Cellular Mechanisms of Subplate-Driven and Cholinergic Input-Dependent Network Activity in the Neonatal Rat Somatosensory Cortex

Early coordinated network activity promotes the development of cortical structures. Although these early activity patterns have been recently characterized with respect to their developmental, spatial and dynamic properties, the cellular mechanisms by which specific neuronal populations trigger coordinated activity in the neonatal cerebral cortex are still poorly understood. Here we characterize the cellular and molecular processes leading to generation of network activity during early postnatal development. We show that the somatosensory cortex of newborn rats expresses cholinergic-driven calcium transients which are synchronized within the deeply located subplate. Correspondingly, endogenous or agonist-induced activation of predominantly m1/m5-assembled muscarinic acetylcholine receptors elicits bursts of action potentials (up states) as a result of suprathreshold activation of the subplate. Tonic activation by ambient nonsynthetically released gamma-aminobutyric acid (GABA) facilitates the generation of up states in the neonatal cortex. Additionally, this network activity critically depends on neuronal gap junctions but not on glutamatergic or GABAAergic synaptic transmission. Thus, an early circuit relying on the integrative function of the subplate as well as on cholinergic-driven tonic GABA depolarization and tight electrical coupling is able to generate coordinated network activity, which may shape the architecture and control the function of the developing cerebral cortex.

Keywords: calcium transients, development, gap junctions, m1/m5 muscarinic receptors, tonic GABA, up state

Introduction

Electrical activity patterns synchronize large populations of neurons into oscillatory networks during early stages of development (Zhang and Poo 2001; Moody and Bosma 2005; Torborg and Feller 2005). In the pre- and neonatal cerebral cortex of various species, these synchronized oscillations occur spontaneously (Yuste et al. 1992; Garaschuk et al. 2000; Corlew et al. 2004; Calderon et al. 2005; Vanhatalo et al. 2005), are triggered by peripheral inputs (Khazipov et al. 2004; Hanganu et al. 2006; Mihl et al. 2006; Minlebaev et al. 2007) or by activation of different metabotropic receptors (Flint et al. 1999; Peinado 2000; Dupont et al. 2006; Khazipov and Luhmann 2006; Wagner and Luhmann 2006; Hanganu et al. 2007). Although these activity patterns differ to some extent in their spatio-temporal dynamics and pharmacological profile, they reveal a striking similarity when the developmental status of the species or preparation is taken into account. In the cerebral cortex of newborn (postnatal day [P] 0–3) mice and rats, spontaneous, periphery-driven and pharmacologically elicited network oscillations, which require activation of voltage-dependent Na⁺ and/or Ca²⁺ channels, synchronize the activity of neurons in a so-called neuronal domain (Yuste et al. 1995; Peinado 2000; Corlew et al. 2004; Calderon et al. 2005; Dupont et al. 2006).

It has been suggested that the formation of neuronal domains depends on the trigger function of a few neurons, which initiate the coactivation and subsequently recruit neighboring cells via gap junctions (Yuste et al. 1995). Recently, we could demonstrate that the transiently expressed subplate neurons (Allendoerfer and Shatz 1994; Kostovic and Judas 2002) are capable of fulfilling this role (Dupont et al. 2006). Their essential role in the establishment of thalamocortical connections and cortical columns (Ghosh et al. 1990; Kanold et al. 2003), their extensive connectivity within the early synaptic network (Friauf et al. 1990; Hanganu et al. 2002; Kanold et al. 2003), their dense gap junctional coupling (Dupont et al. 2006), as well as the abundant innervation by neuromodulatory transmitter systems such as the cholinergic drive arising from the basal forebrain (Calarco and Robertson 1995; Mechawar and Descarries 2001), put them in an ideal position to synchronize the cortical activity during early development.

However, the cellular mechanisms by which subplate neurons drive this synchronized activity are poorly understood. Because recent findings showed that the cholinergic input controls the early neocortical activity in vivo (Hanganu et al. 2007) and that the subplate-driven activity can be elicited by activation of muscarinic acetylcholine receptors (mAChR) (Dupont et al. 2006), we focused on the molecular and cellular mechanisms underlying the early cholinergic-dependent network activity. For this purpose, we performed whole-cell and perforated patch-clamp recordings in combination with single-cell reverse transcription PCR (RT-PCR) and immunocytochemistry. The patterns of correlated activity in the developing cortex were monitored using calcium imaging of large groups of neurons paired with cell-attached patch-clamp recordings. We found that cholinergic-driven m1/m5 mAChR-mediated suprathreshold activation of subplate neurons triggers synchronized network activity dependent on gap junctional coupling. Our data further indicate that the ambient level of extracellular gamma-aminobutyric acid (GABA) boosts the subplate network to generate synchronized activity patterns in the developing cerebral cortex.

Materials and Methods

Slice Preparation

All experiments were conducted in accordance with the national laws for the use of animals in research and approved by the local ethical committee. Brain slices were prepared as described previously.
Fluid (ACSF) containing (in mM) 124 NaCl, 26 NaHCO3, 5 KCl, 1.6 CaCl2, 1.8 MgCl2, 1.3 NaH2PO4, and 20 d-glucose, pH 7.4, after equilibration during preparation and recording procedures in artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 NaHCO3, 5 KCl, 1.6 CaCl2, 1.8 MgCl2, 1.3 NaH2PO4, and 20 d-glucose, pH 7.4, after equilibration.

To ensure the end concentration in the extracellular solution (ACSF). To ensure the end concentration in the extracellular solution (ACSF).

logical properties that did not correspond to those reported previously.

morphological classification of recorded SPn was performed after the end of the experiment. Slices were maintained for at least 1 h at 33 °C and 100% humidity before forming the extracellular solution (ACSF). To ensure the end concentration in the extracellular solution (ACSF).

The maximal time resolution was 2 Hz. Measurements of changes in the intracellular calcium concentration ([Ca2+]i) with CaGreen were performed by using a Nipkow confocal microscope. Emitted fluorescence was filtered with a 500- to 550-nm band-pass filter of the Nipkow spinning disk confocal filter. Off-tissue blanks were used as controls to avoid any artificial contribution of the fluorescence changes. Acquisition protocols generally consisted of 10- to 40-min long time-lapse sequences (1-s exposure time at 2-s intervals), a few acquisitions even being extended up to 120 min. Signals were recorded and analyzed using Methamorph software (Universal Imaging, Downingtown, PA).

**Electrophysiological Recordings**

The videomicroscopic setup consisted of an upright microscope with differential interference contrast (DIC) optics (Axioskop, Zeiss, Oberkochen, Germany) and a CCD camera (C5405, Hamamatsu, Japan). The video image was contrast-enhanced by a video-processor (CZ741, Hamamatsu), visualized on a video-monitor and digitized online using a frame grasper card (Screen machine II, Fast, Munich, Germany). Subplate neurons were visualized using DIC optics (Dott and Ziegler-Gänsbauer 1990) and identified by their location, morphology and electrophysiological properties. Only neurons located between the cell-dense cortical plate/layers with radially oriented neurons and the cell-sparse white matter were investigated. The exact morphological classification of recorded SPn was performed after biocytin-staining. Data obtained from neurons (n = 6) with morphological properties that did not correspond to those reported previously.

(Friauf et al. 1999, Hanganu et al. 2002, Luhmann et al. 2003) or/and with electrophysiological properties that did not fulfill the criteria reported for SPn (resting membrane potential [RMP] negative to –40 mV and input resistance [Rm] > 500 MΩ) (Hanganu et al. 2001, 2002) were not considered for our analysis.

Cell-attached patch-clamp recordings (Hamill et al. 1981) were performed to monitor the action potentials (APs) discharge as capacitive currents (action currents) that charge the membrane under conditions of intact intracellular composition and CaGreen loading. The pipette potential was clamped at 0 mV. Whole-cell patch-clamp recordings were performed according to the procedure described by Stuart et al. (1993).

All recordings were performed at 32–35 °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 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. Or 80 K-glucuronate, 44 KCl, 1 CaCl2, 2 MgCl2, 11 EGTA, 10 K-HEPES, 2 NaATP, and 0.5 NaGTP. For perforated-patch recordings, either high or low [Ca2+]i pipette solution was used when supplied with 240 µg/mL amphoterin B. Both electrode solutions were adjusted to pH 7.4 with 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 (SEC05L, npi elektronik, Tamm, Germany). The signals were amplified and low-pass filtered at 3 kHz, visualized on an oscilloscope (TDS210, Tektronix, Beaverton, OR), digitized online with an Analog-Digital/Digital-Analog board (TCP16, Heka, Lambrecht/ Pfalz, Germany), recorded and processed with WinTida software 4.11 (Heka). All potentials were corrected for liquid junction potentials with –10 mV for the glaconate-based electrode solution (Kidb and Luhmann 2000). The RMP was measured immediately after obtaining the whole-cell configuration. For the determination of Rm, hyperpolarizing 2-s-long current pulses were applied from a holding potential of –70 mV.

