

# Fast synaptic transmission mediated by $\alpha$ -bungarotoxin-sensitive nicotinic acetylcholine receptors in lamina X neurones of neonatal rat spinal cord

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Using patch clamp recordings on neonatal rat spinal cord slices, we have looked for the presence of  $\alpha$ -bungarotoxin-sensitive nicotinic ACh receptors (nAChRs) on sympathetic preganglionic neurones (SPNs) surrounding the central canal of the spinal cord (lamina X) and examined whether they were implicated in a fast cholinergic synaptic transmission. SPNs were identified either by their morphology using biocytin in the recording electrode and/or by antidromic stimulation of the ventral rootlets. The selective  $\alpha 7$ -containing nAChR ( $\alpha 7^*$ nAChR) agonist choline (10 mM) induced a fast, rapidly desensitizing inward current, which was fully blocked by  $\alpha$ -bungarotoxin ( $\alpha$ -BgT; 50 nM) and strychnine (1  $\mu$ M), two antagonists of  $\alpha 7^*$ nAChRs. The  $I$ - $V$  relationship of the choline-induced current showed a strong inward-going rectification. Electrically evoked excitatory postsynaptic currents (eEPSCs) could be recorded. At  $-60$  mV, eEPSCs peaked at  $-26.2$  pA and decayed monoexponentially with a mean time constant of 8.5 ms. The current-voltage relationship for eEPSCs exhibited a strong inward rectification and a reversal potential close to 0 mV, compatible with a non-selective cationic current. The appearance of eEPSCs was entirely suppressed by the application of 100  $\mu$ M ACh or nicotine. Choline (10 mM) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP; 100  $\mu$ M) both reduced the amplitude of eEPSCs, whereas cytisine (100  $\mu$ M) had no effect. Strychnine (1  $\mu$ M) and  $\alpha$ -BgT (50 nM) both suppressed the eEPSCs. Blocking the P2X purinergic and 5-HT<sub>3</sub> receptors had no effect on eEPSCs. DMPP induced four types of current, which differed in their onset and desensitization rate. The most frequently encountered responses were insensitive to the action of strychnine and  $\alpha$ -BgT, and were reproduced by ACh and nicotine but not by cytisine. We conclude that SPNs of the lamina X express several classes of nAChRs and in particular  $\alpha$ -BgT-sensitive nAChRs. This is the first demonstration in a mammalian spinal cord preparation of a fast cholinergic neurotransmission in which  $\alpha$ -BgT-sensitive nicotinic receptors are involved.

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Neuronal nicotinic acetylcholine receptors (nAChRs) are transmembrane oligomeric proteins composed of five subunits (Galzi & Changeux, 1995). Molecular cloning of mammalian neuronal nAChRs from different species have revealed two families of subunits, the  $\alpha$  subtype which contains 7 members ( $\alpha 2$ – $7$ ,  $\alpha 9$  and  $\alpha 10$ ) and the  $\beta$  subtype containing 3 members ( $\beta 2$ – $4$ ) (Sargent, 1993; Elgoyhen *et al.* 2001). Among these subunits, the  $\alpha 7$  subunit differs from the others because it forms a nicotinic receptor with the highest calcium permeability (Bertrand *et al.* 1993; Séguéla *et al.* 1993) and the highest rate of desensitization. From a pharmacological point of view this receptor has a unique profile, namely: (a) it is susceptible to blockade by several neurotoxins ( $\alpha$ -bungarotoxin (Couturier *et al.* 1990), methyllycaconitine (Drasdo *et al.* 1992) and strychnine (Matsubayashi *et al.* 1998)); and (b) the receptor may be activated by choline, the precursor and metabolite

of ACh (Papke *et al.* 1996; Mike *et al.* 2000). As well as possessing these pharmacological differences, nAChRs also differ in their function. It has been proposed that in the central nervous system nAChRs are preferentially located presynaptically and modulate synaptic transmission rather than mediate it (Wonnacott, 1997; MacDermott *et al.* 1999). Indeed, neurotransmission mediated by nAChRs has only been demonstrated in a few mammalian preparations including: rat nucleus ambiguus (Zhang *et al.* 1993), rat and mouse hippocampal CA1 interneurons (Frazier *et al.* 1998; Alkondon *et al.* 1998), rat CA1 pyramidal neurons (Hefft *et al.* 1999) and ferret visual cortex (Roerig *et al.* 1997). So far, the participation of  $\alpha 7^*$ nAChRs ( $\alpha 7$ -containing nAChRs) in a fast cholinergic neurotransmission has been demonstrated in the hippocampus (Orr-Urtreger *et al.* 1997; Frazier *et al.* 1998) and in the supraoptic nucleus (Hatton & Yang, 2002).

In the spinal cord, with the exception of the involvement of nicotinic receptors in the synaptic activation of Renshaw cells by motor axon collaterals (Eccles *et al.* 1954), a fast cholinergic transmission has not yet been demonstrated. We have shown that two populations of neurones of the lamina X express postsynaptic nAChRs; they are located dorsally to the central canal in the ventral half of the dorsal commissure and in the central autonomic area (Bordey *et al.* 1996a, b). Numerous cholinergic neurones are well positioned to synapse onto lamina X cells; these include sympathetic preganglionic neurones (SPN) and cholinergic partition cells (Barber *et al.* 1984; Schäfer *et al.* 1998). Choline acetyltransferase-immunoreactive boutons were indeed found in lamina X (Borges & Iversen, 1986) but a cholinergic transmission in this area has not been demonstrated so far.

This study demonstrates the existence of a cholinergic transmission within the autonomic nucleus of the lamina X. Firstly, we provide pharmacological evidence for the presence of functional  $\alpha$ -BgT-sensitive nAChRs in the central grey of the spinal cord and secondly, we show their involvement in a fast synaptic cholinergic transmission. This is the first demonstration of a fast nicotinic transmission mediated by  $\alpha$ -BgT-sensitive nAChRs in mammalian spinal cord.

## METHODS

### Slice preparation

Neonate Wistar rats (Dépré SARL, France; postnatal day (P)2–7) were decapitated under deep ether anaesthesia. The thoracolumbar section of the spinal cord was removed and transferred into a high-osmolarity, calcium-free, sucrose-based solution bubbled with 95% O<sub>2</sub>–5% CO<sub>2</sub> and maintained at 3 °C (see Richerson & Messer, 1995). This solution contained (mM): sucrose 248; NaH<sub>2</sub>PO<sub>4</sub> 1.25; KCl 2; NaHCO<sub>3</sub> 26; glucose 11.1; NaCl 100; MgSO<sub>4</sub> 2; kynurenic acid 1, pH 7.3. Transverse slices with a thickness of 300–400  $\mu$ m were prepared using a vibratome (Pelco International, USA). Slices were transferred to a storage chamber containing an artificial cerebrospinal fluid (aCSF) containing (mM): NaCl 126; NaH<sub>2</sub>PO<sub>4</sub> 1.25; KCl 2.5; CaCl<sub>2</sub> 2; MgCl<sub>2</sub> 2; glucose 11.1; NaHCO<sub>3</sub> 26; kynurenic acid 2, pH 7.3. After a recovery period of at least one hour, a slice was transferred into the recording chamber and stabilized by two nylon threads glued onto a U-shaped, flattened platinum wire.

### Recordings

The slices were placed on an upright microscope (Axioscop, Carl Zeiss France, S. A. S.) mounted on a Gibraltar X-Y table (Carl Zeiss). Slices were observed through a 40 $\times$  water immersion objective using an infrared sensitive camera (T. I. L. L. Photonics GmbH, Germany). Cells were chosen visually in the lateral part of the lamina X (see Bordey *et al.* 1996b).

