine esterase; KO, knockout; SB, superfusion buffer; MLA, methyllicaconitine; DhβE, dihydro-β-erythroidine.

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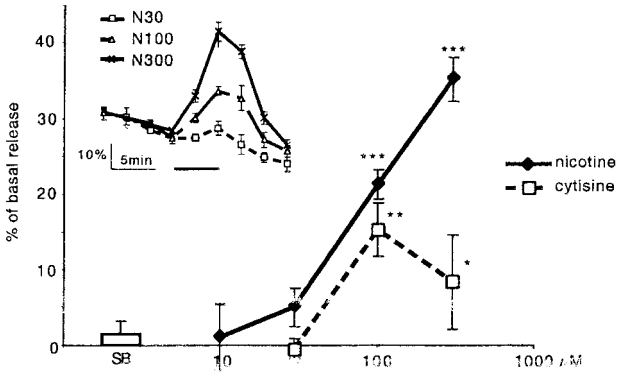

Fig. 1. Dose–response curve of the effects of nicotinic agonists on [^{3}H]5-HT efflux from mouse spinal cord slices (for calculation of the response amplitude see *Materials and Methods*). Nicotine (n = 4–36) and cytisine (n = 4–6) responses were compared to the control, SB (n = 18), response. Statistical analysis according to one-way ANOVA followed by Dunnett's test for treatment vs. control comparisons: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. (*Inset*) Averaged traces for [^{3}H]5-HT efflux elicited by 30, 100, and 300 μM nicotine.

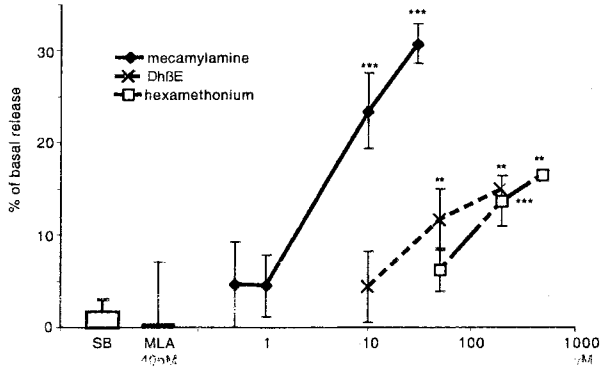

Fig. 2. Dose–response curve of the effects of nicotinic antagonists on [^{3}H]5-HT efflux from mouse spinal cord slices. MLA- (n = 6), mecamylamine- (n = 6–60), hexamethonium- (n = 3–6), and DhβE- (n = 2–11) induced responses were compared to the control, SB, response. Statistical analysis according to one-way ANOVA followed by Dunnett's test for treatment vs. control comparisons: **, $P < 0.01$ and ***, $P < 0.001$.

min at 37°C. The slices were rinsed several times and transferred to the superfusion apparatus.

Superfusion of Slices. Superfusion of slices was performed in home-made Plexiglas chambers (one spinal slice or two hemi-spinal slices per chamber) by using the SB at 37°C and at a rate of 0.5 ml/min. The perfusate collected during the first 30 min was discarded, and subsequent superfusion fluid was collected in 1.25-ml (2.5 min) fractions. Drugs were added at the fourth collection period. When mentioned in the text, a preincubation of antagonist began 15 min before the collection period and continued until the end of the experiment. Fractions of perfusate were counted for radioactivity in a LKB Wallac (1209 RACK-BETA) counter after addition of 8 ml of biodegradable counting scintillant.

Superfusion Data Analysis. Drug-induced release was evaluated at the fifth and sixth collection period. The base line for the fifth and sixth collection periods was calculated from a linear extrapolation of the first four fractions of perfusate. The amplitude of the release is considered as the difference between the observed release and the base line calculated as described above and is expressed in percentage of the basal (mean of the first four fractions) release. Results are expressed as the mean ± SEM of the values obtained in independent experiments, each experiment consisting of at least two replicate chambers for each condition.

