Status epilepticus (StE) in immature rats causes long-term functional impairment. Whether this is associated with structural alterations remains controversial. The present study was designed to test the hypothesis that StE at an early age results in neuronal loss. StE was induced with lithium–pilocarpine in 12-d-old rats, and the presence of neuronal damage was investigated in the brain from 12 hr up to 1 week later using silver and Fluoro-Jade B staining techniques. Analysis of the sections indicated consistent neuronal damage in the central and lateral segments of the mediodorsal nucleus of the thalamus, which was confirmed using adjacent cresyl violet-stained preparations. The mechanism of thalamic damage (necrosis vs apoptosis) was investigated further using TUNEL, immunohistochemistry for caspase-3 and cytochrome c, and electron microscopy. Activated microglia were detected using OX-42 immunohistochemistry. The presence of silver and Fluoro-Jade B-positive degenerating neurons in the mediodorsal thalamic nucleus was associated with the appearance of OX-42 immunopositive activated microglia but not with the expression of markers of programmed cell death, caspase-3, or cytochrome c. Electron microscopy revealed necrosis of the ultrastructure of damaged neurons, providing further evidence that the mechanism of StE-induced damage in the mediodorsal thalamic nucleus at postnatal day 12 is necrosis rather than apoptosis. Finally, these data together with previously described functions of the medial and lateral segments of the mediodorsal thalamic nucleus suggest that some functions, such as adaptation to novelty, might become compromised after StE early in development.

Key words: apoptosis; development; microglia; necrosis; pilocarpine; TUNEL

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Status epilepticus (StE) and prolonged febrile seizures at an early age are associated with brain damage (Sagar and Oxbury, 1987), increased risk of epilepsy, and cognitive impairment in humans (Aicardi and Chevrie, 1970). Similarly, StE or febrile seizures in immature rats causes functional impairments (Sankar et al., 1998; Dubé et al., 2000b; Kubová et al., 2000). For example, uptake of [14C]2-deoxyglucose is reduced in the dorsal and ventral hippocampus and the mammillary bodies in rats with pentyleneetetrazol-induced StE 2 months earlier at postnatal day P10 (Hussein et al., 1995). An association of early StE with a lower seizure threshold and spontaneous seizures was reported by Babb et al. (1995), who injected kainic acid into the hippocampus at P7 and recorded spontaneous seizures with video-EEG (electroencephalogram) 5 months later. More recently, Sankar et al. (1998) recorded spontaneous seizures in 3-month-old rats with lithium–pilocarpine-induced StE during the second week of life. Furthermore, Dubé et al. (2000b) demonstrated that rats with febrile convulsions lasting for ~20 min at P10 have a lower seizure threshold for kainate as adults. Furthermore, as a result of febrile seizures, these animals exhibit increased inhibitory synaptic transmission that lasts into adulthood (Chen et al., 1999). Finally, rats with lithium–pilocarpine-induced StE at P12 exhibit motor impairment in the rotarod and open-field tests at the age of 3 months (Kubová et al., 2000).

Whether the long-term functional consequences induced by StE at an early age in rats (≥P12: corresponds to infancy in humans; Dobbing, 1970) are associated with structural damage has remained controversial. In one study, analysis of hematoxylin–eosin-stained preparations of rats with lithium–pilocarpine-induced StE during the second week of life revealed damage in the hippocampus, amygdala, thalamus, and septum (Sankar et al., 1997). Further analysis of hippocampal CA1 pyramidal cells demonstrated DNA fragmentation [terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)] and apoptotic bodies in electron micrographs of damaged neurons, favoring the idea that apoptosis contributes to the damage (Sankar et al., 1998). In other studies, permanent neuronal damage was not observed in rats experiencing StE or prolonged febrile seizures at or before P12 (Chang and Baram, 1994; Dubé et al., 2000a).