Patch clamp recordings were performed in the whole cell configuration. Patch pipettes were filled with a standard internal solution containing (mM): KCl 130; NaCl 10; Hepes 10; MgCl<sub>2</sub> 1.2; EGTA 5; CaCl<sub>2</sub> 2.5; biocytin 10, pH 7.2. In electrical experiments, the equilibrium potential for chloride was shifted from 2 to

–60 mV by substituting 130 mM potassium gluconate for 130 mM KCl and lowering the [NaCl] from 10 to 5 mM. Pipettes had resistances of between 2.5 and 4 M $\Omega$ . Cells with series resistances of up to 20 M $\Omega$  were discarded. Series resistances were not compensated. Voltage clamp was achieved using an Axopatch 200 B amplifier (Axon Instruments, Inc., USA). Currents were stored on a DAT recorder at 10 kHz (DTR–1201; Biologic) and digitized using a data acquisition board (Digidata 2000; Axon Instruments) operated by pCLAMP 6.0 software (Axon Instruments). Analysis of whole cell currents and electrically evoked currents was performed using Clampfit software. The decay phase of electrically evoked EPSC was best fit by a single exponential function:

$$y = A \exp(-t/\tau) + \text{baseline},$$

where  $\tau$  (time constant) is in ms and  $y$  (current amplitude),  $A$  (peak current amplitude) and baseline (baseline amplitude) are in pA.

Focal bipolar stimulation was achieved using an electrode pulled from a glass theta tube. This electrode was filled with the external solution, had a low resistance (500 k $\Omega$  to 1 M $\Omega$ ) and was positioned 100  $\mu$ m away from the cell in any direction. Stimuli were generated by an isolated stimulation unit (custom made).

### Identification of the recorded cells

It was possible to identify SPNs using two criteria, morphology and antidromic stimulation. Cells were loaded with biocytin (10 mM) using the patch pipette. Only one cell was recorded per slice. After recording, the slices were stored for at least 3 days in Zamboni's fixative (formaldehyde 1.85%; Na<sub>2</sub>HPO<sub>4</sub> 61 mM; NaH<sub>2</sub>PO<sub>4</sub> 39 mM; picric acid 4%). They were rinsed with PBS solution, containing (mM): NaCl 15; Na<sub>2</sub>HPO<sub>4</sub> 1.3; NaH<sub>2</sub>PO<sub>4</sub> 0.25, and soaked overnight at room temperature with ExtrAvidin-FITC conjugate (Sigma-Aldrich Chimie, S. a. r. l., France) diluted 1:400 in a solution containing: glycerol 50%; triton X-100 0.25%; NaCl 7.5 mM; Na<sub>2</sub>HPO<sub>4</sub> 0.65 mM; NaH<sub>2</sub>PO<sub>4</sub> 0.125 mM; thimerosal 0.25 mM. The slices were rinsed three times with PBS, cover-slipped with an antifading medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and stored at 4 °C. They were observed under an epi-illumination microscope (DM RD; Leica Microsystems, France) and digitized using a CoolSNAP camera (Roper Scientific SARL, France). An offline reconstruction of the recorded neurone was made from several photographs using Photoshop software (Adobe Systems).

Some slices still possessed their ventral rootlets after cutting the spinal cord. In these cases, it was possible to evoke antidromic spikes after stimulation of the ipsilateral ventral motor rootlet. The stimulation electrode consisted of a suction electrode filled with extracellular medium. The reference electrode consisted of a silver chloride coated-wire wound round the suction electrode. The antidromic evoked action potentials were all-or-nothing events and occurred with a constant latency (see Fig. 2).

### Solutions

Slices were continuously superfused at a rate of 3–4 ml min<sup>-1</sup> with oxygenated and bubbled aCSF at room temperature (20 °C), supplemented with 0.5  $\mu$ M TTX. Bicuculline (10  $\mu$ M) and kynurenic acid (2 mM) were added to eliminate the GABA-ergic and the glutamatergic components, respectively. For electrical stimulation experiments, TTX was omitted from the superfusion solution and CNQX (10  $\mu$ M) was added to the bath. The volume of the bathing solution was 2 ml.

For pharmacological experiments, we pressure-applied different agonists and antagonists of nAChRs. The pressure ejection pipettes were standard patch pipettes. At least two applications of DMPP, separated by a 5 min interval, were performed to evaluate the run-down of the DMPP response. If the run-down of the control response was significant (current amplitude induced by the second application < 50% of the amplitude induced by the first application), the cell was discarded. If not, a second agonist was applied and its effect compared to the last control, DMPP-induced current (see Fig. 7). Nicotinic antagonists were applied either by bath perfusion or by a pressure pipette. When using a pressure pipette, one pipette was filled with the agonist and the other filled with the agonist together with the antagonist to be tested. Thus, an antagonist could be coapplied during the application of an agonist (see example in Fig. 6A). When two pressure pipettes were used, they were located equidistant from the recorded cell.

### Drugs

$\alpha$ -BgT, bicuculline methiodide, choline, dihydro- $\beta$ -erythroidine (DH $\beta$ E), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), phenylbenzene-*o*-phosphono- $\alpha$ -amino acid (PMBA), (*d*)-tubocurarine (*d*-TC), kynurenic acid, nicotine, atropine and strychnine were obtained from Sigma-Aldrich Chemie. TTX was from Latoxan (France). CNQX and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) were from Tocris Cookson Ltd. (1,2,3,9-Tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)-methyl]-4*H*-carbazol-4-one) ondansetron was from Servier (France).

Results are given as means  $\pm$  S.E.M., with (*n*) representing the number of observations. Statistical differences between results were determined using one-way ANOVA, setting the confidence

interval at 0.05. Statistical differences between results of electrical stimulation were determined using the Welsh test, setting the confidence interval at 0.05.

## RESULTS

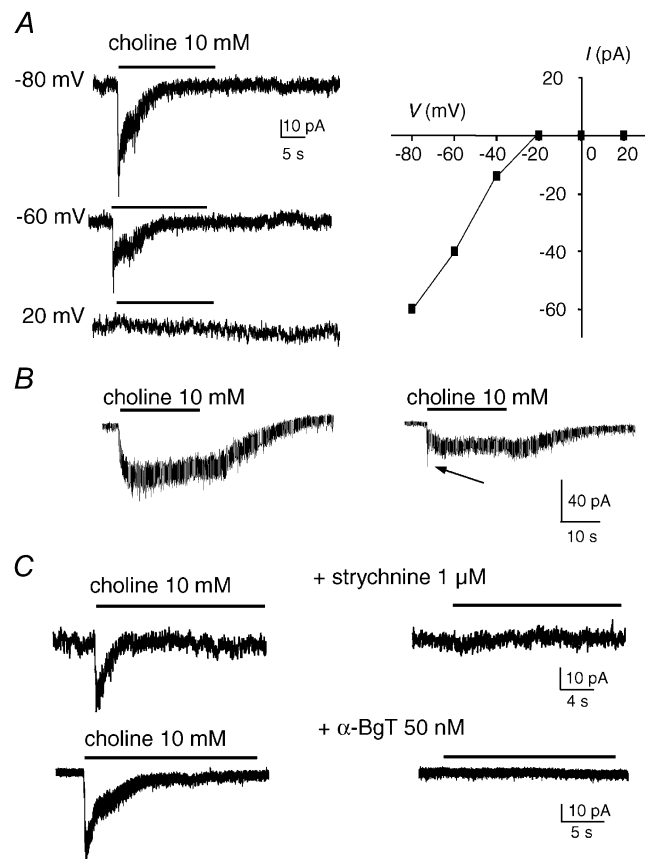
In the first series of experiments we applied choline, a full and selective agonist of  $\alpha 7$ -nAChRs (Papke *et al.* 1996; Alkondon *et al.* 1999), to lamina X neurones and evaluated the potency of the selective  $\alpha 7^*$ nAChR antagonists  $\alpha$ -BgT and strychnine on choline-induced currents. Cells were recorded in the continuous presence of 0.5  $\mu$ M TTX, 10  $\mu$ M bicuculline and 2 mM kynurenic acid.

