Functional Nicotinic Acetylcholine Receptors on Subplate Neurons in Neonatal Rat Somatosensory Cortex

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Hang, Ileana L. and Heiko J. Luhmann. Functional nicotinic acetylcholine receptors on subplate neurons in neonatal rat somatosensory cortex. J Neurophysiol 92: 189–198, 2004. First published March 3, 2004; 10.1152/jn.00010.2004. The establishment of cortical synaptic circuits during early development requires the presence of subplate neurons (SPn’s), a heterogeneous population of neurons capable to integrate and process synaptic information from the thalamus, cortical plate, and neighboring SPn’s. An accumulation of cholinergic afferents and nicotinic acetylcholine receptors (nAChRs) has been documented in the subplate around birth. To assess the developmental role of the cholinergic innervation onto SPn’s, we used whole cell patch-clamp recordings of visually identified and biocytin-labeled SPn’s in neonatal rat somatosensory cortex. Functional nAChRs were present in 92% of the investigated SPn’s. Activation of postsynaptic nAChRs by local application of agonists elicited a brief membrane depolarization associated with a barrage of action potentials and large inward currents reversing around 0 mV. According to our pharmacological data, excitation of SPn’s is mediated by α4β2 receptors. In contrast, functional α7 nAChRs could not be identified on SPn’s. Activation of nAChRs affected neither the spontaneous synaptic activity of SPn’s nor the synaptic connections between thalamus and SPn’s and within subplate. Nicotine, at concentrations reaching the developing brain by maternal smoking, induced a severe desensitization of nAChRs and an increase in the baseline noise. These results indicate that nAChR-mediated excitation of SPn’s may stabilize the developing synaptic circuits and suggest the involvement of nAChRs located on SPn’s in the fetal tobacco syndrome.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs1) are involved in a variety of brain functions, including memory formation, anxiety, and addiction (Jones et al. 1999; Levin and Simon 1998; Perry et al. 1999). They play a critical role in the adult as well as in the developing nervous system by mediating excitatory neurotransmission postsynaptically and by modulating the release of various neurotransmitters presynaptically (Role and Berg 1996). The prevalent functional nAChR subtypes in the mammalian brain are either homomeric, assembled of α7 subunits, or heteromeric, containing mostly α4 and β2 subunits (Le Novere et al. 2002). The cholinergic innervation has been proposed to serve trophic functions and influence brain development, mostly by modulating Ca2+1-dependent proliferation, differentiation, apoptosis, and survival processes (Lipton and Kater 1989; Pugh and Margiotta 2000; Role and Berg 1996). Moreover, nicotine as an exogenous agonist, and the psychoactive component of tobacco is likely to interact with nAChRs and affect their function. Maternal smoking associated with the fetal tobacco syndrome alters normal brain development by disturbing cell proliferation and differentiation (Slotkin 1998).

The major cholinergic innervation originating from the nucleus basalis of Meynert (Mesulam et al. 1983) enters the cerebral cortex around birth (Hohmann and Berger-Sweeney 1998) and is initially confined to the subplate (Candy et al. 1985; Kostovic 1986; Mechawar and Descaries 2001), a transient synapse-rich layer of neurons located directly under the cortical plate (Kostovic and Rakic 1980). The subplate neurons (SPn’s) express different morphologies and neurotransmitter profiles, are actively involved in the pathfinding of corticofugal and corticopetal axonal projections (Ghosh and Shatz 1992b; McConnell et al. 1989; Rakic 1977), and receive distinct functional synaptic inputs from the thalamus, cortical plate and subplate (Friauf et al. 1990; Hanganu et al. 2001, 2002). The presence of a dense cholinergic innervation to the subplate raises the question whether SPn express functional nAChR. Accumulation of α7 and α4 nAChR subunits at the level of the subplate has been reported previously (Broide et al. 1996; Csillik et al. 2002; Fuchs 1989; Ostermann et al. 1995). However, functional nAChR are assembled from multiple subunits, and therefore detection of a receptor subunit mRNA or protein does not necessarily proof the presence of functional receptor-channel complexes. The status of nAChR on SPn and the question whether they play an active role in cortical development is still unknown. In this study, we characterize for the first time the functional status of nAChRs on SPn’s by performing whole cell patch-clamp recordings on visually identified and biocytin-labeled SPn’s in neonatal rat cortical slices. Since nAChRs are also the target for exogenous nicotine in the fetal tobacco syndrome (Lambers and Clark 1996), alterations of receptor function by nicotine concentrations similar to those found during maternal smoking were investigated. We demonstrate that functional nAChRs are present and directly excite SPn’s during the neonatal period of the rat. The strong desensitization of nAChRs in the presence of behaviorally relevant concentrations of nicotine suggests a possible role of SPn’s in the development of pathophysiological disturbances associated with the early tobacco exposure.

METHODS

Slice preparation and identification of SPn

All experiments were conducted in accordance with the national laws for the use of animals in research and approved by the local animal care committee, the City of Mainz, Germany (Approval No.: 35/1996).

