Activity-Dependent Regulation of Neuronal Apoptosis in Neonatal Mouse Cerebral Cortex

A massive neuronal loss during early postnatal development has been well documented in the murine cerebral cortex, but the factors that drive cells into apoptosis are largely unknown. The role of neuronal activity in developmental apoptosis was studied in organotypic neocortical slice cultures of newborn mice. Multielectrode array and whole-cell patch-clamp recordings revealed spontaneous network activity characterized by synchronized burst discharges, which could be blocked by tetrodotoxin and ionotropic glutamate receptor antagonists. The identical neuropharmacological manipulations also caused a significant increase in the number of apoptotic neurons as early as 6 h after the start of drug treatment. Moreover, inhibition of the NMDA receptor subunit NR2A or NR2B induced a differential short-term versus delayed increase in the apoptosis rate, respectively. Activation of L-type, voltage-dependent calcium channels was neuroprotective and could prevent activity-dependent apoptosis during NMDA receptor blockade. Furthermore, this effect involved phosphorylation of cAMP response element–binding protein and activation of the tropomyosin-related kinase (Trk) receptors. Inhibition of electrical synapses and blockade of ionotropic γ-aminobutyric acid receptors induced specific changes in spontaneous electrical activity patterns, which caused an increase in caspase-3–dependent cell death. Our results demonstrate that synchronized spontaneous network bursts activating ionotropic glutamate receptors promote neuronal survival in the neonatal mouse cerebral cortex.

Keywords: apoptosis, cell death, development, neocortex, NMDA, spontaneous activity

Introduction

Programmed cell death (PCD) is an essential process for the proper development of the nervous system. In the developing cerebral cortex, up to 25% of the neuronal population is eliminated during early postnatal stages as revealed by histological analysis (Heumann et al. 1978; Heumann and Leuba 1983; Ferrer et al. 1992; Voyvodic 1996) and labeling of apoptotic DNA fragmentation (Spreafico et al. 1995; Verney 1983; Ferrer et al. 1992; Voyvodic 1996) and labeling of histological analysis (Heumann et al. 1978; Heumann and Leuba 1983). The decision to enter the PCD process depends on several factors such as the proliferative or metabolic state of the neuron as well as the presence of different extracellular signals (Hidalgo and Ffrench-Constant 2003; Benn and Woolf 2004; Buss et al. 2006). However, the question which initial factor drives cortical neurons into apoptosis is still unresolved.

Based on the knowledge from the motor and sympathetic nervous systems, it has been proposed as a general developmental rule that an exuberant number of neurons is produced, from which a subset is selected according to the ability to connect to the appropriate target, that delivers essential trophic factors (Oppenheim 1991; Davies 2003). This model, called neurotrophic theory, has since expanded to take into account competition for afferent inputs (Linden 1994; Sendtner et al. 2000; Banks et al. 2005). Hence, cellular survival would be regulated by competition for retrograde and anterograde trophic factor delivery that depends on electrical activity from the afferents and target neurons. PCD during early development would serve to match the size of interconnected neuronal populations (Galli-Resta and Resta 1992; Posada and Clarke 1999; Buss et al. 2006). As synaptogenesis and refinement of the cortical network precedes (Innocenti and Price 2005), the concomitant neuronal loss could correspond to competition for survival as conceptualized in the neurotrophic theory. In such a model, neurons that integrate into the developing cortical network would develop stable electrical activity and would then be reinforced by trophic factors supply, whereas neurons that would fail to incorporate into the network would lack activity and would be eliminated by entering PCD. Support for the hypothesis of activity-dependent survival comes from in vitro and in vivo studies on cortical neurons that have demonstrated the dependence of survival on the activation of the N-methyl-D-aspartic acid receptor (NMDA-R) and subsequent brain-derived neurotrophic factor (BDNF) synthesis/release (Ghosh et al. 1994; Ikonomidou et al. 1999; Hardingham et al. 2002; Yoon et al. 2003; Hansen et al. 2004; Walz et al. 2006).

Several questions, however, are not yet answered. Whether PCD in the postnatal cerebral cortex in vivo targets neurons has not been unequivocally clarified. Furthermore, the patterns of neuronal activity that drive the survival of early postnatal cortical neurons are largely unknown. It is unclear whether the factors for neuronal survival defined in dissociated cell cultures are also applicable in more intact networks. We aimed at correlating neuronal activity and apoptosis in neurons from the developing network of the cerebral cortex. For this purpose, we have used organotypic slice cultures of somatosensory cortex from newborn mice. By combining measurements of apoptosis and recording of neuronal activity, we have analyzed which activity patterns could promote neuronal survival.