We hypothesized that it is unlikely that widespread long-term sequelae of StE in the immature brain can be attributable merely to the hippocampal damage previously described in detail. Therefore, we investigated the neuronal degeneration in the entire brain 12 hr, 24 hr, 48 hr, and 1 week after induction of StE with...
lithium–pilocarpine at P12 using both silver and Fluoro-Jade B staining. Damage was most prominent in the mediodorsal thalamic nucleus and, therefore, we focused on the mechanisms of damage in the thalamus (apoptosis vs necrosis) using TUNEL (DNA fragmentation), immunohistochemistry for caspase-3 (cytochrome c (mitochondrial electron carrier protein released during programmed cell death), cytochrome c (mitochondrial electron carrier protein released during programmed cell death), and electron microscopy (ultrastructure of damaged neurons).

**MATERIALS AND METHODS**

**Induction of status epilepticus**

Male Wistar albino rats (12-d-old; n = 76; from the facilities of the Institute of Physiology) were used. The day of birth was taken as day 0. Animals were housed in a controlled environment (temperature 22 ± 1°C; humidity 50–60%; lights on 6:00 A.M. to 6:00 P.M.) with *ad libitum* access to food and water. Experiments were approved by the Animal Care Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC and of the U.S. Public Health Service Policy (1985).

To induce Ste, rat pups (n = 54) were injected with an aqueous solution of lithium chloride (3 mmol · ml⁻¹ · kg⁻¹; i.p.; catalog #L-0505; Sigma, St. Louis, MO) on P11 followed by pilocarpine (40 mg · ml⁻¹ · kg⁻¹; i.p.; catalog #P-6503; Sigma; made in saline) 24 hr later (Hirsch et al., 1992). After pilocarpine injection, motor manifestations of seizure activity (swimming of facial muscles, chewing, head bobbing, forelimb clonus, tail erection, “swimming” movements) were monitored by an experienced observer for 2 hr. latency to the first motor seizure after pilocarpine injection was measured, and this time point was considered the beginning of Ste. Two hours later, seizure activity was interrupted with paraldehyde (0.3 ml/kg, i.p.; catalog #76260; Fluka Chemie AG, Buchs, Switzerland). To standardize the experimental group, all 50 animals with motor Ste lasting at least 2 hr were used in the experiments; 16 of these rats died within 24 hr after pilocarpine. Control animals (n = 22) were treated with equal volumes of lithium chloride, but pilocarpine solution was replaced with saline. Paraldehyde was administered 2 hr after saline injection. Animals from each nest were randomly assigned to the experimental and control groups.

**Histologic processing of tissue**

Fixation. Rats were killed at 12 hr (n = 2), 24 hr (n = 11), 48 hr (n = 17), or 1 week (n = 13) after Ste. The brains were deeply anesthetized with 20% urethane (2 g/m/kg, i.p.; catalog #U-2500; Sigma) and perfused as follows: 20 ml of 0.01M sodium PBS, pH 7.4, room temperature, followed 20% urethane (2 gm/kg, i.p.; catalog #U-2500; Sigma) and perfused as

Sections were washed three times (5 min each time) in a solution containing 0.4–0.6% formaldehyde and 0.01% citric acid in 10% ethanol, pH 5.0–5.5, for 1 min and washed three times (10 min each) in 0.5% acetic acid. All steps were performed at room temperature. Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped.

Sections from different treatment groups were analyzed in a blinded manner using a light microscope equipped with bright-field and dark-field optics. All sections (one-in-five series, 30 μm) throughout the entire rostrocaudal extent of the brain back to the occipital pole were inspected. Only shrunken argyrophilic neurons with granular silver deposits were considered to be irreversibly damaged (Fig. 1D). Neuronal damage in silver-stained sections was analyzed side-by-side with adjacent cresyl violet-stained sections.