1 According to the current status of the nomenclature for nicotinic acetylcholine receptors (Lukas et al. 1999), the exact subunit composition of the α7 and α4β2 nAChR is not known.
ethical committee. Brain slices were prepared as described previously (Hanganu et al. 2001, 2002). Briefly, 0- to 4-day-old Wistar rats were anesthetized by hypothermia and decapitated. Whole-brain coronal slices (400 μm thick) including the primary somatosensory cortex were cut on a vibroslicer (Pelco 101, TPI, St. Louis, MO; HR2, SIGMANN Elektronik, Hüffenhardt, Germany) and separated into two hemispheres. Slices were maintained >1 h at 33°C in a storage chamber before being transferred to the submerge recording chamber. During preparation and recording procedures, slices were maintained in artificial cerebrospinal fluid (ACSF) containing (in mM) 26 NaHCO₃, 3 KCl, 1.6 CaCl₂, 1.8 MgCl₂, 1.3 NaH₂PO₄, and 20 n-glucose, pH 7.4, after equilibration with 95% O₂-5% CO₂ (osmolarity 333 mOsm).

The SPn’s were visualized using infrared differential interference contrast (DIC) videomicroscopy (Dodd and Ziegglänsberger 1990). SPn’s were identified by their location, morphology, and electrophysiological properties. Only neurons located between the cell-dense cortical plate with radially oriented neurons and the cell-sparse white matter were investigated. The exact morphological classification of previously recorded SPn’s was performed after biocytin staining. Neurons were excluded from data analysis if their morphological properties did not correspond to those reported previously (Friauf et al. 1990; Hanganu et al. 2002) and/or if their electrophysiological properties did not fulfill the criteria reported for SPn’s (Screen machine II, Fast, Munich, Germany). Whole cell patch-clamp recordings were performed according to the procedure described by Stuart et al. (1993). All recordings were performed at 32-33°C. Recording electrodes (8-15 MΩ) were pulled from borosilicate glass tubing (Science Products, Hofheim, Germany) on a vertical puller (PP83, Narishige, Tokyo, Japan) and filled with standard electrode solution containing (in mM) 117 K-glucuronate, 13 KCl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 K-HEPES, 2 NaATP, and 0.5 NaGTP. For the investigation of GABA₆ receptor-mediated synaptic currents, K-glucuronate was replaced by 117 mM KCl. Both electrode solutions were adjusted to pH 7.4 with 1 M KOH and to an osmolarity of 306 mOsm with sucrose.

Capacitance artifacts and series resistance were minimized using the built-in circuitry of the discontinuous voltage-clamp/current-clamp amplifier (SEÇÖ5L, npi elektronik, Tannn, Germany). The signals were amplified and low-pass filtered at 3 kHz, visualized on an oscilloscope (TDS210, Tektronix, Beaverton, OR), digitized on-line with an AD/DA board (ITC16, Heka, Lambrecht/Pfalz, Germany), and recorded and processed with WinTida software 4.11 (Heka). The bathing solution was connected to the ground via a chlorided silver wire. All potentials were corrected for liquid junction potentials with –10 mV for the gluconate-based electrode solution (Kibl and Luhmann 2000; Mienville and Pesold 1999) and –4 mV for the high chloride electrode solution (Marty and Neher 1995). The RMP was measured immediately after obtaining the whole cell configuration. For the determination of the Rm, hyperpolarizing 2-s-long current pulses were applied from a holding potential of –70 mV.

A bipolar tungsten electrode (5 MΩ; FHC, Bowdonham, ME) was used for the selective electrical stimulation of the thalamocortical afferents (TA), while the stimulus-evoked postsynaptic currents (PSCs) were recorded in SPn’s. The stimulation of TA was performed as described previously (Hanganu et al. 2002). For stimulation of the subplate, a horizontal cut was performed directly below the white matter to eliminate possible inputs from subcortical regions, and the stimulation electrode was placed within the subplate, laterally to the investigated cell. In all experiments, the duration of the electrical stimulus was 70 μs. The intensity of the stimuli varied between 20 and 50 V. Unless otherwise noted, stimuli were delivered at 0.05 Hz. Five stimulus-evoked responses were recorded under control conditions and after drug application.

Data analysis

Agonist-induced currents and spontaneous PSCs (sPSCs) were analyzed using the Mini Analysis Program (Synaptosoft, Leonia, NJ). The sPSCs were captured using a threshold-crossing detector set above the noise level. Events that did not show a typical sPSC waveform were rejected manually and by optimal settings of the program parameters. The evoked PSCs and agonist-induced potentials were analyzed using WinTida Software (Heka). sPSCs, evoked PSCs, and agonist-induced currents and potentials were analyzed in their peak amplitude, 10-90% rise-time, and decay-time. The decay-time constant (τ) was calculated by fitting a single or double exponential function to currents using a simplex algorithm. To determine the current-voltage relationship of the cholinergic currents, voltage steps of 20 mV between –130 and +10 mV were applied. Root mean square (RMS) noise values were calculated using Mini Analysis Software as a measure of the baseline current from the mean. For the evaluation of the agonist-binding constant, the data points were fitted by the equation I/Iₘₐₓ = (c/IC₅₀) / [1 + (c/IC₅₀)], with c = agonist concentration, IC₅₀ = concentration required for half-maximal response, and h = Hill coefficient using a least square algorithm.

