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Changes in neuropeptide Y protein expression following photothrombotic brain infarction and epileptogenesis.

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Abstract

This study characterized morphological changes in the cortex and hippocampus of Sprague-Dawley rats following photothrombotic infarction and epileptogenesis with emphasis on the distribution of neuropeptide Y (NPY) expression. Animals were lesioned in the left sensorimotor cortex and compared with age-matched naïve and sham-operated controls by immunohistochemical techniques at 1, 3, 7, and 180 days post-lesioning (DPL). NPY immunostaining was assessed by light microscopy and quantified by the optical fractionator technique using unbiased stereological methods. At 1, 3, and 7 DPL, the number of NPY-positive somata in the lesioned cortex was increased significantly compared to controls and the contralateral cortex. At 180 DPL, lesioned epileptic animals with frequent seizure activity demonstrated significant increases of NPY expression in the cortex, CA1, CA3, hilar interneurons, and granule cells of the dentate gyrus. In addition to NPY immunostaining, neuronal degeneration, cell death/cell loss, and astroglial response were assessed with cell-specific markers. Nissl and NeuN staining showed reproducible infarctions at each investigated time point. FJB-positive somata were most abundant in the infarct core at 1 DPL, decreased markedly at 3 DPL, and virtually absent by 7 DPL. Activated astroglia were detected in the cortex and hippocampus following lesioning and the development of seizure activity. In summary, NPY protein expression and morphological changes following cortical photothrombosis were time-, region- and pathologic state-dependent. Alterations in NPY expression may reflect reactive or compensatory responses of the rat brain to acute infarction and to the development and expression of epileptic seizures.

Keywords

neuropeptide Y (NPY); brain ischemia; photothrombosis; seizures; immunohistochemistry; stereology

1. Introduction

Neuropeptide Y (NPY) is a 36-amino acid neuroactive peptide found in the cerebral cortex, hippocampus, hypothalamus, thalamus, amygdala, striatum, brainstem, and cerebellum (Allen

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et al., 1983; Dumont et al., 1993; Parker and Herzog, 1999; Wolak et al., 2003). NPY co-localizes with somatostatin, gamma-aminobutyric acid (GABA), and nitric oxide synthase (Bidmon et al., 2001a; Kowianski et al., 2004; Kubota et al., 1994), and can modulate neuronal excitability by multiple pre- and postsynaptic mechanisms under both physiological and pathophysiological conditions. In arterial occlusion models of ischemia, variable short-term alterations of NPY expression in perilesional cortex and subcortical structures have been reported (Allen et al., 1995; Cheung et al., 1995; Grimaldi et al., 1990). In models of temporal lobe epilepsy (TLE), increased NPY mRNA and protein expression has been demonstrated in neocortical and limbic regions, particularly in GABAergic inhibitory interneurons and following its *de novo* synthesis in dentate granule cells and transport through mossy fibers to their terminals (Causing et al., 1996; Gruber et al., 1994; Marksteiner et al., 1989, 1990; Marksteiner and Sperk, 1988; McCarthy et al., 1998; Schwarzer et al., 1995; 1996; Tu et al., 2005). In the epileptic brain, presynaptic release of NPY can reduce epileptiform discharges and inhibit hippocampal seizures (Colmers and El Bahh, 2003; Tu et al., 2005; Woldbye et al., 1996), whereas transgenic mice lacking endogenous NPY exhibit hyperexcitability at the cellular level and increased sensitivity to seizure activity (Baraban et al., 1997; DePrato Primeaux et al., 2000).

Previous studies in our laboratory have shown that cortical photothrombosis can result in poststroke epilepsy characterized by seizures recorded from ipsilateral perilesional cortex associated with motor arrest of the animal (Kelly et al., 2001; Kharlamov et al., 2003). Given the variable expression of NPY in arterial occlusion models of ischemia and the increased expression of NPY following convulsive seizures in models of TLE, we hypothesized that NPY expression would be altered within the first week following cortical photothrombosis and prominent in neocortex following the development of epileptic seizures. Studies were performed to: 1) characterize NPY expression and the associated morphological changes of brain during the first week following photothrombosis; and 2) determine the distribution of NPY expression following the development of epileptic seizures and compare it to that of standard models of TLE. NPY expression was assessed immunohistochemically at 1, 3, 7, and 180 days post-lesioning (DPL); the latter time point included animals with or without epileptic seizures (Kharlamov et al., 2003). Stereological techniques were used to quantify the total number of NPY-positive somata in the entire cerebral cortex, dentate gyrus, CA1, and CA3 subfields. In addition, associated neuronal injury, degeneration, and death, and astroglial response were assessed with cresyl violet, neuron-specific nuclear antigen (NeuN), Fluoro-Jade B (FJB), and anti-glial fibrillary acidic protein (GFAP).