Material and Methods

In Vivo Analysis of Apoptosis

All experiments were conducted in accordance with the national laws for the use of animals in research (Deutsche Forschungsgemeinschaft 2004) and approved by the local ethics committee. Postnatal C56/BalbC mice (P3, P6, and P10) were deeply anaesthetized with an intraperitoneal injection (800 μl) of pentobarbital and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 at 4 °C. The brain was removed from the skull and placed
for 2 h at 4 °C into 4% PFA. After dehydration in graded ethanol baths (3 times for 15 min in 50%, 70%, and 95% and 3 times for 1 h in 100% ethanol), brains were incubated in 100% butanol for 12 h and then immersed at 60 °C with 50% butanol/50% paraffin (Rotiplast, Roth, Karlsruhe, Germany) for 48 h before final incubation in 100% paraffin for 24 h. Paraffin sections of 10 μm thickness were cut on a microtome (SuperCut 2050; Leica, Wetzlar, Germany). The sections were treated with xylol and butanol and rehydrated in ethanol baths (100, 95, 70, 50, and 0%) with a final step in 0.01 phosphate buffer saline (PBS). To improve the efficiency of the Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL), sections were incubated in 0.01 M citrate buffer pH 3 and heated 3 times for 5 min in the microwave at 350 watts. The sections were washed in PBS and incubated with 10% terminal deoxynucleotidyl transferase solution in labeling solution containing deoxyuridine coupled with fluorescein (Roche Applied Science, Mannheim, Germany) for 48 h before final incubation in 100% paraplast (Supercut 2050; Leica, Wetzlar, Germany). The sections were treated with 2% B27 (Gibco), 2 mM glutamax (Gibco) and 10 μg/ml penicillin/streptomycin. After 24 h, 1 μM Arabinoferanosio (Arac, Sigma-Aldrich, Steinheim, Germany) was added to the medium. Culture medium with Arac was renewed every two days, thereafter.

**Pharmacology**

After 4 to 5 days in culture, the medium was replaced by neurobasal medium without B27 and specific antagonists were added. In controls an equal volume of the antagonist solvent was applied. AraC was omitted during pharmacological treatments of the slices. After incubation periods of 6, 12 or 24 h, slices were fixed for 30 min with 4% PFA. The following drugs were used: 1 μM tetrodotoxin (TTX) citrate (Tocris Cookson, Bristol, UK); 10 μM 6-cyano-7-nitroquinoline-2,3-dione (CNQX, Sigma); 80 μM 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H,2,3-benzodiazepine (GYK 52466, a gift from Dr I. Világi, Eötvös Loránd University, Budapest, Hungary); 20 μM (±) 3-(2-carboxyamiphenazin-4-yl)propyl-1-phosphonic acid (CPP, Sigma); 400 nM [(R)-(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydropyridozinolin-5-yl)-methyl]-phosphonic acid (NVP-AM077, Novartis Pharmaceuticals, Basel, Switzerland); 5 μM ifenprodil (Tocris); 10 μM mephedine (Sigma); 100 μM gabazine (SR-95531, Sigma) 25 μM Mefloquine (Roche); 200 nM K252a (Alomone labs, Jerusalem, Israel). Drugs were stored at −20 °C and diluted to the final concentration on the day of experiment.

**Immunostaining for Activated Caspase-3 and Quantification of Apoptotic Cell Death**

An antibody directed against the cleaved, active form of caspase-3 was used (Asp175, Cell Signaling Technology Inc.) (Marín-Teva et al. 2004). This antibody is specific for the activated caspase-3 because it does exclusively recognize the 17/19-kDa fragment of the cleaved caspase-3 form (Lavoie et al. 2005). After fixation slices were rinsed in PBS, treated for 30 min with 0.1% Triton X-100 in PBS, and preincubated in 5% bovine serum albumin (BSA) in PBS for 2 h. Primary rabbit polyclonal antibody against activated caspase-3 (asp175, 1/400, Cell Signaling Technology Inc.) was incubated overnight at room temperature in 5% BSA in PBS. Sections were washed in PBS and incubated with the secondary antibody (biotin-conjugated donkey polyclonal antibody directed against rabbit 1/400, Vector Laboratories) for 2 h at 4 °C, then washed in PBS, and finally incubated with alexa488-conjugated streptavidin (Molecular Probes, Eugene, OR). After washing with PBS, sections were mounted on slides. For double staining with glial fibrillary acidic protein (GFAP), the slices were immunostained for α-spectrin as described above and then fixed again with 4% PFA for 15 min. Immunostaining with rabbit polyclonal antibody directed against GFAP (1/400, Dako, A/S, Denmark) was then processed using the same protocol as for α-spectrin. Slices were analyzed using a confocal system (QLC100; Visitech, Sunderland, UK) attached to a Olympus microscope (BX 51 WI; Olympus, Tokyo, Japan).

Immunostained whole-cortex slices were observed at 600× magnification at an upright microscope (BX51WI) which corresponded to a field of view of 125 μm². Apoptotic cells were counted in 18 fields of view that were assigned to cover all cortical layers in 3 different regions of each slice. Because there were no significant differences between the regions and in between the layers, the number of apoptotic cells from the 18 fields of view was averaged. For each field of view, the focus was manipulated to count cells in the whole depth of the slice. Permeabilization of the slice prior to immunostaining allowed the observation of positive cells in the depth of the slice. Using this standardized protocol, apoptotic cells were manually counted by 3 independent observers naive to the experimental condition (in a blind manner). Slices from at least 3 independent cultures were analyzed. The results are shown as percentage of the respective control condition (control is 100%).

**Electrophysiological Recordings**

All electrophysiological recordings were performed in an artificial cerebrospinal fluid (ACSF) that resembled the ionic and molecular composition of the neurobasal medium without B27 (Breuer et al. 1993). Electrophysiological recordings were performed in organotypic neocortical slices after 4–5 days in culture both at the network level using a multielectrode array (MEA) and at the cellular level using whole-cell patch-clamp recordings. Spontaneous activity was recorded in control conditions, and pharmacological agents were added after at least 4 spontaneous events had been observed.