For analysis of the passive and active membrane properties of SPn and cortical plate neurons (CPn), Rm, and membrane time constant (τ), firing threshold, AP widths, amplitudes, and frequencies were calculated. Voltage-activated currents were recorded in response to positive and negative voltage steps (range –130 to +20 mV, duration 2-s, step amplitude 5 mV). For frequency-current analysis, the mean firing frequency evoked with increasing current steps was measured. For frequency adaptation, the interspike intervals of APs evoked in response to depolarizing current steps were measured and normalized to peak values. For the analysis of Ith, the current–voltage relationship was determined 200 ms after begin and 500 ms before end of the hyperpolarizing step (Kidb and Luhmann 2000). Agonist-induced responses were analyzed in their onset, duration and frequency using WinTida software (Heka). Posttysomatic post synaptic currents (sPSCs) were captured using a threshold-crossing detector set above the noise level and analyzed with Mini Analysis Program (Synaptosoft, Leonia, NJ).

Events that did not show a typical sPSC waveform were rejected manually and by optimal settings of the program parameters. The evoked sPSCs were analyzed in their peak amplitude, 10%-90% rise-time and decay-time constants using WinTida Software. The decay-time constant was calculated by fitting a single or double exponential function to currents using a simplex algorithm. The cumulative probability of the interevent interval and amplitude was compared by Kolmogorov-Smirnov test (Mini Analysis).

**Image Data Analysis**

Individual cell somata were randomly selected and marked with regions of interest (ROIs). Methamorph software was used to determine the area of ROIs in each frame. Raw data, delivered in the form of a linear 12-bit intensity scale, were first plotted as fluorescence intensity versus time. The background fluorescence measured near the ROI was then subtracted from these raw data. Subsequently, data were normalized to the mean fluorescence intensities during a 30- to 120-s control phase and plotted as (AF/AX), allowing the comparison of data across experiments. The onset of Ca2+ transients was detected using the first derivative of the...
the reagents were purchased from Invitrogen GmbH. Water and were run for every batch of neurons. If not otherwise noted, contamination were performed by replacing cellular template with visualized by staining with ethidium bromide. Negative controls for products were separated by electrophoresis in 2% agarose gel and characterized SPn. After the electrophysiological characterization of harvesting cytoplasma from morphologically and electrophysiologically 

Starting the recordings.

Using slight negative pressure, several SPn were aspirated into a patch protocol on total RNA isolated from several visually identified SPn. The expression of messenger RNAs (mRNAs) for all 5 mAChR subunits achieved using the Kolmogorov–Smirnov test that is specially designed to compare the cumulative probability of SPn and CPn was achieved using the Kolmogorov–Smirnov test that is specially designed to decide whether 2 empirical distributions are different. 

**Multi- and Single-Cell RT-PCR**

The expression of messenger RNAs (mRNAs) for all 5 mAChR subunits (m1-m5) in the subplate was assessed using a multicell RT-PCR protocol on total RNA isolated from several visually identified SPn. Using slight negative pressure, several SPn were aspirated into a patch pipette that contains 10 μl of electrode solution and that was gently broken to reach 10-20 μm opening diameter. The expression of m1-m5 mAChR subunits at the single-cell level was assessed by harvesting cytoplasm from morphologically and electrophysiologically characterized SPn. After the electrophysiological characterization of the mAChR-mediated response, the cytoplasma was aspirated under electrical control of seal preservation into the patch pipette containing 10 μl of K-glucosanate-based electrode solution. The content of the patch pipette was immediately frozen at -80°C.

In both multi- and single-cell protocols, oligo(dT)20 (1 μl, 50 ng/μl) and mixed deoxy-nucleotide triphosphates (dNTPs) (1 μl, 10 μM, Amersham Bioscience Europe GmbH, Freiburg, Germany) were added to the harvested material, the mixture heated (5 min at 65°C) and subsequently incubated on ice (>1 min). Single-strand complementary DNA (cDNA) was synthesized from the cellular mRNA by adding Superscript III reverse transcriptase (1 μl, 200 U/μl), RNaseOUT (1 μl, 40 U/μl), dithiothreitol (2 μl, 0.1 M) and either 5× First Strand Buffer (4 μl, 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl2), or 10× First Strand Buffer (2 μl, 200 mM Tris-HCl, 500 mM KCl), and 25 mM MgCl2 (4 μl). The mixture (20 μl) was incubated at 50°C for 30 min and the reaction was terminated by heating (5 min at 85°C) followed by icing. The RNA strand in the RNA–DNA hybrid was removed by adding 0.5 or 1 μl of Ribonuclease H (5 U/μl, USB Corporation, OH), or 2 U/μl, Invitrogen GmbH, Karlsruhe, Germany) and incubating for 20 min at 37°C. The cDNA was used for PCR amplification using a Mastercycler standard (Eppendorf AG, Hamburg, Germany). The primers used for detection of m1-m5 muscarinic receptor subunits were described previously (Yan et al. 2001). To detect individual mRNAs, 2 μl of the single-cell cDNA was used as a template for the PCR. Reaction mixtures (50 μl) contained 10× PCR buffer (5 μl), 3 mM MgCl2, 0.2 mM of each dNTP and 1.25 units HotStarTaq DNA polymerase (Qiagen, Ratingen, Germany). The 15-min preincubation at 95°C was followed by 45 cycles of 30 s at 94°C, 30 s at 58°C and 1 min and 72°C. PCR products were separated by electrophoresis in 2% agarose gel and visualized by staining with ethidium bromide. Negative controls for contamination were performed by replacing cellular template with water and were run for every batch of neurons. If not otherwise noted, the reagents were purchased from Invitrogen GmbH.

**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). Briefly, the slices were fixed in 4% buffered paraformaldehyde (PFA) solution for at least 24 h, rinsed and incubated for 60 min with 0.5% H2O2 and 0.8% Triton X-100 to inhibit endogenous peroxidases. Overnight incubation with an avidin-coupled peroxidase (ABC kit, Vectorlab, Burlingame, CA) was followed by incubation in 0.5 mM diaminobenzidine. The reaction product was intensified with 0.5% OsO4. Slices were dehydrated and embedded in Durcopen (Fluka, Buchs, Switzerland). Biocytin-stained neurons were analyzed in their somatodendritic properties using an Axioskop microscope (Zeiss).

For detection of GAD67 immunoreactivity, the slices were fixed in 4% PFA for 2–24 h, rinsed and incubated in sucrose for 8–72 h at 4°C. Thereafter, they were frozen and resliced at 50 μm. These resliced sections were treated with normal goat (4%) and bovine (3%) serum as well as with 0.3% Triton X-100 to reduce the background staining and permeate the cells. A 48-h-long incubation with mouse anti-GAD67 antibody (MAB5406, Chemicon, Temecula, CA) at 1:1000 in 2% IgG and protease-free bovine serum albumin (BSA) was followed by a 2-h-long incubation with Alexa Fluor 588 goat anti-mouse IgG (Molecular Probes) at 1:400 in 2% BSA. The sections were washed and embedded with Fluoromount. Control sections were processed identically but with the primary antibody replaced by 2% BSA and 0.3% Triton X-100. To identify the exact location of GAD67-positive cells, the sections were further processed for DNA staining using Hoechst 33259 (Sigma) at 1:9000 in PBS (5-min incubation). To characterize the morphology of GAD67-positive neurons, the sections were examined by confocal laser microscope (Nikon confocal module attached to BX51WI Olympus microscope) with a 60×/0.9 NA water-immersion objective.

Data are presented as mean ± standard error of the mean (SEM). For statistical analyses the 2-tailed Student’s t-test, one-way ANOVA test, Wilcoxon test and Kolmogorov–Smirnov test were used. Significance levels of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

**Results**

**Cholinergic Calcium Transients in Neonatal Rat Somatosensory Cortex**

To characterize the network activity in the immature cerebral cortex, optical recordings were performed in 20 coronal slices
from 10 P0 to P4 Wistar rats following incubation with the cell-permeant calcium indicator Calcium Green (CaGreen). Fourteen to eighty neurons (mean 39.6 ± 4) with a healthy morphology were visualized with a 20x/0.4 NA water-immersion objective and randomly selected in each slice at the beginning of the recording. A high proportion of cells within the upper 100 μm of the 400-μm-thick slices were loaded with CaGreen and their location within the cortical layers was assessed by DIC imaging. In order to exclude cells that were damaged by the slicing procedure, only neurons located at a depth of >40 μm were included in this study. Several lines of evidence indicate that the vast majority of cells, which were loaded with CaGreen and further investigated, were neurons. Firstly, the shape of the soma and the orientation of the primary dendrites were easily recognizable at higher magnification (60x, confocal) and facilitated morphological classification of the investigated cells (Fig. 1Aiii, Aiv, Fig. 1F inset) as being similar to our previous DIC optical data (Fig. 1A) (see also Hangau et al. 2002; Albrecht et al. 2005). Secondly, treatment of CaGreen-loaded slices with TTX (n = 6 slices) led to a general decrease of fluorescence intensity and to abolishment of calcium transients (data not shown). Finally, the density of astroglial cells has been reported to be low in this preparation (Misson et al. 1991).