Data are presented as mean ± SE. For statistical analyses, the two-tailed Student’s t-test and one-way ANOVA test were used. Significance levels of P < 0.05, P < 0.01, and P < 0.001 were considered.

Pharmacological procedures

All substances were purchased from Merck with the exception of R(-)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), carbamylcholine chloride (carbachol), (−)nicotine tartrate, choline chloride, methyllycaconitine citrate (MLA), dihydro-β-erythroidine hydrobromide (DHβE), N-methyl-4-(3-pyridinyl)-3-but-en-1-amine hemigalactarate (RJR-2403), kynurenic acid (KYNA), mecaminylamine hydrochloride, neostigmine bromide, and TTX, which were from Sigma-Aldrich (Taufkirchen, Germany), and atropine sulfate, which was from RBI (Natick, MA). Stock solutions of these drugs were prepared as follows: TTX, CPP, nicotine, carbachol, choline, JR-2403, DHβE, KYNA, mecaminylamine, neostigmine, and MLA in distilled water and CNQX in dimethylsulfoxide (DMSO). Stock solutions were stored at −20°C and diluted to their final concentration in ACSF on the day of the experiment. The maximal concentration of DMSO in the superfusate was 0.1%. Receptor antagonists (CPP, CNQX, MLA, DHβE, KYNA, mecaminylamine, neostigmine, and MLA in distilled water and CNQX in dimethylsulfoxide (DMSO)).
Histology and Morphological Analyses

In all experiments, 0.5% biocytin (Sigma-Aldrich) was included in the patch electrode solution for later morphological identification of the recorded cells. The staining protocol for biocytin was described previously (Schröder and Luhmann 1997). Slices were fixed in 4% buffered paraformaldehyde solution for ≥24 h, rinsed, and incubated for 60 min with 0.5% H₂O₂ and 0.8% Triton X-100 to inhibit endogenous peroxidases. An overnight incubation with an avidin-coupled peroxidase (ABC kit, Vectorlab, Burlinghame, CA) was followed by incubation in 0.5 mM diaminobenzidine. The reaction product was intensified with 0.5% OsO₄. Finally, slices were dehydrated and embedded in Durcopan (Fluka, Buchs, Switzerland). Biocytin-stained neurons were analyzed in their somatodendritic properties using an Axioskop microscope (Zeiss). The morphology of a few biocytin-filled SPn’s was reconstructed with Neurolucida software (Microbrightfield, Colchester, VT) and analyzed with the Neuroexplorer software package (Microbrightfield).

RESULTS

Whole cell recordings were performed from 115 SPn’s in somatosensory cortical slices from P0–P4 Wistar rats. As reported previously (Hanganu et al. 2001, 2002), the location of the subplate, the appearance of SPn’s under video-assisted Nomarski microscopy (Fig. 1A), their firing pattern, and their morphological properties after histological processing for biocytin served as criteria to identify the SPn’s. The 86 biocytin-stained SPn’s revealed an extensive arborization of dendritic processes (Fig. 1B). According to previously reported criteria concerning the variable shape of the soma and orientation of the primary dendrites (Hanganu et al. 2002), 27 SPn’s were classified as horizontal bitufted, 7 SPn’s as horizontal monufteted, 22 SPn’s as multipolar, 17 SPn’s as inverted pyramid, 12 SPn’s as tripolar, and 1 SPn as vertical.

The passive and active membrane properties of SPn’s were similar to those reported previously (Hanganu et al. 2001, 2002; Luhmann et al. 1999). Using gluconate-based electrode solution, the RMP and the Rᵢ were −53.4 ± 0.6 mV and 995.1 ± 36 MΩ (n = 98), respectively. Similar values were obtained when using a chloride-based electrode solution (RMP −52.5 ± 1.2 mV and Rᵢ 894 ± 58.5 MΩ, n = 17). All SPn’s were capable of firing one or repetitive overshooting action potentials in response to sustained depolarization by intracellular current injection.

Functional postsynaptic nAChRs on SPn’s

Local pressure application of cholinergic agonists directly onto the recorded cell body by a glass micropipette (Fig. 1A) elicited excitatory responses in 92% of the 91 investigated SPn’s. The remaining 7 SPn’s showed no response to application of cholinergic agonists. No correlation between the morphology and responsiveness to nAChR activation could be observed. To eliminate potential artifacts due to the pressure ejection of agonists, control recordings were performed by applying the extracellular solution (n = 5). Under these conditions, no changes in the membrane potential or the quality of the seal were observed. The presence of functional nAChRs on SPn’s was assessed using the cholinergic agonist carbachol (100 μM). To ensure a complete block of muscarinic receptors activated by carbachol, all experiments were performed in the presence of relatively high concentrations (10 μM) of the competitive antagonist atropine (Margiotta et al. 1987). In current-clamp mode, carbachol caused a transient membrane depolarization from −70 to −54 ± 1.8 mV (n = 4), sufficient to trigger a short-lasting barrage of action potentials (Fig. 2A). When recordings were performed in voltage-clamp mode, carbachol induced a large inward current, with a mean peak amplitude of 81.2 ± 10 pA (n = 33; Fig. 2B). The decay kinetic of the carbachol-induced currents varied from cell to cell but was generally described by the sum of two exponential functions with time constants of 0.8 ± 0.2 and 2.7 ± 0.2 ms, similar to values reported for nAChR-mediated responses during embryonic development (Atluri et al. 2001). Carbachol-elicited responses showed essentially no rundown and could be elicited repeatedly for >40 min with no significant reduction in the amplitude. To ensure a complete recovery, carbachol applications were usually performed at 30-s intervals, although 10-s intervals were equally effective. Carbachol-elicited currents exhibited little or no desensitization when the agonist was applied for longer times ranging from 10 to 60 s (Fig. 2C). The amplitude, but not the kinetics of the carbachol-induced currents, was dependent on the agonist concentration (10 μM, 14.3 ± 7.4 pA, n = 3; 50 μM, 36.3 ± 4 pA, n = 4; 100 μM, 81.2 ± 10 pA, n = 33).