### Effect of choline in lamina X neurones

At a concentration of 1 mM, choline induced no detectable response (*n* = 6). In 42% of the cells tested (89/211 cells), at -60 mV 10 mM choline induced a fast inward current that desensitized entirely during the time of application (Fig. 1A). We referred to this response as the fast choline-induced response. The mean amplitude of the peak of the fast choline-induced current was  $-58 \pm 7.3$  pA (mean  $\pm$  S.E.M., *n* = 38). As illustrated in Fig. 1A, the reversal potential of the fast choline-gated current was close to 0 mV, a value that was compatible with the activation of a non-selective cation conductance. The *I-V* relationship of the choline-induced current showed a strong inward-going rectification, as no outward current elicited by choline was observed. In rarer cases (eight cells), 10 mM

### Figure 1. Choline-induced currents in neurones surrounding the central canal

A left, current traces evoked by 10 mM of choline in a neurone held at -80, -60 and 20 mV. Right, a graph showing the plot of the peak amplitude of choline-induced current (■) versus the membrane potential. The *I-V* relationship shows a strong inward-going rectification as no outward current was recorded. B, current traces illustrating slow-decaying choline-induced currents. In the cell illustrated in the right panel, the slow choline-induced current was preceded by a fast inactivating current, as indicated by the arrow. C, pharmacology of the fast choline-induced current. Upper traces, 5 min bath application of 1  $\mu$ M strychnine totally blocked the choline-induced current. Lower traces, pressure application of 10 mM choline evoked an inward current (left trace). Bath application of 50 nM  $\alpha$ -bungarotoxin ( $\alpha$ -BgT) inhibited the choline-induced current after 20 min application (right trace). All recordings (A, B and C) were made in the presence of 0.5  $\mu$ M TTX, 2 mM kynurenic acid and 10  $\mu$ M bicuculline. Cells in B and C were held at -60 mV.

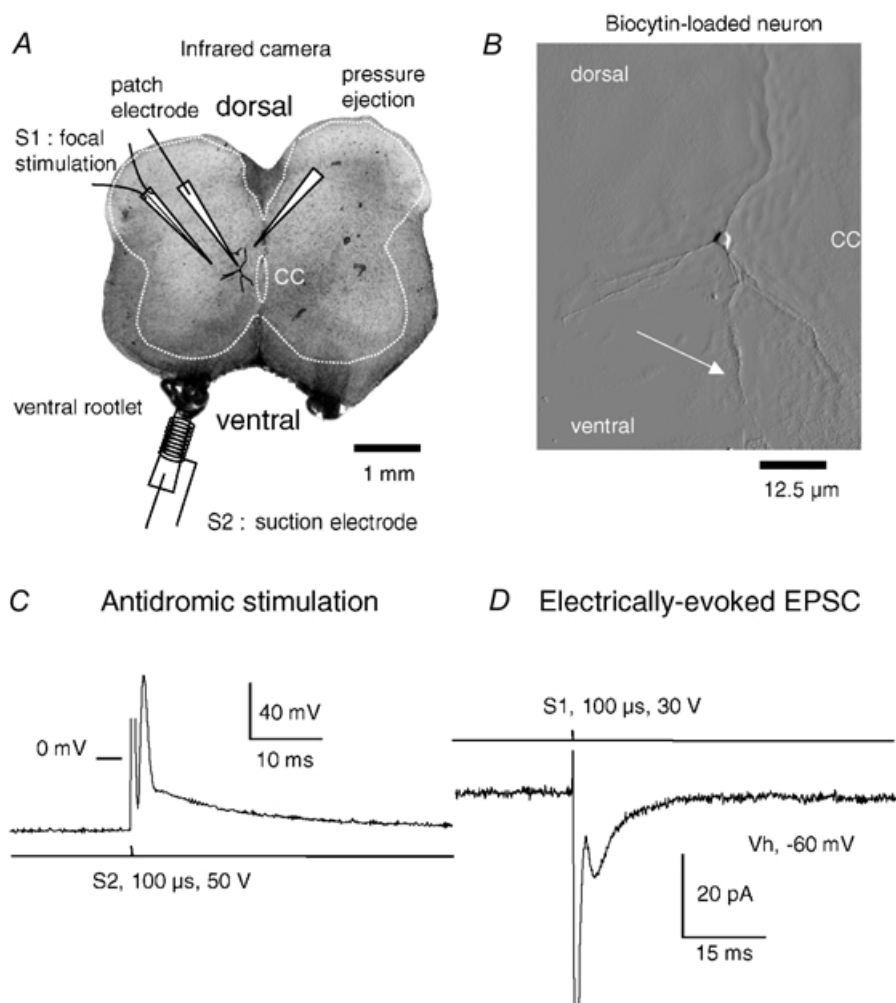


choline induced a slowly decaying inward current. This steady state current had a mean amplitude of  $-37.5 \pm 7.5$  pA at  $-60$  mV and was recorded in isolation in four cells (Fig. 1B, left trace) but in a further four cells was preceded by a fast desensitizing current (Fig. 1B, right trace).

### Effects of selective $\alpha 7^*$ nAChR antagonists on choline-induced fast currents

Choline (10 mM) was pressure-applied every 5 min. In cells showing no tachyphylaxis of response, we bath applied selective  $\alpha 7^*$  nAChR antagonists and evaluated their

ability to inhibit choline-evoked currents. Strychnine, an antagonist of glycinergic receptors, is also known to block the  $\alpha 7$  nicotinic receptors. At  $1 \mu\text{M}$  concentration, strychnine entirely blocked the inward current induced by 10 mM choline ( $n = 5$ ; Fig. 1C, upper traces). The blockade was fast (5 min for a full block) and was apparently irreversible since no recovery was observed after 30 min wash out of the strychnine. In five cells tested, 50 nM  $\alpha$ -BgT totally blocked the choline-induced current.  $\alpha$ -BgT was a slow acting drug since its effect started to be visible after 15 min of application. A full block of the current was



### Figure 2. Neurone identified as a preganglionic sympathetic neurone displays fast eEPSC

A, experimental set-up. Photograph of an upper lumbar (L1–L3) transverse rat spinal cord section showing the different locations of patch electrode, electrical stimulation (S1), pressure pipette and suction stimulating electrode (S2). The recorded neurones were located laterally to the central canal (CC). The dotted white line indicates the limit of the grey matter and the central canal. B, details of the biocytin-loaded neurone revealed with FITC. Typically, neurites extend from the cell body in three directions: (a) towards the intermediate grey; (b) towards the dorsal horn; and (c) towards the ventral rootlet (indicated by the white arrow) and the ventral commissure. C, under current clamp mode, the stimulation of the ventral root whilst (bottom trace,  $100 \mu\text{s}$ ,  $-50\text{V}$ ) using the suction electrode (S2) induced an antidromic spike in the recorded neurone. The voltage trace (top) corresponds to the average of 20 action potentials, which occurred with a constant latency. The bar indicates the 0 mV level. D, under voltage clamp at  $-60$  mV, electrical stimulation (top trace,  $100 \mu\text{s}$ ,  $-30\text{V}$ ) using a focal stimulation electrode (S1) induced an EPSC (bottom trace). Recordings were made in the presence of 2 mM kynurenic acid, 10  $\mu\text{M}$  CNQX and 10  $\mu\text{M}$  bicuculline.  $E_{\text{Cl}}$  was fixed at  $-60$  mV.

achieved after 20–30 min of application (Fig. 1C, lower traces). A partial reversal of the blocking effect of  $\alpha$ -BgT was sometimes observed after 30–40 min wash out of the toxin.