Results

Effects of nAChR Agonists on Serotonin Release. After the incubation of transversal slices of C57 Black6 mice thoracic spinal cord with [^{3}H]5-HT, the application of 25 mM KCl produced an increase (47.5 ± 4.9%, n = 2) of the basal release of radioactivity (result not shown), demonstrating that nerve terminals have efficiently loaded the [^{3}H]5-HT.

Fig. 1 shows that application of nicotine increased the basal release of [^{3}H]5-HT in a dose-dependent manner. The nicotinic response was unchanged by the incubation of the slices with tetrodotoxin (3 μM, result not shown), suggesting a presynaptic mode of action. In a set of experiments, we divided the spinal slices into dorsal and ventral half; nicotine had a comparable effect in both halves (result not shown). We thereafter proceeded with entire spinal slices. The nicotinic agonist cytisine (30–300 μM) produced a dose-dependent increase of [^{3}H]5-HT release (Fig. 1), although the maximal effect was only 43% of the nicotine one.

Endogenously Activated nAChRs. We then tested a number of nAChR antagonists on 5-HT release. Mecamylamine, dihydro-β-erythroidine (DhβE), and hexamethonium, when applied alone, enhanced the release of [^{3}H]5-HT (n = 2–60 depending on concentration) (Fig. 2), whereas methyllicaconitine (MLA, 40 nM; Fig. 2) had no effect. The maximal response was observed after application of mecamylamine 30 μM (30.8 ± 2.2% increase, n = 60). Because mecamylamine is known to also inhibit *N*-methyl-D-aspartate receptors, we tested whether another *N*-methyl-D-aspartate receptor antagonist had a similar effect; 2-amino-5-phosphoentanoic acid (100 μM) failed to modulate the basal release of [^{3}H]5-HT (n = 2, result not shown). The mecamylamine-elicited response was unchanged by the incubation of the slices with tetrodotoxin (3 μM, result not shown).

The fact that nicotinic antagonists have an effect on the basal release of [^{3}H]5-HT suggests that the serotoninergic transmission is tonically controlled, in the basal state, by endogenous activation of nAChRs.

Effects of nAChR Antagonists Preincubation on Nicotine-Elicited Effect. When nicotine was administered after preincubation with mecamylamine (30 μM), DhβE (50 μM), or hexamethonium (200 μM), nicotine-elicited increase in 5-HT release was significantly antagonized ($P = 0.003$, $n = 4$; $P = 0.013$, $n = 5$; and $P = 0.0014$, $n = 8$, respectively) (Fig. 3). In contrast, MLA, at a concentration (40 nM) considered to specifically block α7-subunit containing nAChRs (or α7*-nAChRs, following recommended nomenclature) (13), potentiated the response to nicotine (n = 6) (Fig. 3).

Overall, these experiments suggest the existence of three main populations of nAChRs controlling, directly or indirectly, serotonine release in the spinal cord. The first, activated by agonists application, has an excitatory outcome; the second, tonically activated by endogenous ACh and inhibited by nicotinic antagonists application, has an inhibitory outcome. Finally, the data on MLA suggest that (*i*) the two receptor populations discussed above are composed of MLA-insensitive nAChRs, and (*ii*) there is a third nAChR population, possibly composed of α7*-nAChRs, which is not tonically activated and has an inhibitory effect on 5-HT release.

Effects of the Modulation of ACh Levels on Serotonin Release. To further support the hypothesis of endogenous ACh acting