Local application of the nAChR agonist nicotine (100 μM) excited SPn by eliciting inward currents with a mean amplitude of 32.9 ± 5.7 pA (n = 5; Fig. 2D). The decay-times of the nicotine-elicited currents (25.5 ± 3.4 s, n = 5) were significantly (P < 0.001) longer than those of the carbachol-induced currents, and a second application of nicotine, even after 45 min, produced a much smaller response compared with the initial exposure. A similar pharmacodynamic response pattern (tachyphylaxis) to nicotine has been reported previously for nAChR in rats (Chessell and Humphrey 1995; Wong and...
Gallagher 1989) and humans (Benowitz et al. 1989). Therefore activation of nAChRs in subsequent experiments was performed by carbachol in the presence of atropine.

As previously reported for nicotinic receptor channels (Bertrand et al. 1990; Mathie et al. 1990), carbachol-induced currents reversed at around 0 mV and showed a strong inward rectification in all five investigated SPn’s. Little or no current was seen at positive holding potentials (Fig. 3A).

In the brain, nAChRs may act both postsynaptically by directly exciting the neurons and presynaptically by modulating neurotransmitter release (McGehee and Role 1995; Role and Berg 1996). To detect the location and function of nAChRs on SPn’s, carbachol-elicted responses were recorded under control conditions as well as in the presence of the AMPA/kainate receptor antagonist CNQX (10 μM), the N-methyl-D-aspartate (NMDA) receptor antagonist CPP (20 μM), and TTX (1 μM). Under this condition, local application of carbachol elicited a postsynaptic inward current (26.3 ± 6 pA; n = 5) and a membrane depolarization (-53.8 ± 1.6 mV, n = 3) that was similar to that obtained under control conditions (31.8 ± 4.9 pA, n = 5; -54 ± 1.8 mV, n = 3; Fig. 3B). When carbachol was bath applied for 30 s (n = 7), the induced currents persisted in the presence of TTX, CPP, and CNQX. The reduction in peak amplitude of the inward current from 61.6 to 49.6 ± 7.1 pA (n = 7) was not significant. These results suggest that carbachol-induced responses are mediated by a direct activation of postsynaptic nAChRs and not by a presynaptic facilitation of glutamate release.

To decide whether endogenous acetylcholine (ACh) release could activate functional nAChRs, the effects of the nAChR antagonist mecamylamine (10 μM) on the spontaneous synaptic activity in eight SPn’s was investigated. Mecamylamine significantly (P < 0.05) decreased the sPSC frequency from

![Diagram](https://www.jn.org/)

**FIG. 2.** Responses of SPn to local application of nicotinic acetylcholine receptors (nAChR) agonists. A: nAChR-induced depolarization recorded from a current-clamped P4 SPn in response to local application of carbachol (100 μM). Note that carbachol-induced depolarization was of sufficient magnitude to trigger action potentials. Inset: carbachol-induced action potentials displayed at a larger scale. B: carbachol-elicted currents recorded in voltage-clamp modus from the same P4 SPn as displayed in A. Decay time of current could be described by the sum of 2 exponential functions (τ1 = 0.4 s, τ2 = 2.3 s). C: nAChR-mediated currents recorded after prolonged local application of 100 μM carbachol. Carbachol-elicted currents maintained in a steady-state level in the presence of the agonist. Inset: increased baseline current was not accompanied by an increase in spontaneous synaptic activity. D: nAChR-mediated current recorded from a voltage-clamped P2 SPn in response to local application of nicotine (100 μM). Note the slow kinetic of current (τ = 31.8 s) compared with carbachol-induced current in C. All recordings were performed in the presence of 10 μM atropine to prevent activation of the muscarinic receptors, and holding potential was adjusted to ~70 mV. Local application of carbachol or nicotine marked by arrows was performed for 200 ms (A, B, and D).
0.33 ± 0.06 to 0.22 ± 0.06 Hz in six SPn’s but had no effect in the two remaining neurons. The amplitude of the sPSCs was not affected. As reported previously (Hanganu et al. 2001), SPn’s show fast, AMPA receptor-mediated sPSCs and slow, NMDA receptor-mediated sPSCs. Although mecamylamine decreased the frequency of both populations of events, only the frequency of the fast sPSCs was significantly (P < 0.05) reduced from 0.3 ± 0.06 to 0.18 ± 0.07 Hz (n = 6). In addition, a similar decrease in sPSCs frequency from 0.15 ± 0.01 to 0.11 ± 0.02 was observed in three of four investigated SPn’s when bath application of mecamylamine was preceded by incubation of the slices with the AChesterase inhibitor neostigmine (10 μM). These results suggest that endogenously released ACh acts on the functional nAChR of the SPn’s.