**Fluoro-Jade B staining.** In one series of sections (30 μm; one-in-five series), degenerating neurons were stained with Fluoro-Jade B using the method described by Schmued et al. (1997). Briefly, sections were mounted from 0.1 μm sodium phosphate buffer, pH 7.4, onto gelatin-coated slides and dried at 37°C overnight. Then they were immersed in absolute alcohol for 3 min, followed by 70% ethanol for 2 min, and distilled water for 2 min. The slides were transferred to 0.06% potassium permanganate for 15 min. After rinsing with distilled water for 2 min, the slides were incubated for 30 min in 0.001% Fluoro-Jade B solution (Histo-Chem, Inc., Jefferson, AR) made in 0.1% acetic acid. Slides were rinsed in water, dried at 37°C, dehydrated in xylene, and coverslipped. Sections throughout the entire rostrocaudal extent of the brain were examined using a Leica DM2500 fluorescence microscope (13 filter cube for FITC, excitation band 450–490 nm).

**Immunohistochemistry.** Adjacent sections were processed immunochemically with antibodies raised against caspase-3 (goat polyclonal, dilution 1:2000; detects p20 subunit and precursor of caspase-3; catalog #sc-1225; Santa Cruz Biotechnology, Santa Cruz, CA), cytochrome c (mouse monoclonal, 1:14, catalog #6A7, Invitrogen, CA), caspase-3 (mouse monoclonal, 1:4000; catalog #N0273; Serotec, Oxford, UK) using the avidin–biotin method described previously in detail (Tuunanen et al., 1996). As a positive control, a thalamic section from an adult rat that experienced Ste 24 hr earlier was included into each set of immunostainings.

A separate group of animals (n = 9; five with Ste) was prepared for TUNEL staining. Brains were removed from the skull 48 hr after Ste, frozen in dry ice, and coronal sections were cut with the cryostat (20-μm-thick). Sections were incubated in 0.1 μm sodium citrate at +70°C for 30 min for permeabilization, washed with H₂O (3 × 10 min), and dried. Subsequently, 100 μl of solution containing 1 μl of terminal deoxyxynucleotidyl transferase (TdT; Promega) and 0.5 μl of fluorescein-12-DUTP (Boehringer Mannheim GmbH, Mannheim, Germany) in TdT buffer was applied on each slide, and sections were incubated for 1 hr at +37°C. Thereafter, sections were washed with 0.02 M KBPS, pH 7.4, and incubated for 2 hr in 10% normal horse serum (NHS) and 0.25% Triton X-100 in 0.02 M KBPS. Then, sections were incubated overnight in a solution containing anti-fluorescein monoclonal antibody (1:200; Boehringer Mannheim). 1% NHS, and 0.25% Triton X-100 in 0.02 M KBPS. After washing (3 × 10 min in 1% NHS and 0.25% Triton X-100 in 0.02 M KBPS, sections were incubated with biotinylated anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA), 1% NHS, 0.01% Triton X-100 in 0.02 M KBPS, and then washed with KBPS and incubated with avidin–biotin solution (Vectastain ABC kit; Vector Laboratories) according to the manufacturer’s instructions. The reaction was developed with 0.05% 3’, 3-diaminobenzidine (Pierce, Rockford, IL) and 0.04% H₂O₂ in KBPS. For a TUNEL-positive control, some sections were treated with DNAAse (1 mg/ml in 100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM CaCl₂, and 250 mM KCl; 10 min at 37°C), processed as described above.

To analyze the distribution of TUNEL-positive nuclei, labeled nuclei were plotted from sections with a computer-aided digitizing system (Minnesota Datametrics, St. Paul, MN). The anatomic boundaries were drawn from adjacent cresyl violet-stained sections using a stereomicroscope equipped with a drawing tube.