2. Results

2.1. Photothrombotic brain infarction

Well-demarcated and consistently-sized photothrombotic infarcts of the left frontoparietal cortex were observed during gross examination of the brains of lesioned animals at 1, 3, 7, and 180 DPL. At 180 DPL, the area of cortical infarction appeared as a cystic, scarred area, ~2.5 mm in diameter. No gross abnormalities were detected in the brains of sham-operated or naïve controls. The methods and data for lesion size measurement have been described previously (Kelly et al., 2001; Kharlamov et al., 2003). The experimental design of the present study is presented in Fig. 1.

2.2. NPY expression following photothrombotic brain infarction

2.2.1. NPY expression in control animals—Age-matched naïve and sham-operated control animals showed the same NPY localization and distribution pattern throughout the cortex and hippocampus. NPY immunoreactivity (NPY IR) was present in neuronal somata and dendrites (Fig. 2A). NPY-positive somata were fusiform or multipolar in shape. Because

the number of NPY-positive somata in naïve animals was not significantly different than that of sham-operated controls, only analysis of NPY IR of sham-operated (controls) vs. lesioned animals is presented. Quantitative assessments of NPY-positive somata in the cortex and hippocampus of controls at 1, 3, 7, and 180 DPL are presented in Figs. 3, 4.

2.2.2. NPY expression in the cortex of lesioned animals—Compared to controls and contralateral cortex, there was increased NPY IR of neuronal somata in lesioned cortex at 1, 3, and 7 DPL (Fig. 2B–D). NPY IR was decreased in neuronal dendrites in the areas of frontoparietal cortex that surrounded the infarct core. At 180 DPL, there was greater variation of NPY IR in the cortex of lesioned animals associated with established seizure activity (Fig. 2E, F). Quantitative assessment revealed significant ($p < 0.01$ to $p < 0.001$) increases in the number of NPY-positive somata in the lesioned cortex compared to the contralateral side and controls (Figs. 3, 4).

2.2.3. NPY expression in the hippocampus of lesioned animals—NPY-positive somata were found in all hippocampal subfields of lesioned animals at each post-lesioning time point (Fig. 5). No aberrant mossy fiber sprouting was detected in lesioned animals at 1, 3, 7, or 180 DPL. Lesioned animals that did not display seizure activity by 180 DPL showed a greater variation of NPY expression in the hippocampus than lesioned epileptic animals, ranging from markedly decreased expression to the level of controls. Quantitative analysis revealed a significant increase ($p < 0.01$) in the number of NPY-positive somata in CA1 and CA3 of lesioned animals vs. the contralateral side at 1 DPL and in the dentate gyrus at 3 DPL, whereas significant decreases ($p < 0.01$ to $p < 0.001$) of cell numbers in CA1, CA3, and dentate gyrus were observed at 7 DPL (Fig. 3).

2.3. NPY expression in the cortex and hippocampus of lesioned animals following the development of epileptic seizures

At 180 DPL, 5 of 10 (50%) animals were epileptic. One animal demonstrated 23 seizures, 8 of which were recorded one week prior to sacrifice. Another animal demonstrated 5 recurrent seizures in one recording session one week prior to sacrifice. The other three epileptic animals had a total of 10 seizures, many of which were recorded weeks to months before the time of sacrifice (Kharlamov et al., 2003). The two epileptic animals that exhibited frequent seizures during the last video-EEG recording session demonstrated increased NPY IR in perilesional cortex, CA1, CA3, hilar interneurons, and dentate granule cells (Fig. 2F and Fig. 6C, D). The other three epileptic animals demonstrated NPY IR similar to that of controls. NPY-positive somata of lesioned epileptic animals were stained more darkly than those of controls and lesioned nonepileptic animals. NPY-positive somata with increased NPY IR in dendrites were found in layer V–VI of the cortex at the edge of the photothrombotic infarct. There was no evidence of mossy fiber spouting in either the inner molecular layer of the dentate gyrus or in the mossy fiber terminal region of CA3 pyramidal cells in lesioned epileptic animals. At 180 DPL, epileptic animals demonstrated a significant ($p < 0.05$ to $p < 0.001$) increase in the number of NPY-positive somata compared to controls (cortex, CA1, dentate gyrus), the contralateral side (cortex, CA1, dentate gyrus), and lesioned nonepileptic animals (cortex, CA1, CA3) (Fig. 4).