The calcium transients of large neuronal populations were monitored by recording time-lapse sequences of changes in CaGreen fluorescence intensity. Recordings with a good signal-to-noise ratio were obtained at relatively low excitation intensities and minimal photobleaching (average 0.5 ± 0.1%/min, n = 16 slices, in 7 slices less than 0.1%/min) was observed during the imaging experiments, which lasted for 10–120 min. In standard ACSF at 35 °C less than 15% of the investigated neurons with no preferential localization or clustering in the slice occasionally showed spontaneous sporadic calcium transients with very low frequency (ca. 0.3/min, n = 6 slices). In contrast, a large population of neurons throughout the entire somatosensory cortex showed a prominent and repetitive calcium signal increase (23.8 ± 1.8% from the baseline, n = 228 cells in 16 slices) upon activation of mAChR by bath application of 60 μM (-)-muscarine. These calcium transients could be recorded at a frequency of 0.88 ± 0.12 per min (n = 228 cells in 16 slices) and persisted 5–15 min after muscarine application. A more detailed analysis of the spatiotemporal properties of the calcium transients revealed layerspecific differences in their synchronization pattern. Whereas calcium transients in the deeply located subplate and layer VI were highly synchronized (Fig. 1A-E, Movie SP in Supplementary Material), calcium signals in the cortical plate and layer IV occurred more randomly and in a less synchronized manner (Fig. 1F-I, Movie CP in Supplementary Material). In accordance with this observation, we analyzed the cholinergic calcium transients in subplate and cortical plate neurons separately.

The subplate was identified under DIC optics as a layer with a relatively low cell density located between the cell-dense layer VI and the cell-sparse white matter. As previously reported for biocytin-stained SPn (Hangau et al. 2002, see also Fig. 1Aii), CaGreen-loaded SPn revealed a rather heterogeneous morphology. Whereas 10 out of the 20 in detail investigated SPn showed a spindle-like, horizontally oriented soma and a well-developed dendritic and axonal arborization (Fig. 1Aiii), SPn with inverted pyramidal (n = 4), multipolar (n = 4) (Fig. 1Aiv) or pyramidial shape (n = 2) could be also identified. The consequences of cholinergic activation were investigated in populations of 20–80 SPn. Upon bath application of muscarine (60 μM), SPn revealed prominent repetitive calcium transients occurring repetitively for 5–15 min at a mean frequency of 1.15 ± 0.22/min (n = 107 cells, 8 slices) and showing a mean maximal fluorescence increase of 58.2 ± 27.5% ΔF/ΔF₀ (n = 107 cells, 8 slices) (Fig. 1B,C). This response started 73.2 ± 14.9 s (n = 107 cells, 8 slices). In 29 out of 107 investigated SPn, the initial calcium burst lasted longer (8.07 ± 0.07 s, n = 29 cells, 8 slices) than the subsequent ones. Analysis of 209 SPn in 8 slices from P0 to P4 rats demonstrated that, on average, 58.5 ± 10.5% of the cells showed synchronized repetitive calcium transients. However, the number of synchronized SPn decreased with age from 90 ± 10% at P0 to 48 ± 13.7% at P4 in accordance with the progressive disappearance of the subplate. In order to assess the degree of synchronization within the subplate, the first derivative of the fluorescence signal was calculated and the calcium transients, which exceeded the background “noise,” were converted into binary rasterplots (Mao et al. 2001). These rasterplots revealed that a large fraction of investigated SPn responded with synchronized calcium transients to muscarine application (Fig. 1D).

Similar calcium transients could be observed in SPn when we inhibited the acetylcholinesterase (ACHE) with neostigmine, indicating that these responses can also be generated by endogenously released acetylcholine (ACh). Bath application of neostigmine at a concentration reported to have no unspecific effects (10 μM) (Calabresi et al. 1998; Zheng et al. 1998) induced repetitive calcium transients in a similar fraction of investigated SPn as muscarine (50.4 ± 15.7% of 174 SPn in neostigmine). However, these endogenous calcium transients occurred at a significantly (P = 0.003) lower frequency (0.17 ± 0.04 per min, n = 91 SPn, 5 slices) and displayed a significantly (P = 0.007) longer duration (6.9 ± 0.8 s, n = 91 SPn, 5 slices) compared with the muscarine-induced bursts. We hypothesize that this result is due to the slow accumulation of ACh in the slice during neostigmine application. Interestingly, the calcium transients observed in neostigmine also revealed a high degree of synchronization (Fig. 1E), suggesting that this type of coordinated activity is an endogenous feature of the subplate.

In contrast to the calcium transients recorded in the subplate, muscarine-induced calcium responses in the pyramidal-shaped cortical plate neurons (CPn) (Fig. 1F) showed a lower degree of synchronization. This is mirrored by a significantly (P = 0.006) smaller number of CPn that responded to muscarine (25.4 ± 5%, n = 121 CPn, 8 slices) and by the lower increase in the average fluorescence (10.7 ± 3.4% ΔF/ΔF₀, n = 121 CPn) of CPn when compared with the responses of SPn. Calcium transients recorded in CPn revealed a similar duration (3.9 ± 0.3 s, n = 121 CPn, 8 slices) as in SPn (Fig. 1G,H). The lower degree of synchronization within the developing cortical plate is documented in the binary rasterplots of the calcium transients (cf. Fig. 1D with Fig. 1F). Two methods were used to quantify the degree of synchronization within the subplate versus the cortical plate. Firstly, the fraction of SPn versus CPn showing synchronized calcium transients from the total number of responsive neurons was calculated for each investigated slice (Fig. 2A). Whereas 89.6 ± 5% of SPn revealed synchronized bursts in the presence of muscarine, only 53.3 ± 10.1% of CPn responded synchronously.
Figure 1. Cholinergic input-dependent calcium transients in the subplate and cortical plate of the newborn rat. (A) Morphology of subplate neurons. (i) DIC image of a horizontal SPn in a living neocortical slice from a P2 rat. (ii) Photomicrograph of a biocytin-stained SPn with an arborized dendritic tree. (iii)–(iv) Fluorescence images of horizontal and multipolar SPn loaded with 2.5 μM CaGreen at 33 °C for 15 min. Note that the morphology of the soma and of the primary dendrites (red arrows) can be resolved, allowing morphological classification. Scale bar in (i)–(iv) corresponds to 25 μm. (B) Fluorescence images of calcium transients in the subplate of a P1 rat in response to transient bath application of 60 μM (±) muscarine. The image sequence was captured before (control), during (74 s, 118 s) and after (264 s, 336 s) muscarine application (muscarine application onset was considered as t = 0 s). Note the simultaneous rise in the calcium signal in a large population of SPn. Scale bar corresponds to 50 μm. (C) Changes in the fluorescence intensity of 5 CaGreen-loaded SPn (corresponding to the SPn marked by circles in B). Note the synchronized response pattern in all 5 neurons. Muscarine application is marked by black bar. (D) Binary raster plot of muscarine-induced calcium transients recorded simultaneously in 31 randomly selected SPn from the slice shown in B. Note synchronized responses in all recorded SPn. (E) Binary raster plot of spontaneous calcium transients recorded simultaneously in 35 SPn from a P2 rat after blockade of AChE with neostigmine (10 μM). Note the correlated spontaneous activity in the majority of the SPn. (F) Fluorescence image from immature cortical neurons of a P1 rat, taken with a 10x objective in a focal plane 40 μm below the surface of the coronal slice. The region marked by the red box is shown at higher magnification in (G). Scale bar corresponds to 200 μm. Inset, fluorescence image of pyramidal cortical plate neurons (CPn) loaded with CaGreen. Note that the morphology of soma and primary dendrites can be resolved. Scale bar corresponds to 25 μm. (G) Fluorescence images of calcium transients in CPn in response to transient muscarine application. The image sequence was captured before (control), during (170 s) and after (222 s, 244 s, 340 s, 440 s) muscarine application. Scale bar corresponds to 50 μm. The Ca2+ transients from the cells marked by colored circles are shown in (H). (H) Changes in the fluorescence level of 4 CaGreen-loaded immature CPn marked in (G). Muscarine application is marked by black bar. (I) Binary raster plot of muscarine-induced calcium transients recorded simultaneously in 56 randomly selected cortical neurons from the slice shown in (F) and (G). In (D), (E), and (I) data were reduced to a binary form by using the first derivate of the fluorescence (ΔF/ΔF₀) signal and setting a threshold from the resting fluorescence of 0.03 and 0.01, respectively. Each horizontal line represents a cell and each vertical black bar represents the onset of a calcium transient. The synchronized coactivation of more than 10 neurons is marked by dotted lines. Note that the muscarine-induced activity in the cortical plate (I) reveals a less synchronized pattern than the activity recorded in the subplate (D, E).
to cholinergic activation. Secondly, the deviation from the median value of the first burst onset was calculated for all investigated SPn \((n = 107)\) and CPn \((n = 121)\) (Fig. 2B). The significantly \((P < 0.001)\) narrower distribution of SPn (mean deviation 8.2 s) in comparison to the more scattered distribution in CPn (mean deviation 26.5 s) further supports the higher degree of synchronization in the subplate than in the cortical plate. These data demonstrate that both SPn and CPn respond with repetitive calcium transients to the activation of mAChR. The higher synchronization of these responses within the subplate suggests that SPn are actively involved in the generation of network activity in the neonatal cortex.