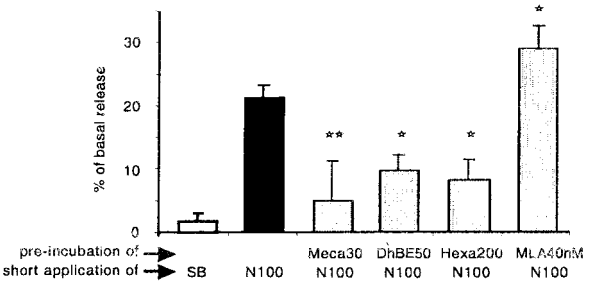

Fig. 3. Effect of preincubation with various nicotinic antagonists on the nicotine-induced increase in [^{3}H]5-HT efflux from mouse spinal cord slices. Concentrations are given in μM unless otherwise specified. SB; Meca, mecamylamine; DhβE; Hexa, hexamethonium, MLA; and N100, nicotine 100 μM. Statistical analysis according to one-way ANOVA followed by Dunnett's test: *, $P < 0.05$ and **, $P < 0.01$ vs. the response induced by 100 μM nicotine without antagonist preincubation.

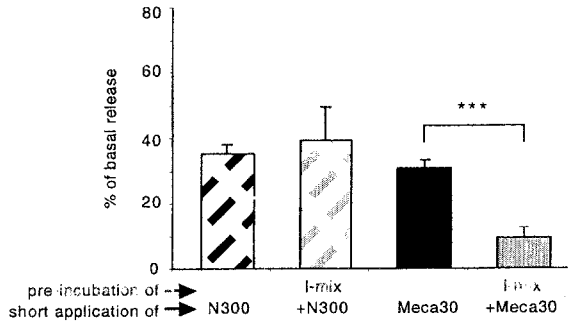

Fig. 5. Effect of inhibitory transmission blockage on 300 μM nicotine (N300)- or 30 μM mecamylamine (Meca30)-induced increase in [^{3}H]5-HT efflux from mouse spinal cord slices. The I-mix is composed of strychnine (1 μM), saclofen (50 μM), and bicuculline (40 μM). Statistical analysis according to one-way ANOVA followed by Dunnett's test: ***, $P < 0.001$ vs. drug effect in the presence of I-mix.

through nAChRs to modulate serotonin release, we modulated ACh levels by either inhibiting or increasing its breakdown. An AChE inhibitor, neostigmine (10 μM), caused a 23% increase in [^{3}H]5-HT release ($\pm$1.4%, $n = 6$) (Fig. 4), suggesting that cholinergic activation of serotoninergic terminals is not maximal. The effects of neostigmine were insensitive to the muscarinic receptor inhibitor atropine (1 μM, $n = 5$) (Fig. 4). These data provide further support to the notion that there exists a population of nAChRs whose activation leads to a potentiation of 5-HT transmission.

On the other hand, the application of AChE (from 10 ng to 30 μg/ml) enhanced the release of [^{3}H]5-HT ($n = 2$–4) (Fig. 4). This result confirms that a population of nAChRs, which inhibits 5-HT transmission, is tonically activated by endogenous ACh.

Block of the Inhibitory Transmission. Given the excitatory nature of nicotinic transmission, the simplest explanation for the results obtained with nAChR agonists and antagonists as well as modulators of ACh metabolism is to assume (*i*) the existence of a population of nAChRs, which, directly or through an excitatory local circuit, activate 5-HT release, and (*ii*) a second nAChR population, which directly or through an excitatory local circuit, inhibits 5-HT transmission. To test this hypothesis, we decided to block the major inhibitory synaptic transmission in the spinal cord by incubating the slices with strychnine (1 μM glycine receptor antagonist), saclofen [50 μM γ-aminobutyric acid type B ($GABA_B$) receptor antagonist], and bicuculline (40 μM $GABA_A$ receptor antagonist).

This solution, that we refer to as I-mix, caused by its own a 59.3% ($\pm$ 15%, $n = 6$, result not shown) increase in the basal release of [^{3}H]5-HT, indicating the existence of a basal inhibitory tone on 5-HT transmission in the spinal cord. Therefore, in experiments in which the slices were incubated with the I-mix, the base line of [^{3}H]5-HT release was higher than in control experiments.