Cultured slices were transferred to 3-D multi-MEA chips (interelectrode distances of 200 μm, electrode diameter of 30 or 40 μm; Ayanda Biosystems, Lausanne, Switzerland) on MEA 1060-INV-BC (Multi Channel Systems [MCS], Reutlingen, Germany), which were mounted on an inverted microscope (Optika microscopes, Ponteranica, Italy). Slices were superfused with an ACSF consisted of (in millimolar) 51.3, 133.6...

For the measurement of the levels of phosphorylated CREB response element–binding protein (CREB), slices were treated for 6 h with CPP, high [K+]o, and k252a as described in the Results and then harvested in a lysis buffer composed of 0.5% Triton X-100, 1 mM EGTA, 6 M Urea in PBS. The slices were allowed to sit on ice for 15 min and were briefly vortexed before use. Enzyme-Linked ImmunoSorbent Assay (ELISA) assay was performed according to manufacturer’s protocol (DuoSet IC Phospho-CREB S133, R&D systems, Minneapolis, MN). Briefly, capture antibody directed against phospho-CREB in PBS was incubated overnight in maxisorp 96-wells plates (Nunc, Wiesbaden, Germany). After washing in PBS and blocking step in 1% BSA in PBS, samples were incubated for 2 h by incubation for 2 h with detection antibody directed against phospho-CREB in 1% BSA in PBS. After washing step, 100 µl streptavidin coupled with horseradish peroxidase was incubated for 20 min. After several washes in PBS, the peroxidase activity was detected by addition of 10% w/v 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma) diluted with 0.025% v/v water peroxide 30% in 0.05 M phosphate-citrate buffer. The reaction was allowed to develop overnight at 4 °C and was measured at 405 nm on an ELISA plate reader (Infinite 200; Tecan, Crailsheim, Germany).

Statistics
For statistical analyses, Student’s t-test and analysis of variance (ANOVA) followed by Newman–Keuls post hoc test were used. The non-parametric Mann–Whitney and Kruskal–Wallis test (nonparametric equivalent of ANOVA) followed by Dunn post hoc test were used when the sample were not following a Gaussian distribution. All data are expressed as mean ± standard error of the mean. Number of apoptotic cells are represented in histograms as percentage of control values, the control being normalized at the value 100%.

Results

Caspase-3 Dependent Apoptosis In Vivo
In order to address the question whether PCD during early cortical development in vivo involves the activation of caspase-3, we performed TUNEL and caspase-3 staining in the somatosensory cortex of postnatal day mice. In agreement with previous studies (Verney et al. 2000), scattered TUNEL-positive cells could be observed in all cortical layers (Fig. 1 A,B). The occurrence of caspase-3-dependent PCD was assessed by immunostaining directed against the a-casp3. A-casp3–positive cells could be detected in all cortical layers (Fig. 1 C,D). The number and distribution of TUNEL stained and a-casp3–positive cells were very similar, suggesting that both methods label the same population of cells. This hypothesis was supported by our double staining experiments, which demonstrated a colocalization of TUNEL and a-casp3 signals (Fig. 1 E).

Because activation of caspase-3 is an early event in PCD (Marin-Teva et al. 2004), a-casp3–positive cells still showed a relatively normal morphology. The detailed microscopic analysis of a-casp3–positive cells revealed a clear neuronal morphology (Fig. 1 F). Pyramidal neurons with typical soma morphology and proximal dendrites could be identified in all cortical layers. However, in some pyramidal neurons, primary dendrites were interrupted and the soma showed a more round shape, indicating that these neurons were already affected by the cell death process.

Our data clearly indicate that neurons in all cortical layers of the newborn mouse cerebral cortex undergo caspase-3–dependent apoptosis in vivo.

Histology and Morphological Analysis of the Recorded Cells
All recorded cells were filled with 0.5% biocytin (Sigma). After recording, slices were fixed in 4% buffered formaldehyde solution for at least 24 h, rinsed in PBS, and incubated for 2 h in alexa488 coupled to streptavidin (1/800, Molecular Probes). Slices were analyzed using a confocal system (QLC100, Visitech) attached to an Olympus microscope (BX 51 WI). Images were collected with a 20× objective (Olympus) and captured using a cooled charged couple device camera (CoolSnap HQ; Roper Scientific, Trenton, NY). Because the field of view was too narrow to cover a whole neuron, mosaic images were assembled.

Measure of Levels of Phosphorylated CREB
For the measurement of the levels of the phosphorylated form of cAMP response element–binding protein (CREB), slices were treated for 6 h with CPP, high [K+]o, and k252a as described in the Results and then harvested in a lysis buffer composed of 0.5% Triton X-100, 1 mM EGTA, 6 M Urea in PBS. The slices were allowed to sit on ice for 15 min and were briefly vortexed before use. Enzyme-Linked ImmunoSorbent Assay (ELISA) assay was performed according to manufacturer’s protocol (DuoSet IC Phospho-CREB S133, R&D systems, Minneapolis, MN). Briefly, capture antibody directed against phospho-CREB in PBS was incubated overnight in maxisorp 96-wells plates (Nunc, Wiesbaden, Germany). After washing in PBS and blocking step in 1% BSA in PBS, samples were incubated for 2 h by incubation for 2 h with detection antibody directed against phospho-CREB in 1% BSA in PBS. After washing step, 100 µl streptavidin coupled with horseradish peroxidase was incubated for 20 min. After several washes in PBS, the peroxidase activity was detected by addition of 10% w/v 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma) diluted with 0.025% v/v water peroxide 30% in 0.05 M phosphate-citrate buffer. The reaction was allowed to develop overnight at 4 °C and was measured at 405 nm on an ELISA plate reader (Infinite 200; Tecan, Crailsheim, Germany).
neurons in our neocortical slice cultures revealed the typical complex morphology with a rich dendritic branching pattern and axons extending over several layers up to 850 μm in length (Fig. 2A,B).