**Electrophysiological Characterization of Muscarine-Induced Responses in Subplate Neurons**

Simultaneous imaging of calcium transients and recording of electrical activity of single CaGreen-loaded neurons in cell-attached patch-clamp configuration (Fig. 3A) was performed in 10 slices and revealed that the muscarine-induced calcium transients correlate with bursts of action currents (Fig. 3Aii). In all investigated neurons, several action currents grouped in a burst-like pattern correspond to a muscarine-induced calcium transient, whereas muscarine-independent single action currents were never detected as calcium increase. The mechanisms underlying the muscarine-induced bursts of APs were analyzed in more detail by performing whole-cell and perforated patch-clamp recordings from 191 visually identified and biocytin-stained SPn in the somatosensory cortex of P0–P4 Wistar rats. Out of 133 biocytin-stained SPn, 16 were morphologically classified as horizontal monounifurled, 38 as horizontal bitufted, 34 as multipolar, 24 as inverted pyramidal and 21 as tripolar neurons (see also Fig. 1 in Hanganu et al. 2002). The passive and active membrane properties of SPn were similar to those described previously (RMP = \(-49.6 \pm 0.4\) mV and \(R_m = 854.3 \pm 19\) M\(\Omega\), AP threshold \(-50.4 \pm 0.6\) mV, AP amplitude 65.7 \(\pm 1.3\) mV, AP width at spike base 10.7 \(\pm 1.2\) ms, \(n = 191)\). At a holding potential of \(-70\) mV all SPn were capable of firing repetitive overshooting APs in response to sustained depolarization by intracellular current injection. More than 70% of the investigated cells fired repetitive APs at RMP. No developmental changes in the membrane properties of SPn were observed between P0 and P4.

As indicated by the cell-attached recordings, the great majority of whole-cell recorded SPn responded to activation of mAChR by a repetitive burst discharge of APs with properties similar to the previously described cortical up state (Steriade et al. 1993; Haider et al. 2006) (Fig. 3B). This response was consistently observed in the presence of 60 \(\mu\)M (±) muscarine, which is in the range of concentrations required in similar investigations (Takayasu et al. 2003; Sookswate and Isa 2006). In contrast, the up states were absent in the presence of 1 \(\mu\)M muscarine \((n = 5)\) and present in only 40% of SPn when 3 \(\mu\)M muscarine was applied \((n = 5)\) (Fig. 3C). A muscarine concentration of 10 \(\mu\)M was sufficient to induce up states in all investigated SPn \((n = 5)\). However, the number of up states and therefore, the total duration of this phase increased with higher concentrations of the agonist from 67.3 \(\pm 10.1\) s for 10 \(\mu\)M muscarine to 239.8 \(\pm 78.8\) s for 60 \(\mu\)M muscarine, leveling off between 30 and 60 \(\mu\)M. Bath application of 60 \(\mu\)M (±) muscarine for 60–90 s at a holding potential of \(-70\) mV induced in 41 out of 49 investigated SPn repetitive up states with a similar time course and developmental profile as the calcium responses described above (Fig. 3Bii). The initial up state started 92.9 \(\pm 7.2\) s \((n = 41)\) after the muscarine application. The mean duration of a single up state was 3.6 \(\pm 0.3\) s \((n = 41)\) and the AP frequency within an up state varied between 2 and 30 Hz (mean 15.5 \(\pm 0.9\) Hz, \(n = 41)\) (Fig. 3Biii). A significant \((P = 0.002)\) decrease in the frequency of APs within the up state was observed from the first \((mean 17.7 \pm 1.4\) Hz, \(n = 30)\) to the later bursts \((12.2 \pm 0.9, n = 30)\). A variable number of up states ranging from 2 to more than 20 were recorded after a single muscarine application. This type of discharge generally persisted for 5–15 min, but in a few neurons it lasted more than 20 min after washout of the cholinergic agonist (Fig. 3Bii). Although spontaneous cholinergic-independent up states discharge could never be observed in SPn, bath application of the AChE inhibitor neostigmine (10 \(\mu\)M) induced in 5 out of 7 SPn repetitive up states with similar properties as the muscarine-induced responses (Fig. 3D). However, these endogenous up states recorded in neostigmine appeared at a lower frequency of 0.8 \(\pm 0.2\) min \((n = 5)\). When SPn \((n = 8)\) were held at their RMP, muscarine application \((60\ \mu\)M) induced a prominent change in the firing pattern from regular single AP firing to repetitive burst discharges with a higher AP frequency (Fig. 3E).

Cholinergic activation induced not only up state discharge in SPn, but also a slow and long-lasting \((236 \pm 15.9\) s, \(n = 29)\) membrane depolarization (LD) from \(-70\) to \(-62.6 \pm 0.7\) mV.
Muscarine increases the firing discharge and modifies the firing pattern in SPn. In all SPn showing both LD and up states, the LD preceded the AP discharge (onset LD 54.3 ± 4.1 s, n = 29, onset up state 92.9 ± 7.2 s, n = 41), but no clear phase correlation between up states and LD was detected.

Previous data revealed that muscarine action relies on several potassium conductances (McCormick 1993). Additionally, extracellularly recorded large-scale oscillations in the immature cortex have been reported to critically depend on the extracellular potassium concentration (Sun and Luhmann 2007). Therefore, the role of extracellular potassium concentration ([K+]e) for the generation of muscarine-induced up states was studied in more detail. Firstly, we reduced the [K+]e from 5 to 2.5 mM. Even though precise measurements of [K+]e within the highly interconnected and synthaptically active subplate (Hangasu et al. 2001, 2002) are missing at this developmental stage, 2.5 mM is considered to mirror the "rest" [K+]e, whereas 5 mM [K+]e corresponds to the "physiological" upper limit resulting from strong discharges and synaptic/extrasynaptic activity (Heinemann and Lux 1977; Somjen 1979). Whereas 41 out of 49 SPn showed up state discharge in 5 mM [K+]e, only 6 out of 22 neurons revealed this activity in 2.5 mM [K+]e, indicating that the generation of up states critically depends on [K+]e. The onset, number and duration of up states as well as the AP frequency within an up state were not significantly different in high and low [K+]e. Secondly, to elucidate the relevance of potassium conductances for the muscarine action in our experiments, we investigated the muscarine-induced effects on voltage-gated currents recorded in TTX (n = 5). Reduction of an inward rectifying potassium current by muscarine was accompanied by effects on other nonspecific cation conductances, as previously reported for the adult cortex (Klink and Alonso 1997; Egorov et al. 2003). Taken together, these data indicate that potassium as well as nonspecific cation conductances account for the effect of muscarine on SPn.