Functional subtypes of nAChR on SPn’s

To analyze the subunit composition of the functional nAChRs, receptor subtype-selective agonists and antagonists were used. Bath application of dehydro-β-erythroidine (DHβE), a selective agonist of the α4β2 subtype of nAChRs (Alkondon and Albuquerque 1993), completely abolished carbachol-induced currents in all 10 investigated SPn’s (Fig. 4A). A low concentration of this antagonist (500 nM) was applied to prevent partial blockade of other nAChR subtypes reported to be achieved with DHβE at higher concentrations (1-10 μM) (Alkondon et al. 1999). In addition, the nicotine-derived compound RJR-2403, an agonist with selectivity for the α4β2 subtype of nAChRs (Papke et al. 2000), depolarized SPn’s in a similar manner as carbachol. Local application of RJR-2403 (30 μM) elicited a long-lasting membrane depolarization to −49.2 ± 5 mV (n = 4) in all four investigated SPn’s, and this depolarization was accompanied by a barrage of action potentials in three of four SPn’s (Fig. 4B). RJR-2403–elicited responses showed similar amplitudes and rise-times compared with carbachol-induced responses, but their decay-times were significantly (P < 0.05) longer (Fig. 4B, inset). The longer decay-times may result from a slower washout of the α4β2 nAChR agonist, but relatively few data concerning the kinetic of the RJR-2403-elicited responses in the slice are currently available.

To assess the presence of functional homomeric α7 nAChR on SPn’s, the selective α7 nAChR antagonist methlylycaconitine (MLA) (Palma et al. 1996) and the α7 nAChR agonist choline (Albuquerque et al. 1998) were used. Bath application of MLA (50 nM) had no significant effect on the amplitude (control: 113.8 ± 10.2 pA, MLA: 92.1 ± 15.8 pA, n = 6) or the kinetic of carbachol-induced currents (Fig. 4C). Moreover, local application of choline (10 mM) elicited no response in any of the five investigated SPn’s. Another series of experiments also failed to show α7 nAChR-mediated responses. The brain metabolite kynurenic acid (KYNA) is known to act as an antagonist at the glycine site of the NMDA receptor (Stone et al. 1972) and inhibit α7 nAChR (Hilmas et al. 2001). Bath application of 10 μM KYNA in the presence of the NMDA receptor antagonist CPP (10 μM) had no effect on the amplitude or the kinetic of carbachol-induced responses (Fig. 4D). Since the fast desensitization kinetic of the α7 nAChR may preclude the identification of the α7 nAChR-mediated responses, the duration of the carbachol application was reduced from 100 to 20 ms. Under these conditions, carbachol-induced responses with a peak amplitude ranging from 6 to 33 pA could be recorded in all three investigated SPn’s and were completely blocked by bath application of DHβE. Taken together, these results indicate that the α4β2 nAChR mediate the excitation of SPn, whereas α7 are not involved.

Pathophysiological effects of low concentrations of nicotine

Maternal nicotine gains access to the fetal compartment via the placenta, arrives more slowly, and acts for a longer time in the immature brain (Benowitz et al. 1989; Slotkin 1998). To better mimic the pathophysiological conditions associated with fetal exposure through maternal smoking, behaviorally relevant concentrations of nicotine were bath applied on SPn. The concentration of nicotine in the venous blood after smoking of