**Electron microscopy.** An additional group of animals (two controls and two with Ste) was perfused 48 hr after Ste with 20 ml of 0.01M sodium PBS, pH 7.4, (room temperature) followed by 2% paraformaldehyde and 2% glutaraldehyde in 0.1 μm sodium phosphate buffer, pH 7.4 (2 ml/gm of body weight, +4°C). The brains were post-fixed for 24 hr in the same fixative. Thereafter, the mediodorsal nucleus of the thalamus was dissected, and the tissue blocks were post-fixed in 2% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) for 2 hr, dehydrated in an ascending ethanol series, and embedded in Durcupan (Fluka, Switzerland). Semithin sections (1 μm) were cut on Reichert
Figure 1. A, Computer-generated plot demonstrating the distribution of silver-positive cells in the different nuclei of the thalamus. Each red dot represents one silver-positive cell. B, A dark-field photomicrograph demonstrating the silver-positive cells (appear as white dots) in the thalamus in a rat that experienced StE 48 hr earlier (case StE10). Note the large number of silver-positive cells in the periphery of the central segment and also in the dorsal aspect of the lateral segment. C, Computer-generated plot demonstrating the distribution of Fluoro-Jade B-positive cells in the different nuclei of the thalamus. Each red dot represents one labeled cell (case StE21). Note the similarity in the distribution with silver-positive (Figure continues)
Table 1. Distribution of silver-positive cells in the different nuclei of the thalamus in rats that experienced pilocarpine-induced status epilepticus 12, 24, 48 hr or 1 week earlier at the age of 12 d

<table>
<thead>
<tr>
<th>Thalamic nucleus</th>
<th>12 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>1 week after status epilepticus</th>
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</thead>
<tbody>
<tr>
<td>Mediodorsal nucleus (MD)</td>
<td></td>
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<tr>
<td>Medial segment</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Central segment</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lateral segment</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lateral dorsal nucleus (LD)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lateral posterior nucleus (LP)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anteroventral nucleus (AV)</td>
<td>o</td>
<td>o</td>
<td>o</td>
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<tr>
<td>Anteroventral nucleus (AM)</td>
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<td>Ventrolateral nucleus (VL)</td>
<td>o</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Ventrolateral nucleus (VLP)</td>
<td>o</td>
<td>o</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Density of silver-stained neurons was scored as follows: o, no damaged neurons; +, <5 silver-positive neurons per nucleus; ++, 5–10 silver-positive neurons per nucleus; ++++, >20 silver-positive neurons per nucleus.

RESULTS

Development and severity of status epilepticus

The development of SE correlated well with the original description by Hirsch et al. (1992). The latency to the first behavioral seizure was 519 ± 151 sec (n = 50). Motor SE lasting for at least 2 hr was observed in 50 of 54 rats, and only these animals were included into the analysis. In this group, 16 of 50 rats died within the first 24 hr. Typically, it was preceded by the loss of a righting reflex and the occurrence of a tonic phase of generalized tonic–clonic seizure. Body weight of experimental animals did not differ from that in control siblings (1 week follow-up).

Distribution of damage in silver staining

Initially, the entire brain back to the level of the brainstem was analyzed (n = 16). The mediodorsal nucleus of the thalamus contained damaged neurons in 13 of 16 rats, and the damaged neurons were present in cases analyzed 12 hr (two of two animals), 24 hr (two of two animals), 48 hr (five of five animals), or 1 week (four of seven animals) after SE (Table 1). The anterior cortical and/or medial nuclei of the amygdala contained damaged neurons in 9 of 16 cases. Cells with granular silver deposits were rare in other brain areas, including the hippocampus. Therefore, the distribution and mechanisms of neuronal damage were explored further in the thalamus. There were no silver-positive neurons observed in the controls.

The thalamus was partitioned into subnuclei according to Paxinos and Watson (1982). The density of silver-positive neurons was highest in the mediodorsal nucleus (>20 silver-positive cells per 30-μm-thick section), next highest in the lateral dorsal nucleus, lateral posterior nucleus, ventrolateral nucleus, ventromedial nucleus, and dorsal lateral geniculate nucleus (5–10 silver-positive cells per 30-μm-thick section), and next highest anteroventral
nucleus, anteromedial nucleus, ventroposterior nucleus, posterior thalamic nuclear group (<5) (Fig. 1A, Table 1).

The mediodorsal nucleus was partitioned into the medial, central, and lateral segments (Krettek and Price, 1977). Most of the silver-positive cells were located at the periphery of the central segment (Fig. 1A,B, Table 1) throughout its rostrocaudal extent. Some damaged neurons with granular silver deposits were also observed in the lateral segment. The density of silver-positive neurons appeared slightly lower (20–25 silver-positive neurons per section) in animals that were perfused 1 week after StE rather than 48 hr. The distribution of damaged neurons within the mediodorsal nucleus was similar in both groups (Table 1).