Besides relying on the potassium conductance, muscarine action has been reported to depend on intracellular metabolic pathways (Lucas-Meunier et al. 2003). To elucidate the

configuration (bottom trace). Note that several bursts of action currents correspond to each calcium transient. (bii) Current-clamp whole-cell recording from a P3 SPn at a holding potential of −70 mV. Note the long-lasting burst discharge (up states) after a single 30 s-long bath application of muscarine (marked by a black bar). The recording marked by the dashed box is shown at higher magnification in (bii). (bii) Muscarine-induced up states from the trace shown in (a) displayed at larger scale. Note the variable duration of the up states. (biii) Frequency power spectrum of the AP discharge within one representative up state from the trace shown in (a). (C) Current-clamp whole-cell recordings from a P2 SPn displaying the effects of 1 μM (i) and 10 μM (ii) (+) muscarine. Note the absence of up state discharge in the presence of 1 μM muscarine and the reduced duration of up state phase when 10 μM muscarine was applied. Inset, effects of different muscarine concentrations on the number of SPn showing muscarine-induced up states. (D) Current-clamp whole-cell recording from a P2 SPn at a holding potential of −70 mV displaying the effects of 10 μM neostigmine. Up state discharge appeared 10 min after the beginning of the neostigmine application. The recording marked by the dashed box is displayed at higher magnification in (Dii). (Dii) Neostigmine-induced up states displayed at larger scale from the trace shown in (Di). (Diii) Frequency power spectrum of the AP discharge within one up state from the trace shown in (Di). Note the similar frequency of the discharge in muscarine- and neostigmine-induced up states. (E) Muscarine increases the firing discharge and modifies the firing pattern in SPn. Current-clamp whole-cell recordings from a P3 SPn at RMP of −56 mV. Note the change in discharge pattern from regular firing to repetitive bursting. Muscarine application is marked by black bar.
contribution of intact intracellular environment to the generation of up states and corresponding Ca transients, we performed amphotericin B perforated-patch recordings in the presence of 2.5 mM [K+]. The passive and active membrane properties of the 24 perforated-patched SPn were similar to those reported for whole-cell recordings (RMP = 49.5 ± 1.2 mV, RIN = 964.5 ± 57 MΩ, n = 24). Bath application of muscarine induced in 22% of the perforated-patch recorded SPn repetitive up states, this activity starting 96 ± 36.4 s after agonist application. Two to 5 up states with a mean duration of 17.2 ± 5.3 s and AP frequency within burst of 9 ± 0.7 Hz were recorded in 5 SPn. These data demonstrate that the muscarine-induced up states show similar occurrence and properties under perforated-patch and whole-cell recording conditions. To test whether calcium release from intracellular stores contributed to the generation of muscarine-induced up states and Ca2+ transients, we depleted the intracellular Ca2+ stores by applying thapsigargin (1 μM for 30 min) in 5 SPn. Neither the occurrence nor the onset (control 43.2 ± 14.6 s, thapsigargin 85.8 ± 14.8 s, n = 5) or the duration (control 370.8 ± 79.7 s, thapsigargin 350.4 ± 56.9 s, n = 5) of the up state phase was significantly modified by thapsigargin. Moreover, thapsigargin did not significantly affect the holding current in all investigated SPn (control 41.2 ± 5.5 pA, thapsigargin 61.4 ± 17.6 pA, n = 5). These results reinforce the conclusion of our perforated-patch recordings and indicate that the influx of extracellular Ca2+, rather than the release of Ca2+ from intracellular stores, is the major source of calcium elevation in response to muscarine activation.

**Pharmacological Profile of Muscarine-Induced Activity**

Supporting the results of calcium imaging experiments, whole-cell current- and voltage-clamp recordings revealed that coordinated synchronized up states can be induced within the subplate by mimicking the cholinergic input (muscarine application) or by blocking the spontaneously released ACh (neostigmine application) (Fig. 4A). Several experimental approaches were used to identify the mechanisms by which the up states and the underlying LD are generated. The extent to which the muscarine-induced responses are network-related was assessed by blockade of the AP propagation with TTX. The muscarine-induced up states were completely abolished by 1 μM TTX (n = 9), indicating that AP-dependent mechanisms are required to generate the up state discharges (Fig. 4B,C). In contrast, the muscarine-induced LD present in 7 out of 9 SPn persisted in TTX (n = 7 SPn) (Fig. 4B,C), indicating that this response is AP independent.

The contribution of glutamatergic and GABAergic synaptic mechanisms in mediating the muscarine-induced responses was studied by the use of selective antagonists (Fig. 4C). Neither the postsynaptic LD nor the network up states were affected by blockade of (±)-α-amino-3-hydroxy-5-methylisoxazole-4-pro-pionic acid (AMPA)/kainate receptors with CNQX (10 μM) (n = 6) or by blockade of N-Methyl-D-aspartic acid (NMDA) receptors with CPP (10 μM) (n = 4). A combined blockade of AMPA/kainate and NMDA receptors did not modify the incidence and properties of the LD (n = 9) and had no effect on the up state discharge (n = 12). These data suggest that a glutamatergic input is not required for the generation of LD and network up states during early postnatal development (P0–P2), but it becomes more relevant with ongoing maturation as previously reported (Dupont et al. 2006).

Bath application of the selective GABAa antagonist gabazine (100 μM) did not abolish the up state discharge, but reduced the total duration of this phase from 270 ± 59.1 s to 140.1 ± 36.4 s (n = 8). Additionally, the effects of gabazine on LD were investigated in 18 SPn. The LD was completely abolished by gabazine in 9 out of 18 investigated SPn (Fig. 4D) and reduced in amplitude from 7.6 ± 1 to 4.5 ± 0.5 mV in the remaining 9 SPn (Fig. 4Di). Similar effects on up states and LD were observed when lower gabazine concentrations were used (n = 25) (Fig. 4E). The up state discharge persisted, but had a significantly (P = 0.043) shorter duration in the presence of 1 or 10 μM gabazine (control 253 ± 68.2 s, 1 μM gabazine 120 ± 4.13 s, 10 μM gabazine 100 ± 46 s). The LD was blocked in 60% of the SPn and persisted at lower amplitude in the remaining 40% when 1 μM, 10 μM gabazine, or bicuculline (10 μM) were applied. To further elucidate the role of GABA in the generation of muscarine-induced responses, the GABA uptake was blocked by bath application of 50 μM tiagabine in 5 SPn (Borden et al. 1994). Blockade of GABA uptake induced by itself a small membrane depolarization by 7.5 ± 1.5 mV (n = 5). Moreover, when muscarine was applied in the presence of tiagabine, the amplitude of the LD increased significantly (P = 0.005) from 7 ± 1 mV to 12 ± 0.4 mV (n = 4) (Fig. 4F). A similar effect on the LD was observed with the GABA uptake blocker nipecotic acid (500 μM) that significantly augmented the muscarine-induced LD from 6.3 ± 0.3 mV to 12.2 ± 2.2 mV (n = 5) (Fig. 4G). The up state discharge was not significantly modified by blockade of GABA uptake, except the shortened bursting phase observed in 4 out of 9 SPn after application of nipecotic acid. These data suggest that ambient GABA, tonically acting on GABAa receptors of SPn, but not synaptically acting GABA, contributes to the LD generation.

To test this hypothesis and selectively assess the contribution of synaptic versus tonic GABA for the LD generation, we used 2 approaches. Firstly, the high sensitivity of tonic GABA currents to low picrotoxin (PTX) concentration and of synaptic currents to high PTX concentration was used (Semyanov et al. 2003) (Fig. 5A, B). Bath application of 1 μM PTX decreased the holding current by 7.3 ± 2 PA (n = 4) (Fig. 5A), but had no effect on the averaged GABAergic sPSCs frequency (control 1 ± 0.5 Hz, 1 μM PTX 0.73 ± 0.2 Hz) or amplitude (control 25.3 ± 1 PA, 1 μM PTX 22.5 ± 2.3 PA, n = 4) (Fig. 5A). More relevant for our hypothesis is the fact that low PTX concentration blocked the muscarine-induced LD (n = 4) (Fig. 5B). In contrast, high PTX concentration (100 μM) abolished the GABAergic spontaneous synaptic activity (Fig. 5A, B) and, as expected, the LD (Fig. 5B). Secondly, we distinguished between muscarine-dependent tonic and synaptic GABA currents by using the stronger effects of lower gabazine concentration (0.2 μM) on the synaptic currents then on the tonic ones (Stell and Mody 2002). As expected, the frequency of GABAergic sPSCs significantly (P = 0.01) decreased from 0.22 ± 0.5 Hz under control conditions to 0.06 ± 0.02 Hz in 0.2 μM gabazine (n = 6) (Fig. 5C). In contrast, the muscarine-induced LD persisted in the presence of low gabazine concentration (n = 5) (Fig. 5D), but a slight decrease in its amplitude (control 6.8 ± 0.8 mV, 0.2 μM gabazine 4.9 ± 0.4 mV) could be observed. High gabazine concentration (10 μM) abolished the muscarine-induced LD (n = 5) (Fig. 5D) and reduced the holding current by 26.7 ± 8.1 PA (n = 3) (Fig. 5C). These results indicate that a substantial tonic activation of GABAa receptors is present on SPn and that this tonic
activation is critically involved in the generation of muscarine-dependent LD.