To study caspase-3 dependent apoptosis in vitro, organotypic neocortical slices were immunostained with an antibody directed against α-casp3 (see Fig. 2D). During the first 2 days in vitro (div), slice cultures exhibited a pronounced α-casp3 immunoreactivity. At 3 div, the number of α-casp3-positive cells markedly decreased and remained constant until 12 div, the latest developmental stage analyzed in the present study. All subsequent experiments were performed on slice cultures between 4 and 6 div because during this period the apoptosis rate had already reached the plateau phase and because neocortical slice cultures at this stage reveal spontaneous activity patterns similar to those observed in the early postnatal rodent cortex in vivo (see below and Khazipov and Luhmann 2006).

We first studied the dependence of the slice cultures on external supply of trophic factors by replacing the culture medium, which was composed of neurobasal medium supplemented with B27, with neurobasal medium without B27. After incubation periods of 6, 12, or 24 h, slice cultures were immunostained for α-casp3 and the number of positive cells was determined. The number of α-casp3-positive neurons remained largely constant over 24 h indicating that this deprivation from the B27 medium supplement did not induce any increase in the number of apoptotic cells (Fig. 2C). In contrast, a previous report in dissociated cortical cultures (Poser et al. 2003; Liot et al. 2004) and our own observations on an increased cell death in dissociated cultures of cortical neurons deprived of B27 medium supplement (A. Golbs and H.J. Luhmann, unpublished data) demonstrate a difference between both culture systems in terms of sensitivity to trophic factors external supply. These observations indicate that neurons in organotypic cultures develop within an environment that allows for the release and constrains the diffusion of trophic factors supporting neuronal survival.

Activity-Dependent Control of Neuronal Apoptosis
To assess whether survival is dependent on electrical activity, slice cultures were treated with 1 μM tetrodotoxin, a blocker of voltage-dependent sodium channels (Fig. 2E). The number of apoptotic cells was significantly \( P < 0.001, n = 12 \) slices for each time point) higher after 6, 12, and 24 h of TTX application as compared with the control slices without TTX (Fig. 2F). This increase in apoptotic cells could be observed uniformly in the whole slice culture, indicating that all cortical layers showed the same sensitivity to neuronal activity. The possibility of cell death in astrocytes was assessed by double staining for α-casp3 and the marker GFAP. In these analyses, we found no evidence for colocalization of α-casp3 and GFAP, suggesting that TTX induced neuronal apoptosis and not glial cell death (Fig. 2G–I). These TTX experiments were performed in the absence of B27 supplement. In culture medium supplemented with B27, TTX application did not induce any significant changes in the apoptosis rate (data not shown), indicating that B27 can compensate for the lack of trophic factors released by neuronal activity. For this reason, all subsequent experiments were performed in neurobasal medium in the absence of B27.

Electrophysiological and Pharmacological Characterization of Spontaneous Network Activity in Neocortical Slice Cultures
Because TTX treatment caused an increase in the apoptosis level, we expected that spontaneous electrical activity patterns sustain neuronal survival. Therefore, we studied the spontaneous network activity in neocortical slice cultures at the network and single-cell level by the use of different electrophysiological recording techniques. The slice culture was
network oscillations propagated with an average velocity of 70.3 ± 0.65 m/s and a maximal amplitude of 679 ± 49.5 mV (n = 8 slices). Each event was characterized by an initial oscillation in the frequency range from 5 to 8 Hz, followed by repetitive wave-like discharges (Fig. 3C). In 5 slices, the activity appeared simultaneously at all recording sites, and in 9 slices, the activity was recorded with 60 extracellular electrodes placed onto a MEA system (Fig. 3A), and the spontaneous neuronal activity was recorded with 60 extracellular electrodes with the MEA system. In agreement with previous in vivo and in vitro observations in newborn rodent cerebral cortex (Kilb and Luhmann 2003; Khazipov et al. 2004; Dupont et al. 2006; Hanganu et al. 2006), neocortical slice cultures showed synchronized network oscillations. Spontaneous network oscillations could be recorded simultaneously over the whole MEA and in all cortical layers (Fig. 3B). The spontaneous network oscillations occurred at an average interval of 6.7 ± 0.8 min (n = 8 slices) had a duration of 9.1 ± 0.65 s and a maximal amplitude of 679 ± 18 μV (n = 8 slices). Each event was characterized by an initial oscillation in the frequency range from 5 to 8 Hz, followed by repetitive wave-like discharges (Fig. 3C). In 5 slices, the activity appeared simultaneously at all recording sites, and in 9 slices, the network oscillations propagated with an average velocity of 70.3 ± 4.9 mm/s over the MEA. Thus, the oscillatory activity in our neocortical slice cultures was highly correlated over a few hundred micrometers (Fig. 3D,E).