After incubation with the I-mix, nicotine-elicited increase in [^{3}H]5-HT release was not significantly different from the response obtained in the absence of the I-mix ($P = 0.11$ $n = 7$, Fig. 5), suggesting that the observed effects of nicotine on 5-HT release are not mediated by inhibitory neurons. On the contrary, mecamylamine-elicited increase in [^{3}H]5-HT release was markedly ($\approx$70%) reduced by the addition of the I-mix ($P = 2.7 \cdot 10^{-6}$, $n = 29$) (Fig. 5), suggesting that the tonically activated nAChRs are mainly located on neurons which inhibit 5-HT transmission.

Effects of Nicotine on Serotonin Release in α4 and β2 KO Mice. Similar experiments were conducted in α4 and β2 KO mice to evaluate possible contributions of these two subunits to nicotine-elicited effects. In both strains, nicotine caused a dose-dependent increase that was not significantly different from that obtained in the corresponding control mice ($n = 3$–7, depending on concentrations) (Fig. 6). These data support the notion that neither subunit is involved in the nicotinic control of 5-HT release in the spinal cord.

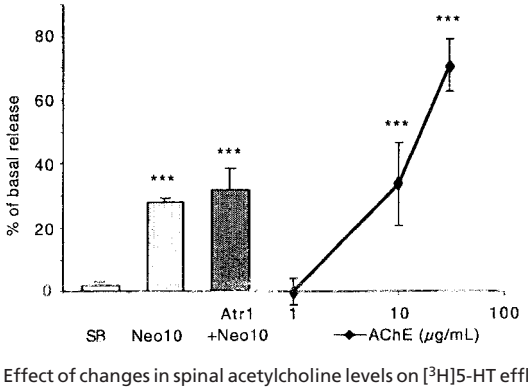

Fig. 4. Effect of changes in spinal acetylcholine levels on [^{3}H]5-HT efflux. The effect of the AChE inhibitor neostigmine (Neo, 10 μM) with or without preincubation of a muscarinic antagonist [atropine (Atr) 1 μM] and the effect of AChE were compared to the control, SB, response. Statistical analysis according to one-way ANOVA followed by Dunnett's test for treatment vs. control comparisons: ***, $P < 0.001$.

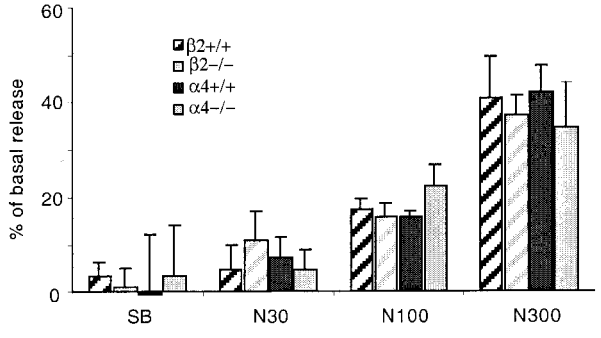

Fig. 6. Effects of 30 μM, 100 μM, or 300 μM nicotine (N30, N100, or N300, respectively) on [^{3}H]5-HT efflux in spinal cord slices of α4 or β2 KO mice. No significant difference between nicotine response in KO vs. wild-type mice was observed.

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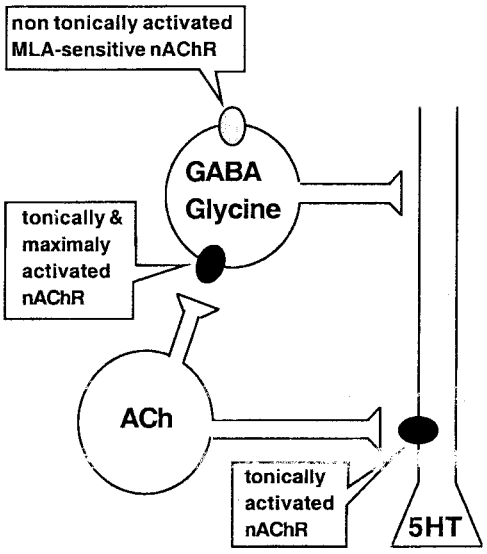

Fig. 7. Minimal anatomical circuit for acetylcholine (ACh) and nicotine regulation of serotonin (5-HT) release in the mouse spinal cord.