2.4. Morphological changes in neurons and astrocytes following photothrombosis

2.4.1. Cresyl violet (Nissl) staining—Control animals did not demonstrate any cytoarchitectural abnormalities in the cortex or hippocampus with Nissl staining. In lesioned animals, Nissl staining showed reproducible infarctions of the left frontoparietal cortex at 1, 3, 7, and 180 DPL (Fig. 7A). The infarct core extended throughout the entire thickness of the cortex and was characterized by widespread cell death at 1, 3, and 7 DPL. In the peri-infarct area, many cells appeared either swollen or distorted; with increased distance from this area,

somata appeared progressively normal (Fig. 7C, D). At 180 DPL, Nissl staining demonstrated alterations in the cytoarchitecture of the lesioned cortex proximal to the infarct. There was no evidence of overt pathological injury to the hippocampus following photothrombosis at any post-lesion time point, including epileptic animals.

2.4.2. NeuN immunopositive neurons—NeuN IR was present in neuronal somata and dendrites throughout both hemispheres; the staining was lighter in the cytoplasm than in the nucleus. In lesioned animals, NeuN IR was markedly decreased in the ischemic core and the perilesional area at 1, 3, 7, and 180 DPL (Fig. 7E, F). NeuN IR highlighted the lesioned area more extensively than that estimated with Nissl staining. NeuN IR was reduced in ipsilateral hippocampus at 1, 3, 7, and 180 DPL (Fig. 8). Hoechst 33258 nuclear staining revealed that the majority of cells in the cortex and hippocampus were present at the investigated time points (Fig. 7B).

2.4.3. GFAP immunopositive astroglia—In controls, GFAP IR was present in astrocyte somata and their processes in the superficial and deep cortical layers and throughout the hippocampus. In lesioned animals, modest enhancement of GFAP IR was seen in the lesioned cortex at 1 DPL, whereas a gradual increase of GFAP-positive somata was found throughout the lesioned hemisphere at 3 and 7 DPL compared to the contralateral side (Fig. 7G, H). Astrocytes exhibited more hyperplasia and hypertrophy within the rim of the ischemic core at 7 DPL (Fig. 7H). GFAP-positive somata were present throughout both cortices and the hippocampus at 180 DPL; lesioned epileptic animals showed strong GFAP IR in the hippocampus.

2.4.4. Fluoro-Jade B labeled somata—FJB-positive staining was not detected in brain sections of control animals. In lesioned animals, FJB-positive somata, labeled fine processes, and terminal-like puncta were observed in the ipsilateral frontoparietal cortex surrounding the infarct core (Fig. 9). FJB-positive somata were maximal in the necrotic core at 1 DPL (Fig. 9A), less at 3 DPL (Fig. 9B), and virtually absent at 7 DPL (Fig. 9C) when only sparse numbers of FJB-positive somata were observed in the peri-infarct area. No FJB-positive somata were found in the cortex at 180 DPL. No FJB-positive somata were observed either in the contralateral cortex, or in CA1, CA3, hilus, and granule cell layer of the dentate gyrus of both hemispheres at any time point following photothrombosis.

3. Discussion

This study detailed NPY protein expression and the associated morphological changes in the brain during the first week and at 180 days following cortical photothrombosis. The main findings of this study were: 1) photothrombosis triggered widespread changes in neurons and glia in neocortex and hippocampus; some of these changes were associated with the development of epileptic seizures by 180 days; 2) NPY expression and cellular changes were time-, region- and pathologic state-dependent (brain ischemia +/- poststroke epilepsy); and 3) epileptic activity was associated with increases in NPY expression in both neocortex and hippocampus.