In agreement with our previous observations in neonatal mouse cerebral cortex (Dupont et al. 2006), both gap junction blockers mefloquine (25 µM) (Cruikshank et al. 2004) and carbenoxolone (100 µM), abolished the muscarine-induced network up states (n = 7 SPn). Blockade of gap junctions suppressed the LD in 5 SPn and reduced its amplitude from

Figure 4. Pharmacology of muscarine-induced responses in SPn. (A) Successive current-clamp (CC) and voltage-clamp (VC) whole-cell recordings from a P2 SPn. Note the up state discharge in response to muscarine application (marked by horizontal bar) in both recording configurations. The back arrow marks the switch between current-clamp and voltage-clamp mode. Inset, voltage-clamp recording from a P0 SPn after 20 min bath application of 10 µM neostigmine. (B) Effects of AP blockade on muscarine-induced responses. Current-clamp recordings of muscarine-induced responses under control conditions (upper trace) and in the presence of 1 µM TTX (lower trace) in a P4 SPn. Note blockade of up state discharge but persistence of LD in the presence of TTX. (C) Pharmacological profile of muscarine-induced responses in SPn. Bar diagram displaying the relative amplitude of the LD and the relative incidence of up states after blockade of APs (TTX), glutamatergic (CNQX, CPP) and GABAergic (gabazine, GBZ) synaptic transmission, as well as after neuronal gap junction blockade (mefloquine, MFQ). Data are expressed as relative values from the total number of SPn investigated for each antagonist. (D) Different effects of GABA_A receptor blockade on muscarine-induced LD recorded in SPn. (i) Current-clamp recordings displaying the blockade of the TTX-insensitive LD in the presence of 100 µM gabazine in a P3 SPn. (ii) Current-clamp recordings displaying the persistence of the TTX-insensitive LD in the presence of 100 µM gabazine in a P2 SPn. (E) Effects of different gabazine concentrations (1, 10, 100 µM) and bicuculline (10 µM) on the LD (black) occurrence and amplitude as well as on the up states (gray) occurrence and duration of bursting phase. Data are expressed as relative values compared with control recordings in the absence of the antagonists. (F) Effects of GABA uptake blockade by tiagabine on the LD in SPn. Current-clamp recordings of the LD recorded in the presence of TTX and after addition of 50 µM tiagabine (TGB) in a P3 SPn. Note the augmentation of the LD after blockade of GABA uptake. (G) Effects of GABA uptake blockade by nipecotic acid (NipAc) on the LD in SPn. Current-clamp recordings of the LD recorded in the presence of TTX and after addition of 500 µM nipecotic acid in a P4 SPn. Note the augmentation of the LD in the presence of nipecotic acid. In (A), (B), (D), (F), (G) muscarine application is marked by black bar.
9.7 ± 1.5 mV to 4 mV in the remaining 2 cells. These effects were not related to side-effects of carbenoxolone and mefloquine on standing-outward K\textsuperscript{+} conductances (K\textsubscript{2P} current) \((n = 13)\), because Spn lack bupivacaine- and pH-sensitive K\textsubscript{2P} currents (data not shown).

Summarizing, the different pharmacology of cholinergic-driven up states versus LD gives us important indications about the generation mechanisms of both activity patterns. Whereas the up state discharge is a network activity pattern requiring APs for its generation and gap junctional coupling for its propagation, the underlying postsynaptic LD critically depends on ambient GABA and tonic activation of GABAA receptors. In addition, the different effects of gabazine on the LD allow us to distinguish between 2 types of Spn. The Spn showing gabazine-insensitive LD (almost 40\%) are likely to receive a direct cholinergic input and to tonically release GABA. We defined these cells as GABAergic Spn. The Spn expressing gabazine-sensitive LD (almost 60\%) are more likely to miss a direct cholinergic input but to depolarize in response to the cholinergic-induced GABA release of the GABAergic Spn. We defined this second cell type as neighboring Spn, because they are sensitive to ambient GABA that is spatially restricted.

To address the question to what extent the properties of the 2 types of Spn differ, we analyzed in detail their morphology, firing pattern and membrane properties. The presence of GABAergic and non-GABAergic Spn was detected after GAD67 and Hoechst 33259 DNA staining (Fig. 6A,B). Our observations are in agreement with previous findings on the presence of GABAergic neurons within subplate (Meinecke and Rakic 1992). Characterization of the soma shape and orientation of primary dendrites showed that none of the Spn morphologies (horizontal, inverted pyramidal, pyramidal, multipolar) is exclusively restricted to GAD67-positive cells (Fig. 6Bi,Bii).

The passive and active membrane properties of GABAergic and neighboring Spn were analyzed and compared with those of the immature Cpn, which also contribute to cholinergic-driven network activity (see calcium imaging data). Both GABAergic and neighboring Spn express similar passive and active membrane properties (Table 1, Fig. 6C–G). They fired repetitively (regular spiking) and expressed an \(I_{\text{A}}\) current in 6 out of 12 cells in response to depolarizing current injection (Fig. 6C). Although the Cpn fired also repetitively, the frequency of AP discharge at RMP was significantly \((P = 0.048)\) lower (3.7 ± 1.7 Hz, \(n = 6\)) when compared with GABAergic (8.3 ± 1.6 Hz, \(n = 6\)) and neighboring Spn (8.8 ± 0.8 Hz, \(n = 6\)). Moreover, the Cpn tend to fire APs with longer duration than the Spn (Fig. 6D). All 3 cell types showed a comparable increase in APs frequency with increasing current injection (Fig. 6E). However, the firing pattern over the depolarizing step varied between Spn and Cpn (Fig. 6F). Whereas the AP frequency in GABAergic and neighboring Spn increased over time when small depolarizing currents were injected, the firing frequency of Cpn was constant or slightly reduced. Strong current injection led in all neurons to AP adaptation over time. Hyperpolarizing current injection led to

GABAergic sPSCs. Inset, voltage-clamp recording displaying the current shift induced by 10 \(\mu\)M GBZ. (D) Current-clamp recordings of the muscarine-induced LD in the presence of 0.2 and 10 \(\mu\)M GBZ. Note the persistence of LD in the presence of 0.2 \(\mu\)M GBZ and its abolishment by 10 \(\mu\)M GBZ. In (B) and (D) muscarine application is marked by black bar.
**Figure 6.** Morphological and membrane properties of the neuronal populations that participate to burst discharge in the neonatal cerebral cortex. (A) Hoechst 33258 DNA staining (i) combined with GAD67 immunocytochemistry (ii) demonstrating the presence of GABAergic cells in the subplate. Scale bars correspond to 100 μm. (B) High magnification digital images of GAD67-positive SPn (marked by white arrows) showing inverted pyramidal (i) and horizontal (ii) morphology. Note the presence of neighboring black spots (marked by asterisk) that are likely to be non-GABAergic SPn. Scale bars correspond to 25 μm. (C) Current-clamp recordings showing the firing patterns of the GABAergic SPn (black traces), neighboring SPn (dark gray traces) and cortical plate neurons (CPn) (light gray traces) in response to injection of small (30 pA, left-hand traces) and large (80 pA, right-hand traces) depolarizing currents. (D) Bar diagram of the averaged AP widths and their individual distributions in GABAergic SPn (black bar), neighboring SPn (dark gray bar) and CPn (light gray bar). Note the scattered distribution of AP widths in CPn when compared with SPn. (E) Diagram displaying firing frequency versus injected current for GABAergic SPn (black dots), neighboring SPn (dark gray dots) and CPn (light gray dots). (F) Adaptation of AP discharge during depolarizing current steps of various amplitudes. The values were normalized to the maximal firing frequency in each neuron. (G) Current-voltage plots for the GABAergic SPn (black), neighboring SPn (dark gray) and cortical plate neurons (CPn) (light gray). Voltage was measured at the beginning (diamond, solid line) and at the end (square, dotted line) of the hyperpolarizing pulses. Inset, current-clamp recordings showing the more prominent Ih voltage-sag in CPn when compared with SPn. Rebound depolarization present in both SPn and CPn is marked by arrows.
a large $I_h$ voltage-sag (mean amplitude $9.6 \pm 1.7 \text{ mV}$, $n = 6$) in CPn, whereas little ($3.4 \pm 1.3 \text{ mV}$, $n = 4$) or none ($n = 8$) $I_h$ could be recorded in GABAergic and neighboring SPn (Fig. 6G). Thus, as expected, the properties of immature CPn are distinct from those of GABAergic and neighboring SPn. However, despite their different contribution to the generation of activity patterns within early cortical networks, the morphological and membrane properties of GABAergic and neighboring SPn are similar and do not allow a reliable distinction between the 2 types of SPn.