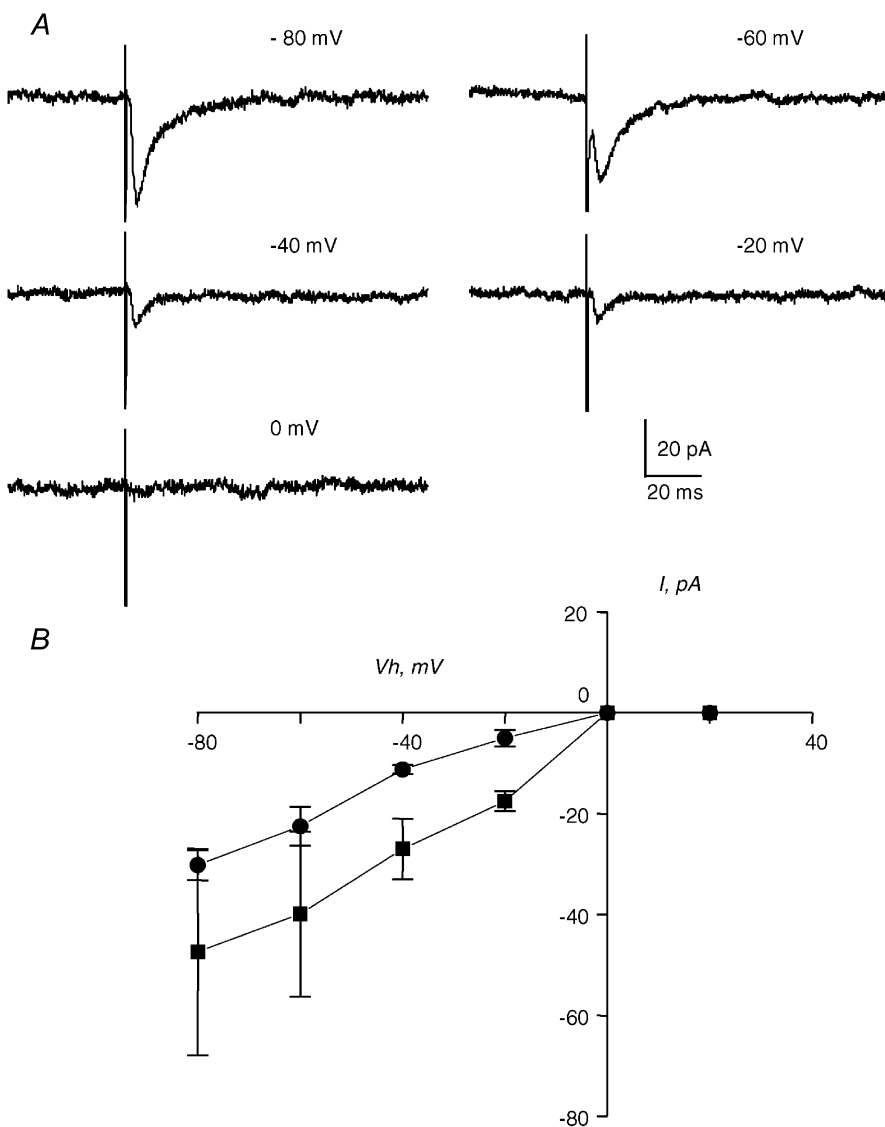
These results supported the idea that classical  $\alpha 7^*$ nAChRs underlie the fast current induced by choline. They also revealed that choline, more rarely, could also induce a non-desensitizing current, suggesting the existence of another class of nAChRs activated by choline. We tested the involvement of  $\alpha$ -BgT-sensitive nAChRs in fast transmission by omitting TTX from the perfusion medium and by using a stimulating electrode to evoke synaptic current.

### Characterization of electrically evoked nicotinic synaptic activity

In subsequent experiments, TTX was omitted from the perfusion medium. Experiments were performed using CNQX, but still in the presence of bicuculline and kynurenic acid. We set the equilibrium potential of chloride ions to  $-60$  mV and voltage clamped the cells at  $-60$  mV to

minimize the contribution of any chloride current to the evoked excitatory postsynaptic current (eEPSC). Under these conditions, an electrical stimulation using a bipolar stimulating electrode located laterally to the recorded cell evoked eEPSCs in 39% of the cells tested ( $n = 272$ ). Of 48 cells showing the typical morphology of SPNs (see Bordey *et al.* 1996b and Fig. 2), fast eEPSCs were evoked in 19 cells (39.6%). It was also possible in some slices with preserved rootlets to test the existence of antidromic spikes when stimulating the ipsilateral ventral root. It was possible to evoke an EPSC in 19 cells out of a total of 48 cells showing antidromic spikes.

These eEPSCs had a mean amplitude of  $-26.2 \pm 2.5$  pA ranging from  $-10$  to  $-130$  pA ( $n = 52$ ). They decayed rapidly and their decaying time course could be fitted by a monoexponential function with a mean time constant of  $8.5 \pm 3.0$  ms ( $n = 45$  cells). We carefully checked that the recorded currents were due to a synaptic transmission by looking at the effects of conditions known to block synaptic activities, e.g. application of low  $Ca^{2+}$ / high  $Mg^{2+}$



### Figure 3. $I$ - $V$ relationship of the eEPSCs

A, eEPSCs were evoked every 3 s (100  $\mu$ s, 20V) in a neurone held at holding potentials varying from  $-80$  to  $0$  mV (in 20 mV steps). Each current trace represents the average of 10 eEPSCs. Recordings were made with  $E_{Cl}$  fixed at 2 mV in the presence of 10  $\mu$ M bicuculline, 2 mM kynurenic acid, 10  $\mu$ M CNQX and 10  $\mu$ M PMBA. B, a graph showing the plot of the mean peak  $\pm$  S.E.M. of eEPSCs versus potential measured in five cells with  $E_{Cl}$  at 2 mV (●, ■), the mean peak eEPSCs  $\pm$  S.E.M. measured in four cells with  $E_{Cl}$  at  $-60$  mV. In both cases, the  $I$ - $V$  relationships show an inward rectification as no outward eEPSCs could be recorded and a reversal potential close to 0 mV.

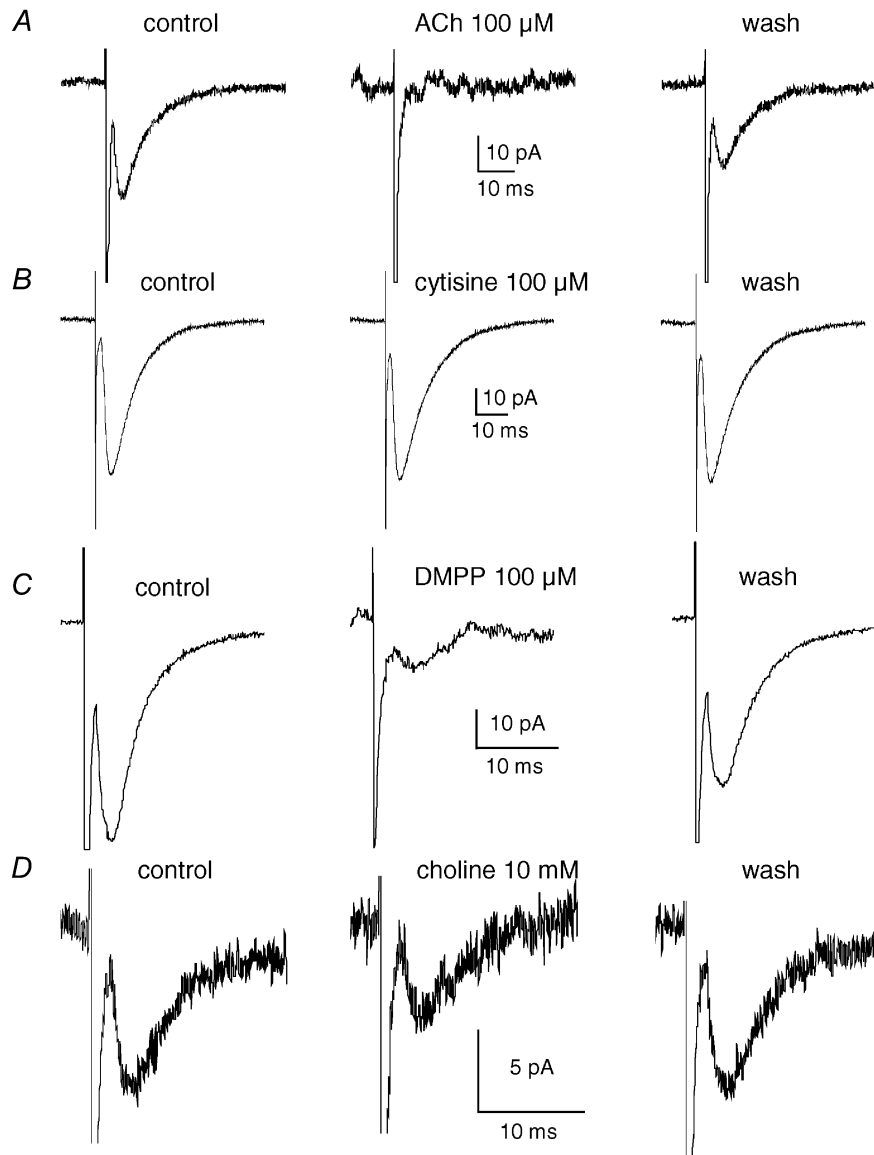
external solution and TTX. Both procedures suppressed the appearance of electrically evoked EPSCs (not shown).