The first objective of this study was to characterize NPY expression and the associated morphological changes of brain during the first week following photothrombosis. In the lesioned cortex, the number of NPY-positive somata was increased significantly at 1 DPL compared to controls and the contralateral side, and remained increased at 3 and 7 DPL, although lower than that at 1 DPL. Variable alterations of NPY-positive neuronal somata were observed in CA1, CA3, and dentate gyrus at 1, 3, and 7 DPL. NPY expression patterns were analyzed in conjunction with cellular markers that provided a clear demarcation of acute neuronal injury and neurodegeneration in and proximal to the infarct core as well as potential

effects remote from the lesion. Although Nissl staining suggested that the hippocampus was spared direct injury by cortical photothrombosis and FJB-positive somata were not detected in the hippocampus at any time following photothrombosis, reduced NeuN IR in the hippocampus suggested some degree of neuronal dysfunction and/or a changed phosphorylation state of the NeuN protein (Lind et al., 2005). The distribution of increased GFAP IR was similar to that previously reported for the photothrombosis model (Bidmon et al., 2001b; Kharlamov et al., 2000; Oermann et al., 2004; Schroeter et al., 1995).

The observed differences in NPY expression in cortical and subcortical structures may be related to increased glutamate release and neuronal hyperexcitability that occur during the first week after photothrombosis (Buchkremer-Ratzmann and Witte, 1997; Schiene et al., 1996). Importantly, there was no loss of GABAergic interneurons in the cortex (other than within the infarct core) and hippocampus during this time (Frahm et al., 2004), suggesting that increased excitation may be associated with impaired GABAergic mechanisms pre- and/or postsynaptically. In arterial occlusion models of ischemia, variable alterations in NPY IR have been reported. NPY IR was increased within perilesional cortex after transient middle cerebral artery occlusion (Allen et al., 1995; Cheung et al., 1995), whereas NPY-positive somata were decreased in frontoparietal cortex at 4 hr, 1 day, and 7 days after global (4-vessel) ischemia, returning to control values at 40 days post-lesioning (Grimaldi et al., 1990). The contrasting results of these studies may be attributable to significant differences in the spatial distribution of cortical ischemia caused by the different methods of arterial occlusion and suggest that these techniques are not directly comparable to the photothrombosis method.

The second objective of this study was to determine the distribution of NPY expression following the development of epileptic seizures and to compare it to that of standard models of TLE. At 180 days after photothrombosis, there was no difference in NPY IR between controls and lesioned nonepileptic animals. Lesioned epileptic animals showed increased NPY IR in the cortex and hippocampus; two of the epileptic animals with frequent seizure activity one week prior to sacrifice demonstrated increased NPY expression in the perilesional cortex, CA1–CA3 region, hilar interneurons, and granule cells of the dentate gyrus of both hemispheres. Decreased NeuN IR and increased GFAP-IR were observed in the cortex and hippocampus of lesioned epileptic animals at 180 DPL corresponding to the alterations of NPY expression. Because animals were not monitored immediately before sacrifice, it could not be determined whether differences in NPY IR might be due to the acute effects of unrecognized seizure activity prior to sacrifice and/or chronic changes associated with the epileptic state.

Numerous studies indicate that the dentate gyrus is frequently involved in the generation of epileptic seizures (Heinemann et al., 1992; Lothman et al., 1992) and may exhibit characteristic histochemical and histopathologic changes, including changes in NPY expression (Sloviter, 1983, 1991; Sloviter et al., 2003). NPY expression was found markedly increased in hilar GABAergic interneurons, granule cells and sprouted mossy fibers (Bellmann et al., 1991; Chafetz et al., 1995; Lurton and Cavalheiro, 1997; Scharfman et al., 1999, 2002; Sperk et al., 1992; 1996; Takahashi et al., 2000; Vezzani et al., 1996). NPY in the dentate gyrus can influence synaptic transmission and neurogenesis, demonstrates remarkable plasticity after seizures (Scharfman and Gray, 2006), and may have an anticonvulsive action (Furtinger et al., 2001; Klapstein and Colmers, 1997; Vezzani et al., 1999). In the present study, epileptic animals demonstrated alterations in NPY IR in both cortex and hippocampus; however, the significance of these changes is not known at present. The absence of mossy fiber sprouting in either the inner molecular layer of the dentate gyrus or in the mossy fiber terminal region of CA3 pyramidal cells may be related to the absence of an initial, induced episode of status epilepticus following focal ischemia (Karhunen et al., 2006; Kelly et al., 2001; Kharlamov et al., 2003); the latter has yet to be validated with long-term monitoring studies using cortical and hippocampal electrodes. However, the present study reveals some of the transient and/or

long-lasting changes of NPY expression demonstrated in other models of acute seizures or epilepsy (Gall et al., 1990; Tonder et al., 1994; Vezzani et al., 2000; Vezzani and Sperk, 2004).