**Characterization of mACh Receptors in Subplate Neurons**

Because the direct cholinergic input leading to LD and consequently facilitating the network up states acts on mAChR of a subpopulation of SPn, we aimed to identify the molecular structure of the involved receptors. For this, the subunit composition of the mAChR was studied in more detail in 15 SPn by multiple- and single-cell RT-PCR as well as by further neuropharmacological experiments. The expression of mAChR subunits was first assessed at the level of the subplate by using a multicellular RT-PCR approach. A large number of SPn were harvested in a pipette with large tip opening (10–20 μm diameter), which was positioned under visual control into the subplate. With this method, mRNA for all 5 receptor subtypes (m1–m5) was detected in the subplate layer (data not shown). The pattern of mRNA expression in individual SPn was determined by single-cell RT-PCR (Fig. 7A). Whole-cell recorded SPn showing muscarine-induced up states and LD were subsequently used for single-cell RT-PCR ($n = 15$) (Fig. 7A,B). All mAChR subunits were expressed in SPn and showed no developmental regulation in P0–P4 pups. However, the mRNA for m1 (69%) and m5 (62%) receptor subunits was most prevalent. Detectable levels of m2, m3, and m4 subunits were identified in only a few SPn. The mAChR subunit composition was very heterogeneous. Whereas 5 SPn expressed detectable levels of only 1 subunit, the majority of cells ($n = 9$) revealed a coexpression of 2–4 subunits and 1 SPn even expressed all 5 subunits (Fig. 7B). These data indicate that SPn express a heterogeneous mAChR subunit composition with a predominance of m1 and m5 subunits.

The functional expression of the m1 subunit was studied in 11 SPn by applying the m1 antagonist pirenzepine in the bathing solution containing TTX. In accordance with the high expression level of the m1 subunit in the subplate, pirenzepine (1 μM) blocked the muscarine-induced LD in the investigated SPn (Fig. 7C). Due to the low expression of the m2, m3, and m4 subunits in SPn and to the absence of reliable and selective pharmacology for each of them, the functionality of these receptor subtypes was not studied in further detail. Despite the abundance of m5 subunits in the subplate, the lack of selective m5 agonists or antagonists precluded further investigation.

**Consequences of Cholinergic Activation on Synaptic Activity of SPn**

The ability of SPn to integrate and process synaptic activity within the immature cortex is reflected by the abundance of received glutamatergic and GABAergic synaptic inputs (Hangasu et al. 2001, 2002). The present results document the minor (if any) contribution of synaptic transmission to the generation of cholinergic-driven LD and up states. However, it is still unknown whether activation of mAChR modifies the synaptic activity of SPn at all. To elucidate this issue, the effects of cholinergic drive mimicked by muscarine application on the spontaneous synaptic currents of SPn were investigated. Bath application of muscarine induced a significant ($P = 0.0003$) and long-lasting increase in the frequency of sPSCs from $0.6 \pm 0.1$ to $2 \pm 0.2$ Hz ($n = 14$) over the entire investigated developmental stage (Fig. 8A). The muscarine-induced PSCs occurred mainly in a burst-like grouped pattern that persisted for several min. Moreover, spontaneous synaptic activity was modulated by endogenous cholinergic mechanisms, because bath application of the broad-spectrum mAChR antagonist atropine (1 μM) caused a significant ($P = 0.037$) decrease in the frequency of sPSCs to $70.8 \pm 6.9\%$ ($n = 7$) without affecting their amplitudes or kinetics.

**Table 1**

| GABAergic SPn | Neighboring SPn | CPn |
|---------------|-----------------|-----|
| RMP (mV)      | 51.5 ± 2.6      | 55.3 ± 2.7 ($P = 0.278$) | 58.8 ± 2.6 ($P = 0.043$) |
| Input resistance (MΩ) | 949 ± 137.4 | 670 ± 58.8 ($P = 0.225$) | 723.3 ± 187.2 ($P = 0.345$) |
| Membrane time constant (ms) | 59.7 ± 1.1 | 44.2 ± 2.3 ($P = 0.248$) | 53.3 ± 5.7 (0.6) |
| Frequency of APs at RMP(Hz) | 8.3 ± 1.6 | 8.8 ± 0.8 ($P = 0.655$) | 3.7 ± 1.7 ($P = 0.019$) |
| Firing threshold (mV) | −56 ± 1.1 | −52.7 ± 1.1 ($P = 0.086$) | −64.7 ± 1.9 ($P = 0.074$) |
| Mean AP amplitude (mV) | 64.1 ± 2.2 | 63.5 ± 1.5 ($P = 0.345$) | 64.7 ± 5.5 ($P = 0.345$) |

Note: Significance values (Wilcoxon test) are indicated in parenthesis and are marked in bold when $P < 0.05$ (significance threshold).
Three populations of sPSCs, consisting of fast AMPA receptor-mediated sPSCs, slow NMDA receptor-mediated sPSCs and chloride-driven GABAA receptor-mediated sPSCs were previously characterized in SPn (Hanganu et al. 2001). The effects of mAChR activation on the glutamatergic and GABAergic populations of sPSCs were investigated by separating them using selective receptor antagonists. GABAA receptor-mediated sPSCs recorded after blockade of glutamatergic synaptic transmission with CNQX and CPP were significantly increased in their frequency from 0.27 ± 0.06 Hz to 1.35 ± 0.23 Hz (n = 16) following muscarine application (Fig. 8B). The significant (Kolmogorov–Smirnov test, P < 0.001) leftward shift in the cumulative probability plot of sPSCs interevent interval (Fig. 8C) documented the muscarine-induced increase of frequency. The amplitude (control 15.6 ± 1.5 pA, muscarine 14.6 ± 1.1 pA) (Fig. 8Ci) rise-time (control 4.5 ± 0.4 ms, muscarine 4.1 ± 0.3 ms), or decay-time (control 31.6 ± 1.6 ms, muscarine 33.2 ± 3.3 ms) of these sPSCs were not significantly changed (n = 16). To address the question to what extent the muscarine effects were dependent on APs, the muscarine-induced GABAergic currents were also investigated in the presence of 1 μM TTX (Fig. 8D). Muscarine had no effect on the GABAergic receptor-mediated sPSCs and neither their frequency (control 0.08 ± 0.02 Hz, muscarine 0.04 ± 0.01 Hz) nor their amplitude (control 25 ± 3.8 pA; muscarine 31 ± 3 pA) were significantly (Kolmogorov–Smirnov test, P > 0.05) changed (n = 10) (Fig. 8B, C). These results indicate that the quantal GABA release is not affected by mAChR activation.

The effects of mAChR activation on spontaneous glutamatergic synaptic activity were investigated after blockade of GABAA receptors using the selective antagonist gabazine. Activation of mAChRs significantly (P = 0.009) increased the frequency of the glutamatergic sPSCs from 0.5 ± 0.1 Hz to 1.5 ± 0.3 Hz (n = 14) (Fig. 8DE) as shown by the significant (Kolmogorov–Smirnov test, P < 0.001) leftward shift of the interevent interval cumulative probability plot (Fig. 8Ei). The mean amplitude (control 27.2 ± 2.4 pA, muscarine 26.6 ± 2.2 pA) (Fig. 8Eii), rise- and decay-time of the sPSCs were not significantly modified by activation of mAChR (n = 14). To differentiate between fast AMPA and slow NMDA receptor-mediated sPSCs,
the 2 types of currents were isolated according to their kinetics. Muscarine significantly (P = 0.025) increased the frequency of fast AMPA receptor-mediated sPSCs from 0.3 ± 0.06 Hz to 1.2 ± 0.4 Hz (n = 14 cells) and of the slow NMDA receptor-mediated sPSCs from 0.11 ± 0.6 Hz to 0.36 ± 0.2 Hz (n = 14). In contrast, muscarine had no significant (Kolmogorov-Smirnov test, P > 0.05) effect on the frequency of the glutamatergic mPSCs isolated in TTX (control 0.36 ± 0.1 Hz, muscarine 0.2 ± 0.06 Hz, n = 9) (Fig. 8D,E), indicating that muscarine does not directly affect the quantal release of glutamate. Summarizing, the augmented frequency of sPSCs in SPn is the consequence of increased AP discharge (up states) in the neonatal cortical circuit, which results from the cholinergic activation. Therefore, the enhancement of synaptic activity in SPn is not a primary effect of mAChR activation, but rather a secondary effect of the up state generation.

Discussion

In the present study we investigated the cellular mechanisms by which the transiently expressed subplate neurons trigger coordinated network activity in the neonatal cerebral cortex. Combining in vitro whole-cell and perforated-patch recordings with calcium imaging, single-cell RT-PCR and immunohistochemistry, we demonstrate that: (1) exogenous (muscarine application) or endogenous (blockade of AChE) activation of cholinergic input induces periodic calcium transients in the neonatal cortex; (2) the cholinergic calcium transients are highly synchronized in the subplate, whereas the degree of synchronization decreases in the immature cortical plate; (3) the cellular correlate of calcium transients in SPn are network-related bursts of APs (up states) underlined by a postsynaptic LD; (4) ambient GABA that is nonsynaptically released by cholinergically innervated SPn as well as gap junctional coupling facilitate the generation and propagation of cholinergic-dependent activity; (5) the mAChR of SPn receiving the functional cholinergic input are mainly assembled from m1 and/or m5 subunits.