This clearly established that motor sympathetic preganglionic neurones from the central autonomic area displayed fast eEPSCs. The ionic basis and pharmacological properties of these eEPSCs were next analysed.

### ***I*-*V* relationship of eEPSCs**

The *I*-*V* curve had to be constructed after blocking glycinergic transmission, as glycinergic chloride currents

may contaminate eEPSCs at membrane potentials different from the equilibrium potential for chloride ions. Strychnine, the classical selective glycinergic competitive receptor antagonist, or its derivatives, could not be used because they also act as competitive antagonists of  $\alpha 7^*$ nAChRs. Instead, we used PMBA (phenylbenzene- $\omega$ -phosphono- $\alpha$ -amino acid) which blocked glycinergic receptors in rat spinal cord (Saitoh *et al.* 1994) without affecting fast eEPSCs (not shown). In the presence of 10  $\mu$ M PMBA, in symmetrical chloride conditions, the *I*-*V* relationship of



**Figure 4. Cross-desensitization of eEPSCs by nicotinic receptor agonists**

A, focally evoked EPSCs were recorded in neurones held at  $-60$  mV in the presence of 2 mM kynurenic acid, 10  $\mu$ M CNQX and 10  $\mu$ M bicuculline. The holding potential was  $-60$  mV and  $E_{Cl}$  was  $-60$  mV. Stimuli (100  $\mu$ s, 20 V) were delivered every 3 s. nAChR agonists were applied for 20 s using a pressure ejection pipette. Control (left panels) and wash (right panels) traces correspond to the average of 40 events before and after the application of nAChR agonists. Traces during the agonist application (middle panels) are the average of 6 events. ACh (100  $\mu$ M; A) reversibly inhibited fast eEPSCs whilst cytisine (100  $\mu$ M) had no effect (B). DMPP (100  $\mu$ M; C) and choline 10 mM (D) reversibly reduced the current by 75% and 42%, respectively.

eEPSCs showed a typical inward rectification observed with nAChR-mediated currents with a reversal potential close to 0 mV (Fig. 3). Shifting the reversal potential for chloride from 2 to -60 mV using an internal medium with a low chloride concentration (see Methods) did not affect the reversal potential, which remained close to 0 mV (Fig. 3). This confirmed that eEPSCs were carried by cations.

### Pharmacology of eEPSCs

Nicotinic agonists were applied in order to induce an inward current and EPSCs were evoked during and immediately after the application of these agonists before recovery of the nAChRs from desensitization. Under these conditions, 100  $\mu\text{M}$  ACh (Fig. 4A) and 100  $\mu\text{M}$  nicotine (not shown) completely blocked the eEPSCs in a reversible manner ( $n = 4$  for each agonist), whereas 100  $\mu\text{M}$  DMPP and choline reduced the amplitude by  $69 \pm 12\%$  ( $n = 4$ ) and  $63 \pm 29\%$  ( $n = 12$ ), respectively (Fig. 4C and D). Cytisine (100  $\mu\text{M}$ ) had no effect on eEPSCs (Fig. 4B). These cross-desensitization experiments strongly suggested the involvement of nAChRs in the eEPSCs. This hypothesis was confirmed by testing nAChR antagonists.

EPSCs were evoked every 3 s in cells held at -60 mV and nAChR antagonists were bath applied. Bath application of 30  $\mu\text{M}$  *d*-TC ( $n = 9$  cells; Fig. 5A), 1  $\mu\text{M}$  strychnine ( $n = 3$  cells; Fig. 5B) and 50 nM  $\alpha$ -Bgt ( $n = 8$ ; Fig. 5C) entirely suppressed the appearance of eEPSCs. The inhibitory effect of  $\alpha$ -Bgt was detectable 5 min after the beginning of application, was maximal after 30 min of application and was partially reversed after 20 min of the wash out of the

neurotoxin (not shown). eEPSCs were not affected by the bath application of 1  $\mu\text{M}$  DH $\beta$ E ( $n = 16$ ; Fig. 5C), whereas bath application of 10  $\mu\text{M}$  DH $\beta$ E decreased the amplitude of the eEPSCs by  $82 \pm 10\%$  ( $n = 14$ ).

PPADS (10  $\mu\text{M}$ ), an antagonist of P2X receptors, and ondansetron, an antagonist of 5-HT<sub>3</sub> receptors (Mair *et al.* 1998), had no effect on the amplitude of eEPSCs ( $P > 0.05$ ; data not shown).

We concluded that EPSCs evoked by focal stimulation were mediated by nAChRs sensitive to  $\alpha$ -Bgt and strychnine, two selective antagonists of the  $\alpha 7^*$  nAChRs.

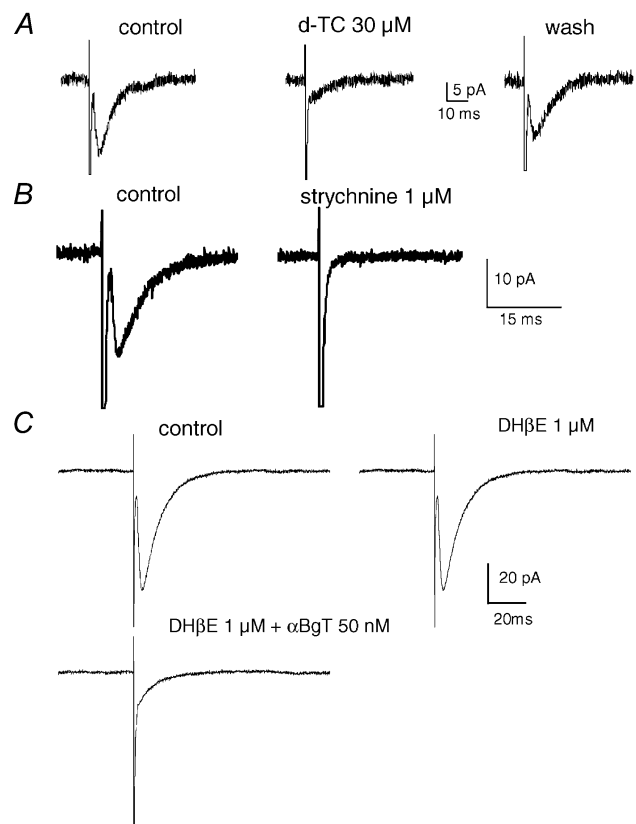
### Lamina X neurones express different types of nAChRs

We have previously reported that SPNs from the central autonomic nucleus showed responses to DMPP which were insensitive to  $\alpha$ -Bgt (Bordey *et al.* 1966b). This might be indicative of the expression of several types of nAChRs. We therefore tested the existence of  $\alpha$ -Bgt-resistant nAChRs by examining the response to DMPP, a less specific nAChR agonist than choline.