In summary, cortical photothrombosis triggered morphologic changes in neurons and glia that may contribute to the development of epileptic seizures. Alteration of NPY expression in the cerebral cortex and hippocampus may be a component of cortical remodeling that occurs after photothrombosis during epileptogenesis and the maintenance of the epileptic state. Future experiments are required to determine the mechanisms of the observed changes in NPY immunostaining and the exact role of NPY receptor subtypes in the cascade of cellular and molecular events in the brain following photothrombosis and epileptogenesis.

4. Material and methods

4.1 Animals and induction of cortical photothrombotic brain ischemia

Animal research experiments were carried out in accordance with the National Institutes of Health Guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee of the Allegheny-Singer Research Institute. Photothrombosis was performed on 2 mo old Sprague-Dawley (SD) rats (Taconic Farms Inc., Germantown, NY), according to the method described by Watson et al. (1985) with minor modifications. Two procedures for photothrombosis were used for these studies (Fig.1). Animals in Study 1 and Study 2 were deeply anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A midline scalp incision was made and the scalp was retracted laterally. In Study 1, the photosensitive dye rose bengal (Sigma, St. Louis, MO), was dissolved in 0.9% saline and injected over 2 min through a catheter placed in the left femoral vein at a dose of 15 mg/kg of animal body weight while the brain was illuminated through the intact skull overlying the sensorimotor cortex for 10 min by an argon laser-activated light beam (Lexel ion laser, model 75, Evergreen Laser Corporation) at a power of 150 mW. The laser was used in the Study 1 due to the necessary replacement of the halogen bulb-based system used in Study 2. In Study 2, rose bengal (30 mg/kg) was administered while the brain was illuminated with a white light beam from a light source that consisted of a power supply, fan, dichroic halogen bulb (12V, 100W), parabolic reflector, focal lens, and optical diaphragm (Olympus Danmark A/S, Glostrup, Denmark) for 20 min (Kharlamov et al., 2003). Both procedures produced a comparably-sized lesion that extended to the cortical-subcortical interface. Sham-operated animals underwent the same experimental procedure except that their skulls were not illuminated. Naïve (age-matched) animals had no surgery, rose bengal injection, or illumination, but were used for an additional comparison of the distribution and the expression of NPY. Body temperature was kept constant throughout surgery at 37°C by use of a thermo-regulated pad. After illumination, the wounds were sutured, and animals were returned to their cages in an environmentally controlled room (23 ± 2°C; 12 hr light /12 hr dark cycle) with free access to food and water. Study 1 used 27 animals: lesioned (n =12), sham-operated (n=9), and naïve (n=6), which were subdivided equally for the 1, 3, and 7 DPL time points. Study 2 used brain sections from 19 animals from our previous study (Kharlamov et al., 2003): lesioned (n=10), sham-operated (n =3), and naïve (n =6) at the 180 DPL time point (Fig. 1). These animals were monitored by intermittent digital video-EEG recordings using skull screw electrodes. Qualitative and quantitative analysis was performed on these recordings, including lesioned epileptic and lesioned nonepileptic animals. The last video-EEG monitoring session was performed one week prior to animal sacrifice (Kharlamov et al., 2003).

4.2 Perfusion, tissue processing, selection of sections, histological approaches

At the time of sacrifice, animals were deeply anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and perfused through the ascending aorta with physiological saline followed by 4% ice-cold paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were removed, post-fixed overnight in the same fixative, and cryoprotected using 30% sucrose solution. Gross examination of the brains was performed to confirm the presence of the photothrombotic infarct. Forty-micron thick sections were cut coronally on a cryostat, collected separately into plastic tissue culture plates containing a cryoprotectant solution (phosphate buffer/ethylene glycol/glycerol) for each staining protocol, and stored at -20°C . Consistency of the methods of tissue preparation was maintained for the different animal groups to minimize potential effects of methodological variation on the results of histological analysis and immunoreactivity. Twenty-four well tissue plates were used for incubation of free-floating sections. For routine histological analysis, brain sections were washed and mounted on gelatinized slides for subsequent cresyl violet staining and for staining of degenerative neurons with FJB (Schmued et al., 1997; Schmued and Hopkins, 2000). After staining, all slides were dehydrated in graded ethanols and xylene and then coverslipped with Permount or DPX (for FJB).