Discussion

This study demonstrates the existence of multiple populations of functional nAChRs in the spinal cord and shows that their activation modulates 5-HT transmission. These receptors are expressed by different neuronal populations within the spinal cord, and some of them are endogenously activated by ACh.

Diversity of Spinal nAChRs Modulating [^{3}H]5-HT Release. Present data give evidence to the existence of at least three different populations of nAChRs in the spinal cord which affect the release of 5-HT.

First, in control conditions, nicotine enhances [^{3}H]5-HT release (Fig. 1). The simplest explanation for this result is that this population of nAChRs is directly expressed on serotoninergic terminals, although nAChRs on excitatory interneurons could have the same effect. These receptors are (*i*) insensitive to MLA (40 nM), (*ii*) are not maximally activated by endogenous ACh as they can still respond to nicotine, and (*iii*) may be subjected to a basal cholinergic tone, because neostigmine treatment enhances 5-HT release.

Second, mecamylamine (10–30 μM), DhβE (50–200 μM), and hexamethonium (200–500 μM) but not MLA (40 nM) enhanced [^{3}H]5-HT release (Fig. 3). This result implies the presence of a population of tonically activated nAChR, which are present on inhibitory neurons (or excitatory neurons impinging on inhibitory neurons) (Fig. 7). In the spinal cord, the main inhibitory transmitters are GABA and glycine. It has even been demonstrated that 64% of GABAergic interneurons also express glycine (14), and there is evidence that both transmitters are liberated at some synapses (15). We therefore decided to inhibit these transmissions to evaluate the contribution of nAChRs present on other neurons. In these conditions, the effect of mecamylamine was markedly reduced (Fig. 6), suggesting that the majority of endogenously activated nAChRs are present on GABAergic and/or glycinergic neurons (or excitatory neurons affecting the inhibitory neurons). These receptors are (*i*) insensitive to MLA, (*ii*) tonically (because both nicotinic antagonists and AChE cause an increase in 5-HT release), and (*iii*) maximally activated by endogenous ACh. This last deduction derives from the finding that the effect of nicotine was not significantly different in the presence or in the absence of I-mix, a mixture of glycine and GABA receptor antagonists (Fig. 6). Therefore, nicotine has no effect on the tonically activated nAChRs affecting inhibitory interneurons, which suggests that these receptors are already maximally activated.

Lastly, MLA (40 nM) alone had no effect on 5-HT release but could potentiate nicotine excitatory action (Fig. 2). This indicates the existence of a third population of nAChRs, which is expressed by inhibitory neurons (or excitatory neurons impinging on them). These receptors are (*i*) MLA sensitive (which suggests an α7* composition) and (*ii*) not tonically activated by endogenous ACh.

Subunit Composition of nAChRs Modulating 5-HT Release in the Spinal Cord. Previous evidence for the expression of nAChR subunit mRNA or protein as well as binding for nicotinic ligands in the spinal cord (6, 7, 16–18) (A. de Kerchove d'Exaerde, personal communication) indicates that most, if not all, subunits are expressed by spinal neurons or by afferents from brain nuclei or dorsal root ganglia. However, detailed information about their intraspinal distribution is largely lacking.

The present data suggest that the nAChRs, which increase 5-HT release, do not contain either α4 or β2 (see data on KO mice) or α7 subunits (see lack of antagonism by MLA). Therefore, they do not correspond to the two major nAChR isoforms present in the rodent brain (19–21), but to minor populations of (non-α4β2*)-nAChRs (12). This result is consistent with the electrophysiological data obtained in α4 and β2 KO mice (12), which revealed the presence of presynaptic (non-α4β2*) nAChRs in the dorsal horn of the spinal cord. Overall, it seems likely that nAChRs involved in the positive control of 5-HT release in the spinal cord have a unique composition and may even contain not yet cloned subunits. These data agree with a previous study on nicotinic binding in membranes from spinal cord, which led Khan and coworkers (22) to consider spinal nAChRs as forming a class of nAChRs different from those expressed in the brain and ganglia.