DMPP responses appeared to be extremely heterogeneous in terms of amplitude, rise time and decay time kinetics. Based on the speed of desensitization of the DMPP current during the application of DMPP, four types of responses could be found. Of a total of 122 responses to DMPP, in 53 cells DMPP induced a slower desensitizing current with a rapid onset; this is referred to as type SD for 'slow desensitizing' (see Fig. 6A and B for two representative

### Figure 5. Effects of nAChR antagonists on eEPSCs

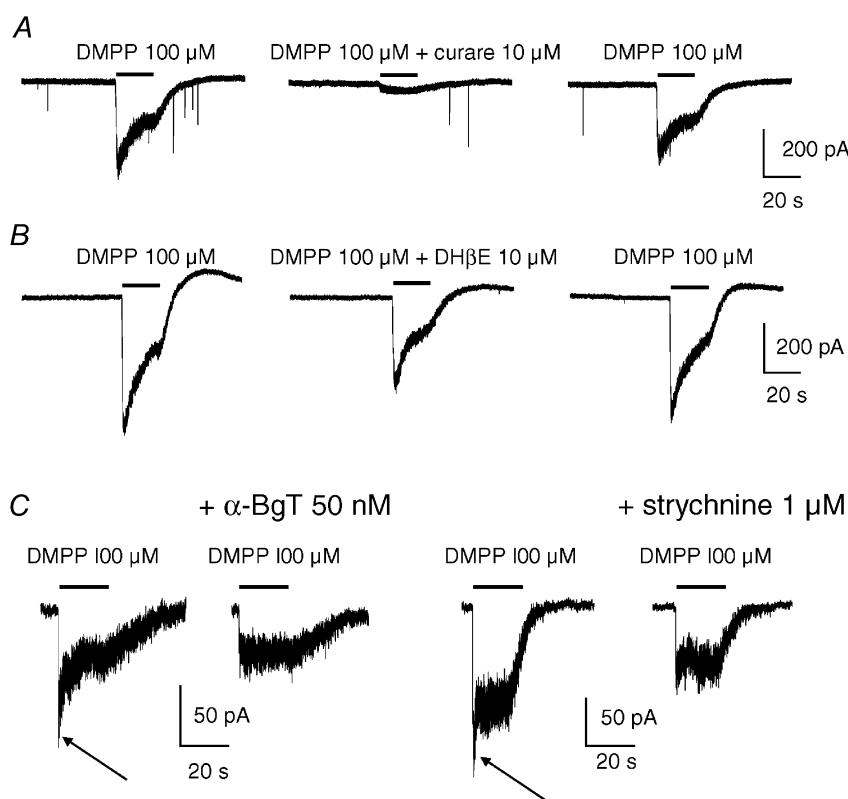
**A**, pressure application of 30  $\mu\text{M}$  *d*-TC for 20 s inhibited the appearance of eEPSCs. Control and wash represent the average of 40 eEPSCs. The middle trace was obtained by averaging six traces made during the application of *d*-TC. Washout of the effect of *d*-TC was obtained 3 min after the application of *d*-TC. **B**, bath application of 1  $\mu\text{M}$  strychnine entirely blocked the eEPSCs. Control and strychnine traces were obtained by averaging 40 traces before and 5 min after the beginning of application of strychnine. **C**, bath application of 1  $\mu\text{M}$  DH $\beta$ E had no effect, whilst  $\alpha$ -Bgt inhibited eEPSCs. Traces illustrate the average of 40 events before (control), during the application of 1  $\mu\text{M}$  DH $\beta$ E (right trace) and after 30 min application of a mixture of 50 nM  $\alpha$ -Bgt and 1  $\mu\text{M}$  DH $\beta$ E (bottom trace). Recordings were made at -60 mV in the presence of 2 mM kynurenic acid, 10  $\mu\text{M}$  CNQX and 10  $\mu\text{M}$  bicuculline.  $E_{\text{Cl}}$  was -60 mV.



responses). In 60 cells, DMPP induced a slowly developing and almost non-desensitizing (ND) current that lasted throughout the application of DMPP (see Fig. 7A, top response to DMPP). It is worth noting that this second type of response was more frequently encountered when the recorded neurones were deep in the slice, whilst type SD was encountered when the recorded neurones were near the surface of the slice. These two types of DMPP responses were found in cells showing fast eEPSCs. On very rare occasions, DMPP only induced a fast-decaying current (in three cells, not shown) or a response that clearly appeared as the succession of a fast-decaying current followed by a more sustained phase ( $n = 6$  cells, see example in Fig. 6C).

### Pharmacological properties of DMPP-induced currents

Pharmacological characterization was conducted on type SD and ND responses, which were those most frequently encountered (Table 1). The DMPP-induced currents produced by pressure application of DMPP were compared to the current induced by pressure coapplication of DMPP and the antagonist to be tested, contained in a second pressure pipette. DMPP responses were reversibly and almost fully blocked by coapplication of  $10 \mu\text{M}$  *d*-TC (see Fig. 6A). On the other hand, coapplication of DH $\beta$ E ( $10 \mu\text{M}$ ), which is classically described as a non- $\alpha 7^*$ nAChR antagonist, reversibly reduced the amplitude of the DMPP peak current from a control value of  $-417.5 \pm 108.8$  to  $-173.3 \pm 53.1$  pA ( $n = 6$ ; Fig. 6B). This corresponded to an



**Figure 6. Effect of nicotinic receptor antagonists on DMPP-induced current**

A,  $100 \mu\text{M}$  DMPP was pressure-applied onto cells held at  $-60$  mV and produced SD responses (left traces in A and B). The coapplication of  $10 \mu\text{M}$  curare (*d*-TC) with DMPP ejected from a second pressure pipette (middle trace) induced a current of smaller amplitude. The effect of *d*-TC was reversible (right trace). The inhibition of the peak of the DMPP-induced current as compared to the control trace was 91%. B, dihydro- $\beta$ -erythroidine (DH $\beta$ E;  $10 \mu\text{M}$ ) was pressure-coapplied with DMPP and reversibly (right trace) reduced (middle trace) the DMPP-induced current compared to the control trace (left trace). The inhibition of the peak of the DMPP-induced current was 34.8% and the inhibition of the DMPP-induced current measured at the end of the application was 43%, indicating a parallel reduction of the DMPP-induced current induced by DH $\beta$ E. All traces in A and B were made at 5 min intervals. C, antagonists of  $\alpha 7^*$ nAChRs were tested in two cells showing composite responses. DMPP was pressure-applied at  $100 \mu\text{M}$  and induced a biphasic current. Bath application of  $50$  nM  $\alpha$ -BgT for 30 min (left traces) and bath application of  $1 \mu\text{M}$  strychnine for 5 min (right traces) blocked the appearance of the fast inward current indicated by the arrows in control conditions. All recordings (A, B and C) were made in the presence of  $0.5 \mu\text{M}$  TTX,  $2$  mM kynurenic acid and  $10 \mu\text{M}$  bicuculline in neurones held at  $-60$  mV.

**Table 1. Diversity and pharmacological properties of DMPP responses in neurones of the lamina X of rat spinal cord**

|                       | Amplitude,<br>mean $\pm$ S.E.M.<br>(pA) | Frequency of<br>occurrence<br>(%) | $\alpha$ -BgT-<br>sensitive          | DH $\beta$ E (10 $\mu$ M)<br>inhibition<br>(%) |
|-----------------------|---|-----------------------------------|--------------------------------------|--|
| SD                    | -278.7 $\pm$ 26.8<br>( <i>n</i> = 53)   | 43.44                             | no                                   | 58.5<br>( <i>n</i> = 6)                        |
| ND                    | -75.1 $\pm$ 9.4<br>( <i>n</i> = 60)     | 49.18                             | no                                   | 66.5<br>( <i>n</i> = 4)                        |
| Fast<br>desensitizing | -95 $\pm$ 18.8<br>( <i>n</i> = 6)       | 4.91                              | yes                                  | —  |
| Composite             | -45 $\pm$ 27.6<br>( <i>n</i> = 3)       | 2.46                              | yes (peak)<br>no (sustained current) | —  |