4.3. Immunohistochemical staining for visualization of NPY, neurons, and astrocytes

To minimize variability of the immunolabeling conditions, level-matched sections from the different groups of animals were processed together. For NPY immunostaining, brain sections were removed from the cryoprotectant solution and rinsed in 0.1 M PB followed by a 30 min incubation in 0.3% hydrogen peroxide in PB. After several rinses in 0.1 M Tris-buffered saline (TBS, pH 7.4), tissue was incubated for 30 min in TBS containing 3% normal goat serum and 0.25% Triton X-100 (TX-100) to block non-specific binding. Tissue sections were incubated in primary antibodies against NPY (rabbit anti-NPY, polyclonal, 1:2000, Peninsula, Belmont, CA) overnight at 4°C in TBS containing 1% goat serum and 0.25% TX-100. After several rinses, the tissue was incubated for 2 hr at room temperature in biotinylated goat anti-rabbit immunoglobulin (1:400; Vector, Burlingame, CA) in TBS containing 1% goat serum. Sections were rinsed and incubated in avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Lab., CA) for 1 hr. The ABC reaction was visualized by incubation in imidazole acetate buffer (pH 9.6) containing 0.05% diaminobenzidine (DAB, Sigma), 2.5% nickel ammonium sulfate, and 0.005% hydrogen peroxide for 6 min and was halted by several washings in TBS.

Identification of neuronal and astrocyte somata was made by application of the primary antibodies NeuN (1:1000; Chemicon) and GFAP (1:500; Sigma), respectively. Some sections were counterstained with Hoechst 33258 (50 ng/ml; Sigma).

The applied protocols yielded complete penetration of the antibodies through the tissue section and high quality staining suitable for morphological and quantitative assessments. Immunostaining specificity was assessed by either the omission of primary or secondary antibodies. No positive immunoreactivity or recognizable background staining was observed under these conditions. Sections were mounted on gelatinized slides, dried, dehydrated in series of graded ethanol solutions, immersed in xylene, and coverslipped with Permount (Fisher).

4.4. Quantitative analysis

The number of NPY-positive somata in the cortex, dentate gyrus, CA1, and CA3 subfields of each experimental cohort was quantified. The studies were conducted blindly with the respect to the animal group, including the outcomes of those animals monitored by digital video-EEG (Kharlamov et al., 2003). Original animal identification numbers were used as an experimental code. The coding was such that experimental groups were not known during quantitative procedures; however, coronal sections from the same animal were identified. Presence of a

cortical lesion was obvious for the animals that underwent photothrombosis; in some analyzed sections, the infarct core area was excluded from the counting because of the absence of NPY-positive somata.

Stereological analysis was performed using the Bioquant computer-based imaging system (Bioquant Image Analysis Corporation, Nashville, TN), a microscope (Nikon Eclipse E600, Japan) equipped with a motorized stage (Applied Scientific Instrument, ASI) and a “Qimaging” color camera (“Qimaging”, Canada). A low power (4x0.2) objective was used for tracing the sampling area, while stereological analysis was undertaken with a 40x0.95 objective. The motorized stage for the movement in x-y-z axes was interfaced between the microscope and the computer and operated by a joystick-control box. The digital input of the camera was displayed on the color monitor of a HP Compaq computer. Each brain region was defined under the microscope according to cytoarchitectural landmarks (Paxinos and Watson, 1998). The contour of the analyzed brain area was delineated using the tracing function of the imaging system. NPY-positive somata were counted using the optical fractionator technique (Gundersen et al., 1988; West, 2001). This technique combines the optical dissector and fractionator sampling scheme (West et al., 1991). The optical dissector involved counting NPY-positive somata in a three-dimensional probe placed systematically throughout the entire region of interest. The fractionator sampling scheme calculated total number of NPY-positive somata as the sum of somata counted, multiplied by the reciprocal of the fraction of the reference space that was sampled (Gundersen et al., 1988). The sampling parameters were established in a pilot experiment and were varied depending on the distribution of NPY-positive somata throughout the analyzed region. These parameters and fractions include: the section sampling fraction (ssf; number of sections sampled/total number of sections in the cortex or hippocampus, the area sampling fraction (asf; counting frame/grid square area) and the thickness sampling fraction (tsf; optical dissector height (15 μm)/mean tissue thickness (40 μm), as represented by: $N = \sum Q^- (1/ssf)(1/asf)(1/tsf)$ where N is the estimate of the number of NPY-positive somata and $\sum Q^-$ is the number of counted NPY-positive somata through the entire reference space. The software generated unbiased stereological estimates of the number of NPY-positive somata (N), the coefficient of error of N (CE; acceptable < 0.1), and the estimated variance due to the nugget effect (nugget percent, Nug %; at least 0.95). Small regions at the most rostral tip of each cerebral hemisphere that included part of the isocortex and olfactory cortex were not investigated. Analyzed regions were smaller in most anterior brain sections than those taken from more intermediate and posterior regions, where NPY-positive somata were counted from a larger number of frames. NPY-positive somata were counted when these somata came into focus (40x0.95 objective) at the upper surface of the section as the plane of the microscope focus was moved vertically by the computer-driven motorized stage. The following sampling scheme was applied to the investigated regions: grid area for NPY counting in the cortex 810000 μm^2 , frame area 2500 μm^2 ; grid for CA1 62500 μm^2 , frame 6400 μm^2 ; grid for CA3 62500 μm^2 , frame 6400 μm^2 ; grid for dentate gyrus 22500 μm^2 , frame 2500 μm^2 .