**Fig. 4.** Pharmacological identification of functional nAChR subtypes on SPn. A: carbachol-induced responses recorded in a voltage-clamped P3 SPn before (black trace) and during bath application of 10 μM DHβE (gray trace). B: nAChR-mediated response elicited in a current-clamped P1 SPn by local application of RJR-2403 (30 μM). Note the long-lasting decay phase of the response. Inset: amplitude, rise-time, and decay-time of the RJR-2403–elicited responses averaged from 4 SPn’s and normalized to data recorded under control conditions. C: carbachol-induced responses recorded in a voltage-clamped P2 SPn before (black trace) and in the presence of 50 nM MLA (gray trace). Inset: amplitude of carbachol-elicited currents averaged from 6 SPn’s under control conditions (black bar) and in the presence of MLA (gray bar). D: carbachol-induced responses recorded in a current-clamped P2 SPn under control conditions (black trace) and during bath application of 10 μM kynurenic acid (KYNA, gray trace). Control recordings were performed in atropine (A–C) or in 10 μM CPP and 10 μM atropine (D). Local pressure application of agonists is marked by arrow, and all recordings were performed at a holding potential of −70 mV.
several cigarettes ranges from 60 to 300 nM (Benowitz et al. 1989), and in the arterial blood, which better represents the level of nicotine in the brain, reaches ~600 nM (Henningfield et al. 1993). In addition, the developing brain tends to have higher nicotine concentrations than the smoking mother because of its higher lipid content and the lower clearance of nicotine from the fetal compartment (Lambers and Clark 1996; T. A. SLOTKIN, personal communication). In this study, SPn’s were exposed to nicotine at bath concentrations ranging from 10 nM to 1 μM. Although little or no detectable change in the membrane potential was induced by nicotine, the amplitude of carbachol-induced currents was decreased by nicotine, the amplitude of carbachol-induced currents was decreased in the presence of nicotine in all 14 investigated SPn’s. Bath application of 100 and 500 nM nicotine applied for 5-10 min reduced the maximal amplitude of carbachol-induced currents by 31.4 ± 5.4% (n = 4, P < 0.05) and 66.6 ± 12% (n = 6, P < 0.005), respectively (Fig. 5A). This effect was accompanied by a slight decrease in the decay-time of the currents (control: 2.7 ± 0.6 s, 500 nM nicotine: 0.7 ± 0.2 s, P < 0.05). A more consistent reduction of the carbachol-induced currents by 86.4 ± 7.7% was obtained after application of 1 μM nicotine. In all investigated SPn’s, desensitization of the nAChR on SPn’s in the presence of nicotine was accompanied by a significant (P < 0.01) decrease in Rm by 37.1 ± 6.9% (n = 4) and in the membrane time constant by 44.7 ± 6.3% (n = 4).

Because the half-life of nicotine in the body is about 2 h (Benowitz et al. 1989), the brain of smokers experiences long exposure to nicotine. When SPn’s were exposed to 500 nM nicotine for >20 min, carbachol-induced currents were almost abolished (Fig. 5A, inset), indicating that a greater fraction of the nAChR desensitizes. Recovery from desensitization was not achieved even after 60 min of washout. Similar results were reported for nAChR present on midbrain dopamine neurons (Pidotlichko et al. 1997).

Behaviorally relevant concentrations of nicotine also affected the baseline current noise in SPn’s. A significant (P < 0.05) increase in the variance of the baseline current to 278.7 ± 45.1% (n = 4) was obtained using 500 nM nicotine, whereas the effects induced by 50 nM, 100 nM, and 1 μM nicotine were not significantly different (Fig. 5B). These results indicate that, after long-lasting exposure similar to that experienced after tobacco exposure, the majority of α4β2 nAChRs present on SPn’s was functionally inactivated.

![Figure 5](image-url)  
**FIG. 5.** Effects of behaviorally relevant concentrations of nicotine on SPn. A: dose–response relationship for nicotine-induced inhibition of carbachol-induced responses. Peak amplitude of carbachol-induced currents in the presence of nicotine was normalized to that of the control and plotted against log concentrations of nicotine. Values were obtained from 4-6 SPn’s. Solid line passing through symbols represents best fit of data to a Hill equation. Inset: consecutive carbachol-induced responses recorded from a P3 SPn under control conditions and under prolonged application of 500 nM nicotine. Time interval between displayed responses was 30 s under control conditions and 120 s in the presence of nicotine. B: effects of nicotine on baseline current noise of SPn. Baseline noise was calculated for current traces recorded under control conditions (black squares) and in the presence of nicotine concentrations ranging from 50 nM to 1 μM (gray squares). Mean values were obtained from 4-6 SPn’s. Inset: current traces recorded under control conditions (black traces) and in the presence of various nicotine concentrations (gray traces). Note significant increase in baseline noise in the presence of 500 nM nicotine. C: effects of nicotine on glutamatergic (black squares) and GABAergic (gray squares) spontaneous synaptic activity of SPn. Frequency of spontaneous postsynaptic currents (sPSCs) was expressed as number of events per 24-s bin, and values were averaged from 4 SPn’s before and during nicotine (500 nM) application. D: effects of nicotine on thalamocortical synaptic function. PSCs were evoked by electrical stimulation of thalamocortical afferents at 0.05 Hz and recorded under control conditions (black trace) and in the presence of 500 nM nicotine (gray trace) from a P2 SPn. All control recordings were performed in the presence of 10 μM atropine, and GABAergic synaptic activity was recorded using high-chloride electrode solution and extracellular solution containing 10 μM CNQX and 10 μM CPP.

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SPn’s are desensitized by concentrations of nicotine that are too low to activate the receptor directly. However, a small fraction of the total number of nAChR stochastically opened in response to bath application of nicotine, producing an increase in the baseline current.