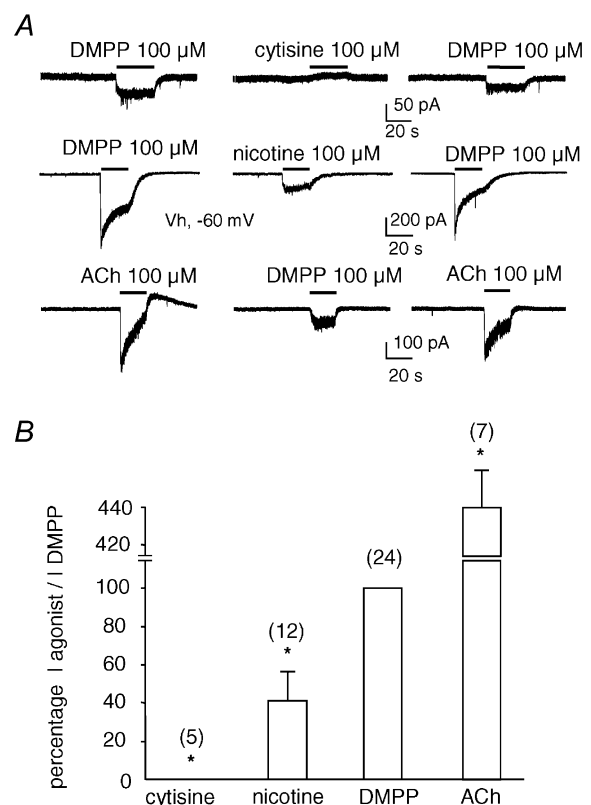
SD, slow desensitizing; ND, non-desensitizing; Composite, fast decaying + slow decaying.

inhibition for the SD type of 58.5%. Similarly, in ND type cells, 10  $\mu$ M DH $\beta$ E coapplied with DMPP reduced the DMPP-induced current from a control value of  $-216.25 \pm 52.9$  to  $-72.5 \pm 39.6$  pA (66.5% inhibition, *n* = 4 cells; not shown). Although DH $\beta$ E was probably not at equilibrium because it was coapplied with the agonist, the percentage inhibition is probably well estimated because bath application of 10  $\mu$ M DH $\beta$ E induced a similar percentage reduction in the DMPP-induced current ( $61 \pm 38\%$ , *n* = 10). Type SD and ND were insensitive to bath application of 50 nM  $\alpha$ -BgT for 30 min (four cells tested for each type), whereas  $\alpha$ -BgT and strychnine suppressed only the fast component in the rare cells showing a response to DMPP consisting of the succession of two phases (Fig. 6C).

We also made pair-wise comparisons of the effectiveness of other nAChR agonists (cytisine, nicotine and ACh) to that of DMPP in SD and ND cell types (see Methods). ACh was applied in the presence of 1  $\mu$ M atropine in the bath to prevent the activation of muscarinic acetylcholine receptors. All agonists were tested at a concentration of 100  $\mu$ M. Cytisine did not induce any measurable currents (*n* = 5). Nicotine produced type SD and ND responses, with amplitudes which were always inferior to that produced by DMPP. The opposite was observed with ACh, as ACh induced currents of SD and ND types that always had an amplitude much greater than that produced by DMPP. The comparison of the effectiveness of these nAChR agonists are presented and summarized in Fig. 7. The apparent order of effectiveness was ACh  $\gg$  DMPP  $>$  nicotine.

### Figure 7. Relative potency of classical nicotinic receptor agonists

A, the potency of 100  $\mu$ M cytisine (upper traces), 100  $\mu$ M nicotine (middle traces) and 100  $\mu$ M ACh (lower traces) was compared to that of 100  $\mu$ M DMPP. All applications (20 s) were separated by 5 min intervals to allow recovery from desensitization. ACh was applied in the presence of 1  $\mu$ M atropine, a muscarinic antagonist. Cells were held at  $-60$  mV and recordings were made in the presence of 0.5  $\mu$ M TTX, 2 mM kynurenic acid and 10  $\mu$ M bicuculline. B, histogram representing the percentage of the peak of the agonist-induced current compared to that induced by DMPP in cells displaying SD and ND responses. The agonists were tested at 100  $\mu$ M. The number of cells tested is given in parentheses. Error bars and \* indicate the S.E.M. and significance, respectively. The apparent order of potency was: ACh  $\gg$  DMPP  $>$  nicotine. Cytisine was inefficient.



## DISCUSSION

The new findings of this study are the identification of  $\alpha$ -BgT-sensitive nAChRs on neurones surrounding the central canal and their participation in fast synaptic transmission. To our knowledge, this is the first such demonstration in a mammalian spinal cord preparation, as fast nicotinic transmission employing  $\alpha$ -BgT-sensitive nAChRs has so far been found only in the brain (Alkondon *et al.* 1998; Frazier *et al.* 1998; Hefft *et al.* 1999; Hatton & Yang, 2002).

### Do lamina X neurones express $\alpha 7^*$ nAChRs?

The fast choline-induced current in the spinal cord showed the typical pharmacology of  $\alpha 7^*$ nAChRs, including a full blockade by two selective  $\alpha 7^*$ nAChR antagonists, i.e.  $\alpha$ -BgT and strychnine.  $\alpha$ -BgT was found to act slowly, a full block occurring after 30 min of perfusion. The reversal of its inhibitory effect was difficult to observe. This current appears very similar to fast-decaying type IA nicotinic current, which is known to involve  $\alpha 7^*$ nAChRs and which is evoked by choline in CA1 interneurons of rat hippocampus (Alkondon *et al.* 1999). In mammals,  $\alpha 9$  and  $\alpha 10$  subunits also display affinity for  $\alpha$ -BgT but none of these subunits has so far been detected in the spinal cord and it is, therefore, likely that  $\alpha$ -BgT-sensitive current is related to  $\alpha 7^*$ nAChRs. Indeed a low level of  $\alpha 7$  transcripts was localized by *in situ* hybridization in the area around the central canal (Séguela *et al.* 1993).

### Fast synaptic nicotinic transmission involved $\alpha 7^*$ nAChRs

Electrical stimulation in the absence of TTX indicated the involvement of nAChRs in a fast neurotransmission. The finding that the eEPSCs were mediated by nAChRs was confirmed by the lack of action of antagonists of other known fast ligand-gated ion channels (glutamate, 5-HT<sub>3</sub>, P2X, GABA<sub>A</sub> and glycine). With regard to the blockade of glycine receptors, PMBA has been shown to be a valuable tool, as it blocked glycinergic transmission without affecting nicotinic receptor-mediated current. Strychnine or its derivatives could not be used because of its ability to totally block  $\alpha 7^*$ nAChR-mediated response and other neuronal nAChR-mediated currents (Matsubayashi *et al.* 1998; Garcia-Colunga & Milei, 1999). Thus, it is probable that the use of strychnine in some preparations to block glycinergic transmission had also blocked  $\alpha 7^*$ nAChRs, thus preventing the recording of fast nicotinic EPSCs mediated by this type of nAChR.

It is likely that only  $\alpha 7^*$ nAChRs contribute to the eEPSCs because eEPSCs were completely abolished by 1  $\mu$ M strychnine and 50 nM  $\alpha$ -BgT. DH $\beta$ E is a competitive antagonist of both homomeric and heteromeric nAChRs, although its IC<sub>50</sub> is higher for the  $\alpha 7^*$ nAChRs. eEPSCs were indeed insensitive to 1  $\mu$ M DH $\beta$ E but sensitive to 10  $\mu$ M DH $\beta$ E (84% reduction in amplitude). It is worth