At least two nAChR populations are expressed on GABA/glycine neurons; one is sensitive to MLA (40 nM), whereas the other is insensitive to MLA but antagonized by mecamylamine, DhβE, and hexamethonium. According to current knowledge, the former population may be composed of α7*-nAChRs, although recent data show that other subunit combinations (without α7) may be sensitive to nM concentrations of MLA (23). The presence of α7*-nAChR in the spinal cord is in agreement with previous reports of α-bungarotoxin binding in this region (6, 7, 16–18). The subunit composition of the latter population is still difficult to infer.

Location of nAChRs Modulating 5-HT Release in the Spinal Cord. The major population of cholinergic cells of the spinal cord are the motorneurons of the ventral horn. In addition, several experiments have shown the presence of a supraspinal cholinergic projection and cholinergic interneurons.

Cholinergic neurons projecting to the spinal cord are found in the medial medullary reticular formation, lateral vestibular nucleus, dorsolateral pontine tegmentum, and the red nucleus (24). Most choline acetyltransferase (ChAT) immunoreactivity throughout the spinal cord corresponds to axons and nerve terminals (25), but occasional cholinergic interneurons are found in lamina III and more frequently in deeper laminae (25, 26). Electron microscopy studies showed synaptic triadic arrangements, in which a single ChAT-immunolabeled profile is presynaptic to both a central varicosity of a sensory afferent, and a dendrite postsynaptic to the central bouton (27).

Although it is known that spinal cord is rich in GABA/glycine-containing interneurons and 5-HT nerve terminals (28), the relationships between these cell structures and cholinergic structures are unknown. The simplest explanation for the functional data presented above requires two cholinergic inputs, one on a

GABAergic structure, which in turn influences a 5-HT terminal, and another directly on a 5-HT terminal. An anatomically plausible model for this circuit comprises a cholinergic innervation of a GABAergic interneuron innervating a 5-HT terminal and a direct cholinergic innervation of a 5-HT terminal (see Fig. 7). A functionally equivalent circuit can be implemented by a local circuit, comprising cholinergic, GABAergic, and serotoninergic terminals. The existence of such a microcircuit would be supported by the evidence that tetrodotoxin (i.e., blockade of axonal impulse flow) does not modify the outcome of the release experiments. In these microcircuits, ACh may not act as a classical synaptic transmitter but rather as a volume transmission signal, as already postulated in many central regions (29, 30). More refined anatomical investigations could help us distinguish between these two hypothesis.

Functional Aspects of Nicotinic Modulation of Serotoninergic Transmission. Serotonin descending pathways to the spinal cord are classically thought to represent a main modulator of pain transmission, with complex state-dependent effects (31–33). A major relevance of cholinergic nicotinic involvement in the spinal cord for analgesia also has recently received strong experimental support (12). Regulation of pain sensation is therefore a possible functional outcome of the spinal nicotinic/serotoninergic circuit characterized in this paper. The experimental evidence on this circuit is still too limited to make strong inferences about its functional effects. However, besides nicotine agonist-elicited analgesia, an apparently paradoxical evidence for analgesia is induced by blockage of cholinergic system (see references in ref. 30). The existence of a population of nAChRs that exerts a tonic negative modulation on [^{3}H]5-HT release through the activation of GABAergic and/or glycinergic interneurons thus represents a possible neuronal substrate for the latter pharmacological effect.

In conclusion, we have demonstrated the existence of multiple nAChR populations exerting tonic or phasic control on 5-HT transmission in the spinal cord. These receptors may be a major target for nicotine effects on antinociception. In addition, the presence of a tonic nicotinic modulation of 5-HT release indicates that cholinergic nicotinic transmission plays a role in the physiological regulation of descending serotoninergic pathways to the spinal cord.

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