Distribution of damage in Fluoro-Jade B staining
In general, the distribution of Fluoro-Jade B-stained neurons was similar to that of silver-positive cells (Fig. 1C,F). Twelve hours after StE (two of two animals), Fluoro-Jade B-stained neurons were observed throughout the entire rostrocaudal extent of the mediodorsal nucleus of the thalamus, most of which (5–10 neurons per section) were located in the central segment, and occasionally in the lateral segment. At this time point, there was no labeling in the other thalamic nuclei. At 24 hr after StE, the density of Fluoro-Jade B-positive neurons in the mediodorsal nucleus increased to 20–30 labeled neurons per section, and at 48 hr, to 30–40 per section. Approximately 80% of the Fluoro-Jade B-positive neurons in the mediodorsal nucleus were located in the central segment and the rest in the lateral segment. In addition, there was a lower density of labeled neurons in the lateral dorsal nucleus and the lateral posterior nucleus (5–10 neurons per section). There were a few positive neurons in the ventromedial nucleus, the ventrolateral nucleus, and the posterior thalamic nuclear group (<5 neurons per section).

TUNEL
TUNEL-positive nuclei were rare in the thalamus in rats with StE 48 hr earlier (n = 5; 5 ± 1 cells per section) as well as in controls (n = 3; 9 ± 2 cells per section). Furthermore, unlike in silver preparations, the few TUNEL-positive nuclei appeared randomly scattered without any accumulation in the mediodorsal thalamic nucleus.

Caspase-3, cytochrome c, and OX-42 immunohistochemistry
We did not observe any caspase-3 or cytochrome c-immunopositive cells in the mediodorsal nucleus of the thalamus (analysis 12, 24, or 48 hr after StE). There were, however, a substantial number of OX-42-positive microglial cells with amoeboid morphology (activated microglia) in the central and lateral segments (Fig. 1G). Activated microglia were also observed in other thalamic areas with silver-positive neurons (data not shown).

Electron microscopy
Ultrastuctural analysis of neurons with a damaged appearance after light microscopic inspection revealed that the damaged cells had a condensed or lysed cytoplasm, and the cellular components were undergoing disintegration (Fig. 1H). In many cases, cells with microglial characteristics were observed in close proximity to lysed neurons (Fig. 1H). We did not identify any neurons with an apoptotic ultrastructure (nuclear condensation and fragmentation, cell surface protrusions, and formation of membrane-bounded apoptotic bodies) in the mediodorsal thalamic nucleus.

DISCUSSION
Recent data demonstrate that StE or recurrent febrile seizures occurring at an early age in rats (<P14) cause long-term functional impairment without any clear histologically assessed neuronal loss (de Feo et al., 1986; Nehlig and Pereira de Vasconcelos, 1996; Dubé et al., 2000,a,b) except in the hippocampus (Sankar et al., 1998). The present study was designed to test the hypothesis that StE at an early age leads to neuronal degeneration in brain areas that have not previously been explored in such detail. There was no hippocampal damage in animals that were perfused for histology from 24 hr up to 1 week after StE, and there was only an occasional degenerating cell in the granule cell layer of the dentate gyrus 12 hr after StE. There was consistent neuronal degeneration, however, in the thalamus, which is consistent with the findings of Sankar et al. (1997). The results of the present study extend previous observations by demonstrating that the damage is already present at 12 hr and can still be detected up to 1 week after StE. Second, neuronal degeneration is most prominent in the central and lateral segments of the mediodorsal nucleus of the thalamus. Third, the mechanism of thalamic damage in 12-d-old rats with StE is necrosis rather than apoptosis.