The pattern and pharmacology of the spontaneous network activity in organotypic neocortical cultures was also studied at the cellular level by performing whole-cell patch-clamp recordings from visually identified and biocytin-stained pyramidal neurons (see Fig. 2B). All recorded neurons (n = 40 neurons) exhibited relatively uniform membrane properties (Fig. 4A-C). The average resting membrane potential was −49.5 ± 1.5 mV and the input resistance amounted to 0.78 ± 0.12 GΩm (n = 34 neurons). All neurons were capable of firing repetitive action potentials in response to sustained depolarization (Fig. 4A). Action potential threshold was found to be at −29.5 ± 1.5 mV. Average action potential amplitude and half-maximal durations were 39.7 ± 1 mV and 7.4 ± 0.5 ms (n = 36), respectively (compare Fig. 4B). Current-voltage relationship showed no rectification (Fig. 4C). Taken together, these properties correspond to recordings of immature pyramidal neocortical neurons, as determined in acute slice preparations (Luhmann et al. 2000).

In 20 out of 28 neurons recorded for at least 20 min, spontaneous burst discharges with an average spike frequency of 7.4 ± 0.5 Hz and duration of 5.8 ± 0.9 s could be observed at intervals of 6.7 ± 0.7 min (Fig. 4D). The properties of these cellular events resembled those of the extracellular network bursts recorded with the MEA. In the remaining 8 neurons, we did not observe any spontaneous activity within the observation period of at least 20 min. Spontaneous synaptic events were also recorded in voltage-clamp mode (Fig. 4E).
Spontaneous synaptic currents occurred in bursts with an average duration of $7.09 \pm 0.48$ s ($n = 19$ slices). The first synaptic current always showed a much higher maximal amplitude with an average of $65.5 \pm 4.6$ pA and a half-maximum duration of $333 \pm 35$ ms ($n = 15$). Both, the postsynaptic potentials as well as the postsynaptic currents consisted of multiple components, indicating that this spontaneous activity was generated by a polysynaptic network.

Finally, we studied with the patch-clamp technique the effect of TTX, which caused a prominent increase in neuronal apoptosis (see Fig. 2D–F), on the spontaneous activity at the single-cell level. In all investigated neurons ($n = 3$) spontaneous burst discharges were completely blocked by TTX (Fig. 4F).

**Role of Ionotropic Glutamate Receptors**

The putative role of AMPA/kainate-type glutamate receptors was studied by incubating slice cultures in $10 \mu M$ of the antagonist CNQX (Fig. 5A). After 6, 12, or 24 h of incubation, CNQX-treated slices showed a significantly ($P < 0.001$, $n = 9$ slices for each time point) higher number of apoptotic cells as compared with the untreated controls. The specific role of AMPA receptors was further assessed by application of the AMPA receptor antagonist GYKI 52466 (Fig. 5B). For all 3 incubation times, $80 \mu M$ GYKI induced a highly significant ($P < 0.001$, $n = 6$ slices for each time point) increase in the apoptosis rate as compared with untreated controls demonstrating that the activation of AMPA glutamate receptors is essential for neuronal survival. The role of NMDA-R in the regulation of activity-dependent apoptosis during early cortical development was studied by the use of the NMDA-R antagonist CPP (Fig. 5C–F). For all 3 incubation times blockade of NMDA-R caused a significant increase in the number of apoptotic cells ($P < 0.001$, $n = 9$ slices for each time point, Fig. 5F). High-magnification microscopic analysis of the a-casp3–positive cell confirmed the neuronal nature of the apoptotic cells (Fig. 5E).

Next, we studied by whole-cell voltage-clamp recordings the role of the different ionotropic glutamate receptors in generating the spontaneous synaptic activity. Spontaneous postsynaptic burst discharges recorded in neurons from 5 DIV slice cultures were completely blocked by CNQX ($n = 4$ slices, Fig. 5G) and CPP ($n = 4$ slices, Fig. 5H), demonstrating the functional role of AMPA/kainate receptors and NMDA-Rs, respectively, in generating spontaneous network activity in neocortical slice cultures.

**Activation of L-type Ca$^+$ Channels as a Secondary Survival Pathway**

Our results suggested that calcium influx via NMDA-Rs is a critical determinant for neuronal survival in our slice cultures. To address the question whether other sources of calcium influx can substitute for the NMDA-R–dependent survival response, cells were depolarized by elevating of the extracellular potassium concentration ($[K^+]_e$) to $25 \text{ mM}$, thus activating voltage-gated calcium channels. When the CPP-treated slices were incubated in culture medium containing high $[K^+]_e$, the number of apoptotic cells was reduced to control values observed in the absence of CPP and low $[K^+]_e$ (Fig. 6D). These data indicate that high $[K^+]_e$ induces an alternative survival pathway that can overcome the blockade of NMDA-R.