noting that choline-induced current and eEPSCs have the same sensitivity to  $\alpha 7^*$ nAChR antagonists, namely full block with strychnine and  $\alpha$ -BgT. Nicotine and ACh entirely abolished the eEPSC, whilst DMPP only reduced its amplitude. This is not surprising because ACh and nicotine have the same efficacy on  $\alpha 7$ nAChRs whilst DMPP is less efficient (Gopalakrishnan *et al.* 1995; Virginio *et al.* 2002). The situation regarding choline and cytosine was less clear. Choline did not entirely suppress eEPSCs, but reduced their amplitude. This is quite surprising because choline, like ACh and nicotine, is a full agonist of  $\alpha 7^*$ nAChRs and was expected to totally suppress eEPSCs. A simple explanation would be that the concentration of choline reached in the synapse was probably reduced. Using choline microsensors above and lowered into a brain slice, it was calculated that the superfusion of a slice with 100  $\mu$ M choline produced an increase in choline concentration of 10  $\mu$ M at 100  $\mu$ m depth into the slice (Xin & Wightman, 1997). This suggested that the choline concentration reaching the recorded cell may be greatly overestimated and is probably at least 10 times lower than the concentration of choline in the pressure pipette. This could be due to a problem with diffusion of choline into the slice from the ejection pipette, which is a major and common problem encountered with all drugs applied to a slice. This might also be due to the participation of low- and high-affinity choline transporters, which might reduce the concentration of choline in a slice. It would be interesting to block choline transporters and observe how the choline response would be modified. We were also unable to detect a reduction in the amplitude of eEPSC by cytosine. To our knowledge, there is no previous report suggesting that cytosine can be actively transported and, although we cannot exclude this possibility, it is more likely that cytosine is a weak agonist of native  $\alpha 7$ -nAChRs present in the lamina X. Cytosine has indeed less efficacy and potency in activation of IA current in hippocampus (Alkondon & Albuquerque, 1993) and of nicotinic currents in *Xenopus* oocytes expressing the  $\alpha 7$  homomers (Gerzanich *et al.* 1994).

### Heterogeneity in the nAChRs expressed by lamina X neurones

The strongest evidence for the presence of non- $\alpha 7^*$ nAChRs came from the observation of  $\alpha$ -BgT-resistant, DMPP-induced current. This was in agreement with our previous study, which failed to detect a DMPP response sensitive to  $\alpha$ -BgT (Bordey *et al.* 1996b). This does not necessarily imply that  $\alpha 7^*$ nAChRs do not participate in the DMPP current, as the suppression by  $\alpha$ -BgT of current of a few picoamperes in amplitude may be difficult to detect. This would correspond with a variation of 10% in the amplitude of the current, which is of the order of the variation we can observe between successive applications of DMPP. Thus, the contribution of an  $\alpha$ -BgT component, if present, must be weak, particularly in SD and ND types.

Obvious  $\alpha$ -BgT- and strychnine-sensitive components of the DMPP current were indeed observed in the DMPP response when this consisted of the succession of two phases (Fig. 6C). These cells were rarely observed and this might explain why they were not detected in our previous work.

Responses to DMPP in this study also differed in two respects from previous responses to DMPP found in neurones recorded from the same area and having the same morphology (Bordey *et al.* 1996b). ACh was previously found to be the least effective agonist, while in this study it was the most effective, and DMPP current amplitudes were 10 times smaller in our previous report. The main differences between the two studies lay in the protocols used. In the previous study, drugs were pressure-applied at a concentration of 1 mM for relatively short times (100–500 ms), whereas in this study nAChR agonists were applied at lower concentration (100  $\mu$ M) but for a longer time (20 s). These differences would affect the degree of desensitization, the amount of blockade by the agonist itself, which occurs at high agonist concentration (Arias, 1996), and the effective dose of agonist that reached the cells. All these factors might explain differences in the kinetics, amplitude of DMPP-currents and order of potency of various agonists seen in these two studies. In addition, previous experiments have been performed in neonatal rats (P2–10) and mostly in very young rats (P2) (Bordey *et al.* 1996b). In this study, we noticed that nAChR responses and the presence of fast nicotinic eEPSCs were more frequently encountered and were more robust in older (P7) than in younger rats (P2). This may indicate that nAChRs are developmentally regulated in the spinal cord. nAChRs are known to be developmentally regulated and, for example, in the rat cortex  $\alpha$ 4 and  $\beta$ 2 mRNA appeared more abundant at P14 whereas  $\alpha$ 7 mRNA peaked at P7 (Shacka & Robinson, 1998). It is possible that spinal nAChRs are similarly regulated and it would be interesting to follow the appearance of nicotinic EPSCs with the increasing age of the animal.

Nevertheless, it is reasonable to admit that neurones expressed different subtypes of nAChRs on their surfaces;  $\alpha$ 7\*nAChRs would predominantly be found within the synapse and non- $\alpha$ 7\*nAChRs would probably be extrasynaptically located. The strongest argument in favour of this differential localization came from the observation that cells showing small-amplitude,  $\alpha$ -BgT-sensitive, fast eEPSCs also displayed DMPP responses of several hundreds of picoamperes that resisted the action of  $\alpha$ -BgT (SD and ND types). Determining the nature of these non- $\alpha$ 7\*nAChRs solely on a pharmacological basis is a difficult task; there are, however, some arguments favouring the implication of  $\beta$ 2\*nAChRs. This subunit has been detected in the central grey matter (Wada *et al.* 1989) and  $\beta$ 2\*nAChRs are virtually insensitive to cytisine and poorly

activated by nicotine (Luetje & Patrick, 1991). In this study, cytisine and nicotine were indeed less effective than DMPP. DH $\beta$ E is classically used as a non- $\alpha$ 7-nAChR antagonist and showed some specificity toward  $\alpha$ 4\* heteromeric subtypes. The inhibition of DMPP-induced current by DH $\beta$ E might indeed suggest the involvement of  $\alpha$ 4 subunits, but one must be cautious for at least two reasons. Firstly, DMPP current was sensitive to DH $\beta$ E at a relatively high dose, i.e. 10  $\mu$ M. Bath application of 1  $\mu$ M DH $\beta$ E slightly reduced DMPP-induced current (10% reduction, data not shown) but it was sometimes difficult to discriminate between rundown of the current and the effect of the antagonist. For comparison, type II current recorded in hippocampal neurones that involve  $\alpha$ 4 $\beta$ 2\* nAChRs is sensitive to 10 nM DH $\beta$ E (Alkondon & Albuquerque, 1993). Secondly, in our hands, DH $\beta$ E did not discriminate between  $\alpha$ 7\* and non- $\alpha$ 7\*nAChRs, since at 10  $\mu$ M it inhibited both the  $\alpha$ -BgT-sensitive eEPSCs and DMPP current.

In conclusion, although there are some indications favouring the existence of  $\alpha$ 4 $\beta$ 2\*nAChRs, much more work would be needed to identify the composition of the extrasynaptically located nAChRs.

### Physiological relevance

The identification of the cells showing eEPSCs as SPNs was based on morphological criteria. They were located dorso-laterally to the central canal, as previously reported (Barber *et al.* 1984; Bordey *et al.* 1996b). It was possible in some cases to follow a neurite to its entry to the ventral root, as expected for an autonomic preganglionic motor neurone. We confirmed and extended this morphological identification by showing that these neurones displayed antidromic spikes when the ipsiventral rootlet was stimulated. This confirms that the recorded neurones were indeed motor neurones. The identity of the cholinergic cell synapsing on the recorded neurones remains unknown. There are several candidates: (a) spinal preganglionic cholinergic neurones, which are found predominantly in three autonomic nuclei: the intermediolateral column, the intercalated cell group and the central autonomic nucleus; (b) partition cells located in the intermediate spinal region; and (c) central canal cluster cells encircling the central canal (Barber *et al.* 1984). Potentially all these cholinergic neurones are well placed to establish synapses onto lamina X neurones. In particular, the three main autonomic nuclei communicate by a dense dendritic meshwork, which gives the 'sympathetic' spinal network the typical appearance of a ladder. The most frequently described dendritic extensions arise from the intercalated cells and run medially toward the lamina X (Cabot, 1990). This organization allows communication between the autonomic nuclei within a spinal segment and communication between autonomic nuclei of different spinal segments. How the activities of these autonomic centres integrate and globally define the

control of autonomic functions is not known. This is a complex issue and so far only electric synapses have been demonstrated between preganglionic neurones (Logan *et al.* 1996). We can hypothesize that the fast cholinergic neurotransmission demonstrated here may participate in such communication. If this is true, this will be the first functional demonstration of chemical communication between autonomic nuclei.

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