SPn’s receive glutamatergic synaptic inputs from the thalamus, CP, and subplate (Hanganu et al. 2002). Therefore we studied the effects of carbachol and low concentrations of nicotine on spontaneous as well as on evoked glutamatergic synaptic activity. In agreement with our previous data (Hanganu et al. 2001), 90% of the SPn’s showed glutamatergic sPSCs at a frequency of 0.19 ± 0.02 Hz (n = 19 cells) and with an amplitude of 15.9 ± 0.6 pA (n = 19 cells). Carbachol (100 μM) failed to modify the frequency, amplitude, and kinetics of the sPSCs. When nicotine at bath concentrations ranging between 50 nM and 1 μM was applied, neither the amplitude nor the kinetics of glutamatergic sPSCs was significantly modified (Fig. 5C). Since fast sPSCs are mediated by AMPA receptors and slow sPSCs by NMDA receptors (Hanganu et al. 2001), and since neither carbachol nor nicotine elicited significant changes in the kinetics of these sPSCs, our observations suggest that neither AMPA nor NMDA receptor-mediated sPSCs were affected by activation of nAChRs. In addition, in five of five investigated SPn’s, glutamatergic PSCs evoked by electrical stimulation of the thalamocortical afferents were not affected by nicotine in their amplitude (control: 28 ± 9.4 pA, nicotine: 26.6 ± 5.6 pA), rise-time (control: 5.5 ± 1.4 ms, nicotine: 5.3 ± 0.8 ms), or decay-time (control: 36.6 ± 1.9 ms, nicotine: 37.8 ± 2.6 ms; Fig. 5D).

A functional intrinsic GABAergic synaptic circuit is present within the subplate (Hanganu et al. 2002). To assess the interactions between nAChRs and the local GABAergic synaptic circuit within the subplate, the effects of nAChR activation on spontaneous as well as on evoked GABAergic activity were investigated. Long-lasting GABAergic sPSCs (decay-time 58.3 ± 8.3 ms) could be recorded at a low-frequency (0.09 ± 0.04 Hz) in all 11 investigated SPn’s using chloride-based electrode solution and an extracellular solution containing 10 μM CNQX and 10 μM CPP to block glutamatergic synaptic transmission. Bath application of nicotine at concentrations ranging from 100 nM to 1 μM did not affect the amplitude or the kinetics of the sPSCs (Fig. 5C). Moreover, GABAergic PSCs evoked by electrical stimulation of the subplate were also not affected by nicotine (Fig. 5E).

These findings suggest that neither glutamatergic synaptic transmission of the thalamocortical input nor GABAergic synaptic interactions between SPn’s are affected by carbachol or nicotine at behaviorally relevant concentrations.

**Discussion**

An accumulation of cholinergic afferents (Kiss and Patel 1992; Kostovic et al. 1989; Mechawar and Descarries 2001) and nAChR subunits (Bina et al. 1995; Broide et al. 1996; Csillik et al. 2002) has been previously shown in the subplate, but the receptor assembly and function are largely unknown. The present in vitro electrophysiological study in neonatal rat somatosensory cortex shows and characterizes for the first time functional nAChRs on SPn’s, presumably mediating the excitation induced by endogenously released ACh. Activation of nAChRs by local pressure application of cholinergic agonists excited the large majority of the investigated SPn’s. Cholinergic excitation seems to be mediated exclusively by postsynaptic α4β2 nAChR. Neither spontaneous synaptic activity nor the glutamatergic synaptic input from the thalamus or the GABAergic synaptic input from other SPn’s seems to be modulated by nAChR. Our results further show that nicotine, reaching the developing brain by maternal smoking or by early postnatal exposure, causes a severe desensitization of nAChR on SPn’s. These data indicate that SPn’s and immature cortical circuits involving SPn’s may be critically modified by low doses of nicotine during pre- and neonatal development.

**Expression of functional nAChR on SPn’s**

The large majority of the investigated SPn’s were excited by nAChR agonists, indicating that the expression of functional nAChR is a common property of SPn’s. Since a large number of SPn’s corresponding to all previously described morphological types was investigated, it is unlikely that a particular subtype of SPn’s was excluded from the present study.

Activation of nAChRs excited the SPn’s to fire action potentials and mediated fast, slowly desensitizing inward currents reversing near 0 mV. Our observations strongly suggest that the nAChRs on SPn’s are located exclusively postsynaptically, in contrast to the presynaptic role of nAChR reported previously (Aramakis and Methnerate 1998; Gray et al. 1996; McGehee and Role 1995).

Although the exact subunit composition of the receptors is not known, the pharmacological tools used in this study offer the best currently available specificity for the nAChR subtypes. Several lines of evidence indicate that the nAChRs on SPn’s are assembled from α4 and β2 subunits. The presence of the α4 subunit at the level of the subplate was shown by immunoperoxidase labeling (H. Schröder, unpublished observation). The presence of functional α4β2 nAChRs on SPn’s is also supported by the slow desensitization rate of the responses to nicotinic agonists, the blockade of the nicotinic responses by DHβE, and the efficiency of RJR-2403 to mimic the carbachol-induced responses. Coexpression of additional subunits, like α5, α3, or β4, as described in heterologous expression systems (Conroy and Berg 1998; Ramirez-Latorre et al. 1996) cannot be excluded, but seems unlikely, since neither α3 nor β4 subunits mRNA have been detected in the subplate (Zoli et al. 1995).