To understand the activity pattern in CPP and high $[K^+]_e$ that promotes survival, MEA multichannel recordings were performed from slices cultures treated with $10 \mu M$ CPP and $25 \text{ mM}$

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**Figure 3.** Synchronized spontaneous network activity in neocortical slice cultures. (A) Photograph of a 7 DIV organotypic neocortical slice culture placed on a 60-channel MEA. Black dots represent extracellular recording electrodes, separated by $200 \mu m$. (B) Simultaneous 60-channel MEA recording from neocortical slice culture shown in (A). Four channels are damaged and were switched off. (C) Spontaneous oscillatory activity recorded with channel 45 and corresponding wavelet analysis. (D) Color plot illustrating the correlation coefficient calculated from cross-correlation analyses between channel 45 and all other channels. (E) Delay of activity onset recorded with channel 45 as compared with the activity onset in the other channels.
In agreement with our single cell recordings, spontaneous events were completely blocked by CPP (n = 5 slices, Fig. 6B). Upon addition of 25 mM extracellular potassium to the CPP-containing bathing solution, a pronounced increase in activity with a maximal amplitude of 332 ± 97 μV could be observed (n = 3 slices, Fig. 6C). However, this network activity with an average duration of 51.6 ± 16.2 s was observed only transiently during the washing phase of 25 mM potassium. Thereafter, no further network activity could be recorded for observation periods up to 1 h. It has been demonstrated previously in neonatal mouse cerebral cortex that spontaneous, synchronous electrical activity could be blocked by the antagonist of L-type calcium channels nifedipine (Corlew et al. 2004). The functional role of these voltage-dependent calcium channels on the network activity in neocortical slice cultures was studied by application of 10 μM nifedipine. Under control conditions in normal extracellular solution, nifedipine had no significant effect on the apoptosis rate (Fig. 6D).

However, in cortical slice cultures treated with 25 mM extracellular potassium and CPP, nifedipine caused a significant (P < 0.01, n = 4 slices) increase in the number of apoptotic cells, demonstrating that under blockade of NMDA-R and increased neuronal activity, L-type calcium channels have a prosurvival influence (Fig. 6D).

We then addressed the question, whether the high [K⁺]c-induced increase in electrical activity could rescue the neurons via the release of trophic factors. Slices were treated with 200 nM k252a, an inhibitor of tyrosine kinases of the neurotrophin receptors TrkB and TrkC. In comparison to CPP-treated slices, the addition of 25 mM of [K⁺]c rescued the neurons, but the prosurvival effect of high [K⁺]c was prevented in the presence of 200 nM k252a (Fig. 6F). Hence, the high [K⁺]c-induced rescue effect is dependent on the activation of tyrosine kinases of the neurotrophin receptors TrkB or TrkC.

It has been previously shown in dissociated cell cultures that high [K⁺]c activates survival signaling pathways that involve the
phosphorylation of CREB (Dolmetsch et al. 2001). We measured in our organotypic neocortical slice cultures, the level of CREB phosphorylation at the Serin 133 site (P-CREB). The addition of high [K\textsuperscript{+}] increased P-CREB levels, and this increase was not observed in the presence of 10 lM nifedipine (Fig. 6F). Hence, the activation of L-type calcium channels by high [K\textsuperscript{+}] induces the activation of the factor CREB, which has been shown to be involved in pathways promoting neuronal survival (Hardingham and Bading 2003).

The NMDA Receptor Subunits NR2A and NR2B Have Different Effects on Neuronal Survival

To elucidate the contribution of different NMDA-R subunits in the NMDA-R-dependent apoptosis process, we blocked the NR2A subunit with 400 nM NVP-AMM077 (Berberich et al. 2005) and the NR2B subunit with 3 lM ifenprodil (Williams 1993). The NR2A subunit antagonist NVP-AMM077 induced a complete blockade of the spontaneous network activity recorded in normal extracellular solution (n = 3 slices, Fig. 7A). In accordance with these electrophysiological results, blockade of the NR2A subunit also induced a significant (P < 0.001, n = 12 slices for each time point) increase in the apoptosis rate after 6, 12, and 24 h when compared with the untreated controls (Fig. 7B). Interestingly, the number of apoptotic cells was highest after 6 h treatment with NVP-AMM077 and significantly decreased with longer application times (P < 0.001 in between each time point), indicating that a NR2A-independent process is upregulated after 12--24 h. Indeed, synchronous activity was observed in slices (n = 4) preincubated for 24 h in NVP-AMM077, which indicates that changes in the mechanisms that generate spontaneous activity occur to compensate for the blockade of NR2A-containing NMDA-R (data not shown). The NR2B subunit specific antagonist ifenprodil had no significant effect on the spontaneous network activity (Fig. 7C) and on the apoptosis rate after 6 h application time (P > 0.05, n = 9 slices), but the number of apoptotic neurons increased significantly (P < 0.001, n = 9 and 14 slices) after 12 and 24 h application of ifenprodil (Fig. 7D). However, spontaneous activity was still present in slices (n = 2) preincubated for 24 h with ifenprodil (data not shown).