The cholinergic system strongly influences the dynamic properties of adult cortical networks and induces a variety of oscillatory membrane potential fluctuations or discharge patterns by activation of muscarinic receptors (Lucas-Meunier et al. 2003; Cobb and Davies 2005). Our recent findings indicate that the cholinergic system is actively involved in the generation and modulation of network activity also during early development (first postnatal week) (Dupont et al. 2006; Hanganu et al. 2007). As shown by anatomical data, the cholinergic input arises from the basal forebrain and by birth selectively innervates the deeply located subplate, before growing into cortical layers (Johnston et al. 1979; Kostovic 1986; Calarco and Robertson 1995; Mechawar and Descarries 2001). Accordingly, our calcium imaging experiments revealed the highest amount of synchronously activated neurons within the subplate. In contrast, the percentage of cortical plate neurons expressing synchronous cholinergic-induced calcium transients was lower, but similar to that previously reported (Garaschuk et al. 2000; Corlew et al. 2004). The presence of such synchronized calcium transients reported to peak during a developmental time near birth (Corlew et al. 2004; Platel et al. 2007) supports their relevance for the establishment and refinement of connections, synapse formation and activity-dependent maturation of ionic mechanisms (Catalano and Shatz 1998; Liu and Kaczmarek 1998; Kasyanov et al. 2004).

The synchronized calcium transients within the subplate reflect the pronounced increase in the SPn firing rate and the up state discharge induced by activation of mAChR on SPn (Fig. 9A). m1 muscarinic receptors, which are selectively blocked by pirenzepine, and most likely also m5 receptors, both of which have been detected by us in SPn by the use of single-cell RT-PCR analysis, lead to K⁺ channel closure and membrane depolarization (Calderon et al. 2005). Both application of muscarine and blockade of the endogenous AChE with neostigmine elicit repetitive up states most likely by the muscarine/ACh-induced suppression of the M-type K⁺ current (Yue and Yaari 2004), by activation of nonspecific K⁺ conducting cationic channels (Egorov et al. 2003) or by activation of Ca²⁺ conductances (Gloveli et al. 1999). A very similar type of network activity can be observed in the neonatal rodent cerebral cortex spontaneously (Sun and Luhmann 2007) and after electrical stimulation of the subplate (Dupont et al. 2006) when the recordings were performed in vitro using thicker slices (up to 1000 μm) with a higher amount of preserved connectivity or in vitro (Hanganu et al. 2006, 2007).

The generation of this network activity in the newborn rodent cortex depends on the initial suprathreshold activation of SPn and can be blocked by TTX. Similarly, a blockade of spontaneous calcium transients by TTX has been previously reported in the perinatal mouse cerebral cortex (Corlew et al. 2004). Ionotropic glutamate receptors play only a minor role in the initiation or propagation of this cholinergic network activity in the newborn rodent cerebral cortex. Although the application of muscarine elicited an increase in glutamatergic sPSCs, AMPA/kainate, and NMDA receptors are not critically involved in the muscarine-induced network activity, because blockade of both ionotropic glutamate receptors reduced neither the LD amplitude nor the number of SPn showing up state discharges. The increased frequency of the muscarine-induced glutamatergic sPSCs simply reflects the sustained AP discharge during up states. This effect is a consequence but not the origin of the up state discharge in the neonatal circuit. Ionotropic glutamate receptors become more important at the end of the first postnatal week, when AMPA/kainate and/or NMDA receptors interfere with the maturation of thalamocortical connectivity (Fox et al. 1996; Kanold et al. 2003) and are required for propagating network oscillations (Garaschuk et al. 2000; Kilb and Luhmann 2003; Dupont et al. 2006; Minlebaev et al. 2007). A comparable developmental "switch" in the mechanisms driving immature neuronal networks has been also demonstrated in the spinal cord (Saint-Amant and Drapeau 2000), retina (Syed et al. 2004), and hypothalamus (Arumugam et al. 2005).

Even though blockade of GABA₄ receptors did not profoundly influence the burst discharge in SPn, gabazine caused a blockade of the LD or a reduction in the LD amplitude. Our data using GABA uptake blockers indicate that a tonic GABA release contributes to the generation of the LD and that this depolarizing GABAergic drive may promote the generation of network up states (Fig. 9B). This conclusion is supported by the effects of high and low concentrations of picrotoxin and gabazine on the LD. A tonic GABAergic activation by ambient GABA has been previously demonstrated in adult hippocampal neurons (Nusser and Mody 2002; Semyanov et al. 2003) and in newly generated granule cells in the dentate gyrus of the adult
hippocampus (Ge et al. 2006), where GABA initially exerts an excitatory action due to the high intracellular chloride concentration. In the immature hippocampus, tonic activation of GABAergic receptors is present prior to synapse formation (Demarque et al. 2002). Recent data indicate that ambient GABA and the corresponding GABA transporter GAT-1 control the excitability of immature hippocampal neurons and networks (Marchionni et al. 2007; Sipila¨et al. 2007). Here we show that a similar process is taking place in the immature neocortex. Low concentrations of ambient GABA can generate a tonic conductance by persistently activating high-affinity, nondesensitizing GABAA receptors that are localized remote from synapses (Semyanov et al. 2004; Farrant and Nusser 2005). The source of this tonic GABA release in the neonatal cerebral cortex is currently unclear, but our present findings indicate that a subpopulation of previously characterized GABAergic SPn neurons (Del Rio et al. 1992; Meinecke and Rakic 1992) is the most likely candidate. The GABA release from these GABAergic SPn in response to cholinergic input generates the LD in neighboring SPn (arrows in Fig. 9B).

Beside this nonsynaptic release of GABA, electrical coupling via gap junctions in the subplate and cortical plate contributes to the propagation of the burst discharge and cortical network activation following muscarinic activation of the subplate. As in electrically coupled GABAergic neurons in the juvenile rat thalamic reticular nucleus (Long et al. 2004), burst discharge may propagate from the GABAergic SPn to neighboring SPn via neuronal (Cx36 assembled) gap junctions (Landisman et al. 2002) (Fig. 9C). We have previously demonstrated that SPn are densely connected via gap junctions and that the Cx36 specific gap junctional blocker mefloquine (Cruikshank et al. 2004) does indeed block the cholinergic network activity (present data, Dupont et al. 2006). The question arises, how 2 different mechanisms, gap junctional coupling and ambient GABA, contribute to the generation of cholinergic-driven network activity, calcium transients and bursts of APs in the neonatal cerebral cortex? One possible explanation is that the depolarizing GABA action as result of tonic activation may operate as a supportive mechanism to ensure adequate activity levels within the immature cortical network. This hypothesis is supported by the fact that blockade of GABA uptake with tiagabine or nipecotic acid induced in SPn membrane depolarization and augmented responsiveness to muscarine application. Oscillatory neuronal activity, occurring spontaneously (Garaschuk et al. 2000; Sun and Luhmann 2007), triggered by spontaneous peripheral signals (Khazipov et al. 2004; Hangaru et al. 2006) or by activation of metabotropic receptors (Peinado 2000; Calderon et al. 2005; Dupont et al. 2006; Wagner and Luhmann 2006; Hangaru et al. 2007) may construct early functional networks before any sensory-driven activity from the external world reaches the developing cerebral cortex (Feller and Scanziani 2005; Khazipov and Luhmann 2006). These self-organizing processes can be observed in rodents during the perinatal period when the transiently expressed subplate plays an important role in the development of the neocortex (Lein et al. 1999; Kanold et al. 2003). Similar processes may take place in the human cerebral cortex before birth, when the prominent subplate is heavily innervated by various fiber systems (Kostovic and Judas 2002). Indeed, conventional electroencephalography (EEG) (Milh et al. 2006) and DC-coupled EEG recordings (Vanhatalo et al.
2005) have recently been used in premature human neonates to demonstrate the presence of cortical activity patterns, which share many functional similarities with the cholinergic oscillations described in the newborn rodent cortex (Dupont et al. 2006; Hangar et al. 2006; Khazipov and Luhmann 2006; Hangar et al. 2007). The characterization of the cellular correlates of network oscillations as well as the elucidation of their underlying mechanisms as achieved in the present study are essential steps toward understanding not only the physiological, but also the disease-related development of cortical circuitry (Jones 1995; Bunney et al. 1997).

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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