The highest density of degenerating neurons was observed in the periphery of the central segment and in the lateral segment of the mediodorsal thalamic nucleus. A question arises whether the damaged neurons are local inhibitory neurons or projection neurons. Previous studies indicated that the central and lateral segments contain very few glutamic acid decarboxylase-immunopositive neurons (Kuroda and Price, 1991), which suggests that the damaged silver-positive cells are projection neurons rather than inhibitory interneurons. Tract-tracing studies show that the central segment provides substantial inputs to various regions of the ventral lateral prefrontal cortex, including the lateral orbital cortex and the ventral agranular insula (Krettek and Price, 1977; Groenewegen, 1988; Ray and Price, 1993). The lateral segment innervates nonoverlapping portions of the prefrontal cortex, including the dorsolateral orbital cortex, dorsal anterior cingulate cortex, medial precentral cortex, and the lateral frontal polar cortex (Krettek and Price, 1977; Groenewegen, 1988; Ray and Price, 1993). Reciprocal connections between the thalamus and the cortex develop prenatally or during early postnatal life in rodents (Lund and Mustari, 1977; Crandall and Caviness, 1984a,b; Minciachchi and Granato, 1988). Therefore, the somata of neurons in the central and lateral segments of the mediodorsal nucleus innervating the prefrontal cortex form a candidate neuronal population damaged by StE at P12.

Why are the thalamic neurons so sensitive to damage in the lithium–pilocarpine model of StE? One explanation could be a direct toxic effect of cholinergic muscarinic receptor activation by pilocarpine. An anatomic basis supporting this idea comes from electron microscopic studies demonstrating that an input from the dorsal tegmental region to the lateral segment of the mediodorsal nucleus is cholinergic and makes asymmetric, presumably excitatory, contacts with the proximal dendrites of target neurons (Kuroda and Price, 1991). Indirect evidence arguing against cholinergic toxicity comes from experiments in which hippocampal cultures were exposed to the acetylcholinesterase inhibitor, soman, and no effect was observed on neuronal viability (Deshpande et al., 1995). Furthermore, using dissociated retinal ganglion cells, direct toxicity of pilocarpine was documented only after incubation at a very high concentration (≥0.4 mM; Vorwerk et al., 1999). In addition, rats that were treated with a high dose of pilocarpine without...
segments of the mediodorsal nucleus in most of the rats with StE, however, were observed in the central and lateral Our data cannot be explained by the inability of the developing ischemia causes caspase activation (Hu et al., 2000), and therefore these data suggest that the damage to the mediodorsal thalamic nucleus after lithium–pilocarpine-induced StE at P12 is irreversible and occurs via necrotic rather than apoptotic mechanisms.

What are the implications of the damage caused by StE to the mediodorsal thalamic nucleus in immature brain for the long-term functional outcome? A local infusion of glutamate receptor antagonists or GABA_A agonists into the mediodorsal nucleus leads to seizure suppression in adult rats (Patel et al., 1988; Cassidy and Gale, 1998). Therefore, the presumed loss of projection neurons in the mediodorsal thalamic nucleus, which converge both glutamatergic and GABAergic inputs (Ray et al., 1992) and are proposed to be involved in suppression of seizure activity (Cassidy and Gale, 1998), might result in a change in the seizure threshold. Otherwise, the mediodorsal nucleus acts as a critical link between the basal forebrain and the prefrontal cortex (Krettek and Price, 1977). Particularly, tasks assigned to the central and lateral segments of the mediodorsal nucleus include the olfactory-related functions, memory, and eye movements (McCrea and Baker, 1985). Adult rats surviving lithium–pilocarpine-induced StE have impaired learning and retention in radial maze, which is related to the severity of seizure-induced damage of the mediodorsal nucleus (Harrigan et al., 1991). These data are consistent with our recent findings in immature rats showing that animals surviving StE at P12 exhibit learning deficits in Morris water maze when tested 2–3 months later (H. Kubová, unpublished observations).

In conclusion, the present study provides evidence that pilocarpine-induced StE causes neuronal damage in selective populations of neurons in the mediodorsal thalamic nucleus as early as on P12. The mechanism of neuronal damage appears to be necrosis rather than apoptosis.

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