Figure 5. Activation of ionotropic glutamate receptors is essential for neuronal survival. (A) Blockade of AMPA/kainate glutamate receptors with 10 lM CNQX induces increase in the number of apoptotic cells compared with control condition. This increase is observed after 6, 12, and 24 h of treatment with CNQX (Mann-Whitney test, all P < 0.0001). (B) The specific AMPA receptor blocker GYKI (80 lM) also induces an increase in apoptosis rate after 6, 12, and 24 h of treatment (Mann-Whitney test, all P < 0.001). (C, D, E) In comparison to untreated control slices (C), slices treated with the NMDA receptor antagonist CPP show a homogeneous and prominent increase in the number of apoptotic cells stained for a-casp3 (D). Higher magnification of a-casp3-positive cells allows the identification of neurons with a typical somatodendritic morphology (E). As indicated by arrows, some neurons show relatively intact morphology (arrow), whereas other neurons reveal typical signs of apoptosis with round soma and degenerating processes (arrowhead). (F) Quantification of apoptosis rate by counting a-casp3-positive cells after NMDA receptor blockade reveals significant increase after 6, 12, and 24 h of treatment with 10 lM CPP (Mann-Whitney test, all P < 0.0001). Scale bar in (C) and (D) = 200 mm; in (E) = 10 mm. (G-H) Spontaneous activity recorded at single-cell level by patch-clamp technique in voltage-clamp mode was completely blocked by the addition of either 10 lM CNQX (G) or 10 lM CPP (H).
Role of GABA-A Receptors in Spontaneous Network Activity and Survival

The potential role of γ-aminobutyric acidergic synaptic mechanisms was also assessed. In control condition, spontaneous events recorded with the MEA system showed a pattern consisting of a prominent activity in the frequency range from 5 to 8 Hz (see Fig. 3C and arrow in Fig. 8A). After treatment with 100 µM gabazine, a specific antagonist of ionotopic γ-aminobutyric acid (GABA) receptors, the amplitude of this activity was significantly reduced (control: 142.1 ± 13.4 µV; gabazine 36.9 ± 3.1 µV; P < 0.0001 Mann-Whitney test, 15 spontaneous events from n = 3 slices, Fig. 8B). This change in activity pattern also induced a significant increase in apoptosis after 6, 12, and 24 h of 100 µM gabazine treatment (P < 0.001, n = 12 slices for 6 and 24 h; P < 0.01, n = 12 slices for 12 h).

Role of Neuronal Gap-Junctions in Spontaneous Network Activity and Survival

In agreement with previous observations on newborn rodent, acute slice preparations (Yuste et al. 1995; Kandler and Katz 1998) and intact cortices (Dupont et al. 2006), synchronized network activity in neocortical slice cultures from newborn
mice required intact gap junctional coupling. Application of 25 μM mefloquine, an inhibitor of neuronal, connexin 36 containing gap junctions (Cruikshank et al. 2004), blocked the synchronized network activity (n = 5 slices, Fig. 9A,B). Interestingly, the number of action potentials recorded extracellularly with the entire MEA in a 5-min observation period increased significantly (P < 0.05) from 31.5 ± 7.2 under control conditions to 138.1 ± 42.3 in mefloquine (n = 3 slices), indicating that spontaneous activity was still present, but no longer synchronized. In agreement with these electrophysiological results, 25 μM mefloquine also caused a significant increase (P < 0.001, n = 12 for 6h and n = 18 slices for both 12 and 24 h) in the number of apoptotic neurons, but only after application for 24 h (Fig. 9C).

Discussion

The main conclusions of our study on the role of electrical activity in regulating caspase-3-dependent apoptosis in neonatal mouse somatosensory cortex can be summarized as follows: 1) Caspase-3-mediated apoptosis can be demonstrated in neurons of all cortical layers in vivo. In organotypic cultures from neonatal neocortex; 2) Blockade of action-potential-dependent neuronal activity with TTX or blockade of ionotropic glutamate receptors induces an increase in the number of apoptotic neurons. 3) NMDA-R containing the NR2A or NR2B subunit show a different time course on the activity-dependent regulation of neuronal survival. 4) Calcium influx through nifedipine-sensitive high voltage-activated calcium channels provides an additional mechanism to promote neuronal survival, which involves CREB phosphorylation and Trk receptors. 5) The inhibition of ionotropic GABA receptors induced a change in spontaneous activity that suggests a link between a specific pattern of activity and survival. 6) Gap junctional coupling promotes long-time survival. 7) Extracellular multichannel and whole-cell patch-clamp recordings reveal spontaneous synchronized network activity with a pharmacological profile that correlates with the caspase-3-dependent apoptosis.

Our data demonstrate for the first time that spontaneous neuronal activity mediated by neuronal gap junctions and by glutamatergic synapses plays an important role in regulating caspase-3-dependent neuronal apoptosis in the developing cerebral cortex. Because the spontaneous electrical activity recorded in organotypic neocortical slice cultures resembles in many aspects the spontaneous activity patterns observed in vivo, our observations are probably also relevant for the
activity-dependent regulation of neuronal apoptosis during early cortical development in vivo.

**Cortical Neurons Undergo Caspase-3-Dependent Apoptosis In Vivo**

Although several studies have assessed the occurrence of apoptosis in the developing cerebral cortex (Ferrer et al. 1992;...
Neuronal Survival during Early Neocortical Development Is Activity Dependent

Previous studies have demonstrated in various model systems that electrical activity plays an important role in promoting neuronal survival (for review see Mennerick and Zorumski 2000). We used organotypic slice cultures to study in more detail the mechanisms that regulate activity-dependent neuronal survival during early neocortical development. These slice cultures showed a distribution of caspase-3-dependent apoptosis that was similar to what was observed in vivo. The pattern of spontaneous neuronal activity recorded at the single-cell level with whole-cell patch-clamp recordings and at the network level with MEAs resembled the in vivo activity pattern observed in the rodent cerebral cortex during the first postnatal week (Khazipov et al. 2004; Hangaru et al. 2006) (for review see Moody and Bosma 2005; Khazipov and Luhmann 2006). Spontaneous TTX-sensitive bursts and synchronized network activity could be blocked by ionotropic glutamate receptor antagonists (Minleheau et al. 2007) and during earlier developmental stage with gap junction blockers (Adelsberger et al. 2005; Dupont et al. 2006).