In contrast to the previously reported accumulation of α7 mRNA and α-bungarotoxin binding sites in the subplate (Bina et al. 1995; Broide et al. 1995; Csillik et al. 2002; Naeff et al. 1992), MLA did not affect cholinergic responses, and choline did not elicit α7 nAChR-mediated currents in SPn. This discrepancy between a molecular biological demonstration and a lack of a functional proof for the presence of α7 nAChR is not unique for SPn’s. Previous studies (Frazier et al. 1998; Radcliffe and Dani 1998) suggested that the number of α7 subunit-binding sites exceeds the number of functional α7 nAChRs. Neither in situ hybridization experiments nor the α-bungarotoxin binding in sections and lysates of brain tissue are capable of distinguishing clearly between receptors located intracellularly or at the membrane surface. The presence of intracellular pools of subunits that are not assembled into functional receptors was reported for neuronal nAChRs (Jacob et al. 1986). The mechanisms of receptor trafficking to the membrane and their
assembling depend on cell- or receptor-specific factors (Dineley and Patrick 2000; Wang et al. 2002), which differ for heteromeric and homomeric receptors (Wang et al. 2002). The possibility that rapid desensitization of α7 nAChRs (Aramakis and Metherate 1998) masks the functional detection of these receptors on SPn’s cannot completely be ignored, but according to our data, seems to be unlikely. A decrease of the application pulse duration to 20 ms did not induce an α7 nAChR-mediated response. However, previous studies identified α7 nAChR-mediated responses by using slow bath perfusion of agonists (Gil et al. 1997). Moreover, fast application of the α7 nAChR agonist choline, which has an advantage in that receptor desensitization is much less pronounced compared with nicotine (Vogt and Regehr 2001), also failed to elicit α7 nAChR-mediated responses in SPn’s.

**Physiological significance of the nAChRs on SPn’s during cortical development**

The neurotransmitter ACh plays an important role in the manifestation and modulation of activity-dependent processes in the developing brain (Feller 2002). In the immature cerebral cortex, the cholinergic system has a prominent modulatory influence on a number of structural and functional modifications underlying age-dependent synaptic plasticity (Aramakis et al. 2000; Bear and Singer 1986; Ego-Stengel et al. 2001; Hohmann and Berger-Sweeney 1998). Both the endogenous ACh release and the presence of functional nAChRs on SPn’s indicate that the cholinergic system may modulate immature cortical neurons and circuits transiently expressed during early development. The cholinergic projection from the nucleus basalis of Meynert innervates the subplate (Mesulam et al. 1983), and on activation, causes a pronounced excitation of the SPn’s. Since SPn’s are key elements in the development of cortical afferents and efferents and in the establishment of the columnar architecture (Ghosh and Shatz 1992a; Ghosh et al. 1990; Kanold et al. 2003; McConnell et al. 1989), the functional modulation of SPn’s by the cholinergic input may also profoundly influence these developmental processes. A simultaneous activation of the subthreshold glutamatergic input from the thalamus (Hanganu et al. 2002) and the excitatory cholinergic input mediated by nAChRs may cause a suprathreshold activation of SPn’s and a Hebb-like stabilization of immature cortical synapses and circuits. These connections may form the template for the generation of the mature cortical circuits and architecture.

Besides modulating the activity-dependent refinement of early cortical circuits involving SPn’s, the nAChRs may also influence the fate of this transient neuronal population. In rodents, a subpopulation of SPn’s disappears by apoptosis, whereas other SPn’s seem to survive and transform themselves into other cell types within the white matter or cortical layer VIb (Woo et al. 1991). A lack of nicotinic binding sites has been previously associated with an increased neuronal degeneration (Zoli et al. 1999), and a neuroprotective action resulting from activation of α4β2 nAChRs was reported for neonatal brain insults (Laudenbach et al. 2002). The data indicate that nAChRs may regulate the survival of SPn’s in the developing cerebral cortex.

**Pathophysiological role of nAChRs during early cortical development**

Since the rat cerebral cortex is relatively immature at birth compared at the cellular level with the cortex of a full-term human infant (Romijn et al. 1991), the findings of this study concerning the action of nicotine on the newborn rat SPn’s can be compared with the situation of the prenatal human cortex. Maternal smoking during pregnancy has been reported to cause tobacco-induced abortions, premature deliveries, low birth weight, and an increased incidence of sudden infant death syndrome (Lambers and Clark 1996; Slotkin 1998). The cellular mechanisms of these processes are not completely understood, but exposure to nicotine during prenatal stages may disturb cell division and neuronal differentiation.

Since nicotine at concentrations experienced by smokers caused a pronounced desensitization of the α4β2 nAChRs in SPn’s, exogenous nicotine may profoundly influence cortical development. The desensitization of nAChRs on SPn’s precludes the neurotrophic and -modulatory function of acetylcholine in the cortex. Moreover, this desensitization may up-regulate the number of nAChRs as shown to occur in vivo (Buisson and Bertrand 2001; Flores et al. 1992; Shacka and Robinson 1998). To which extent the desensitization of nAChRs may prevent the excessive stimulation and the potentially excitotoxic death of SPn’s remains to be elucidated.

In conclusion, the present electrophysiological data show that SPn’s express functional nAChRs that are assembled most likely from α4 and β2 subunits and shape the excitability of immature synaptic circuits present in the cortex around birth. The nAChRs are desensitized by long exposure to nicotine at concentrations similar to those found in cigarette smokers.

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**References**