When action potentials and evoked transmitter release were blocked by TTX, the number of apoptotic cells was significantly increased, demonstrating that spontaneous electrical activity is required for neuronal survival. Our results are in good agreement with previous observations of massive cell loss in knockout mice deficient for voltage-gated sodium channels (Planells-Cases et al. 2000) and for munc13 (Verhage et al. 2000). The pattern observed in the rodent cerebral cortex during the first postnatal week (Mooney and Miller 2000) and the neocortex reveals a significantly higher expression levels of Bax and active caspase-3 than other brain structures (Menshanov et al. 2006).

Role of Glutamatergic Receptors and Postynaptic Calcium Level

The action of this anti-apoptotic activity code requires activation of ionotropic glutamate receptors. Blockade of AMPA/kainate as well as NMDA-R caused a 2- to 2.5-fold increase in the apoptosis rate. An essential role of NMDA-R in activity-dependent survival during early cortical development has been previously demonstrated in culture (Hwang et al. 1999; Yoon et al. 2003) and in vivo (Ikonomidou et al. 1999; Tome et al. 2006). NMDA-R deficient mice also reveal an approximately 2-fold increase in developmental cell death, which depends on caspase-3 and Bax (Adams et al. 2004; Rivero Vaccari et al. 2006). Our data demonstrate for the first time a contribution of different NMDA-R subunits in the control of caspase-3 dependent apoptosis. Interestingly, NR2A and NR2B subunits seem to play a different role in activity-dependent survival. Blockade of NR2A-containing NMDA-Rs causes an immediate increase in PCD, which gradually declines over 12-24 h. In contrast, blockade of the NR2B subunit induces a delayed onset in apoptosis which remains constant over at least 24 h. Activation of NMDA-Rs promotes a short-term as well as a long-lasting neuroprotection, both mediated by independent pathways (Papadia et al. 2005; Soriano et al. 2006). The NR2B subunit is specifically coupled to the ERK pathway (Krapivinsky et al. 2003) and extracellular signal-regulated kinase (ERK) is involved in long-lasting neuroprotection by regulating CREB phosphorylation (Hardingham and Bading 2003; Papadia et al. 2005). Therefore, we postulate on the basis of our results that long-lasting activity-dependent cell survival is mediated by NR2B, whereas short-term survival depends on NR2A. Further support for this hypothesis comes from recent studies (Zhou and Baudry 2006; Chen et al. 2007), which demonstrate distinct roles for NR2A and NR2B in neuroprotection.
Our data indicate that high-threshold L-type calcium channels are also capable to activate a prosurvival pathway. Under blockade of NMDA-R, an increase in \([K^+]_e \) elicited a transient synchronized network activity that was sufficient to prevent the CPP-induced apoptosis. Nifedipine blocked this high \([K^+]_e \)-induced prosurvival process but did not influence the apoptosis rate under normal experimental conditions. These data clearly indicate that an intracellular calcium rise via L-type calcium channels represents an alternative survival pathway (Ghosh et al. 1994; Tao et al. 1998). Although calcium influx via NMDA-Rs and L-type calcium channels activate different calcium-binding proteins (Marshall et al. 2003), both pathways activate kinases leading to CREB dependent BDNF synthesis (Ghosh et al. 1994; Dolmetsch et al. 2001). In our model, the activation of L-type calcium channels was associated with an increase in the level of P-CREB and the rescue of the neurons from the blockade of NMDA-R was dependent on TrkB/C receptors, which are activated by BDNF and NT-3. Our results demonstrate that in a physiological neuronal network both pathways can complement each other. This supports the hypothesis that postsynaptic calcium currents are one essential component of the survival (for review see Harter and Snyder 2007). Interestingly, \(Ca^{2+}\)-influx via NMDA-Rs or L-type voltage-gated calcium channels are the 2 key mechanisms to elicit activity-dependent release of BDNF and NT-3 in central neuronal cultures (Kolarow et al. 2007). It is thus tempting to speculate that release of neurotrophins driven by network activity is a possible link between activity and neuronal survival in our model.

Conclusion

We demonstrate that during early development, neocortical neurons undergo activity-dependent, caspase-3-mediated apoptosis. Synchronized spontaneous network bursts activating ionotropic glutamate receptors and high-threshold calcium channels as well as gap junctional coupling promotes neuronal survival. NMDA-Rs containing NR2A and NR2B subunits show a differential and time-dependent influence on the regulation of apoptosis indicating that different calcium-dependent pathways are activated. Our data suggest that disturbances of early apoptosis indicating that different calcium-dependent pathways activate kinases leading to CREB dependent BDNF regulation and limbic epileptogenesis. J. Neurosci. 27:542-552.

References