4.5. Data analysis

For quantitative analysis of NPY-positive somata, an average of 14–16 serial sections was used for each animal for the cortex, and an average of 12–14 sections for the dentate gyrus, CA1, and CA3 subfields. The numbers of NPY-positive somata calculated by the software for each animal were used to estimate the mean and standard error for each experimental group and/or in each analyzed region. Data for NPY-positive somata for the different DPL time points were compared using a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparisons post test. Comparison of regions of interest in the same animal (lesioned vs. contralateral hemisphere) was analyzed by paired Student's t-tests. All data were presented as

mean values $\pm$ standard errors. A p value of less than 0.05 was considered statistically significant.

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Abbreviations

FJB	Fluoro-Jade B
GABA	gamma-aminobutric acid
GFAP	glial fibrillary acidic protein
DPL	day post-lesioning
NeuN	neuron-specific nuclear antigen
NPY	neuropeptide Y
IR	immunoreactivity
TLE	temporal lobe epilepsy

Fig.1.
Schematic illustration of the experimental design.

Fig.2.

Representative photomicrographs of NPY immunoreactivity (NPY IR) in the cortex of control (A) and lesioned (B–F) animals at 1 (B), 3 (C), 7 (D), and 180 (E, F) DPL. Increased NPY expression was present in the cortex of lesioned animals at 1 DPL (B), and at 180 DPL following the development of epileptic seizures (F). Scale bar: 100 μ m (A–F).

Fig.3.

Quantitative analysis of NPY-positive neuronal somata in the cortex and hippocampus of control and lesioned animals at 1, 3, and 7 DPL. Values are means and standard errors of the number of NPY-positive somata. In lesioned cortex, the number of NPY-positive somata was increased compared to controls and the contralateral side at 1, 3, and 7 DPL. In the hippocampus, the number of NPY-positive somata was increased in CA1 and CA3 of lesioned animals compared to the contralateral side at 1 DPL, increased in the dentate gyrus at 3 DPL compared to controls and the contralateral side, and decreased in CA1, CA3, and dentate gyrus at 7 DPL compared to controls and/or the contralateral side. Significant differences are indicated by an asterisk: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Fig.4.

Quantitative analysis of NPY-positive neuronal somata in the cortex and hippocampus of control, lesioned non-epileptic, and epileptic animals at 180 DPL. At 180 DPL, the number of NPY-positive somata was significantly increased in the cortex, CA1, CA3, and dentate gyrus of lesioned epileptic animals compared to controls, lesioned non-epileptic animals, and/or the contralateral hemisphere. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Fig.5.

Low and higher power photomicrographs of NPY IR in the hippocampus of control (A) and lesioned (B–F) animals. An asterisk (*) marks the area of infarct (B). NPY-positive somata and dendrites are shown at higher magnification (C).

Representative distribution of NPY IR in CA1 (D), CA3 (E), and the hilus (F) of lesioned animals at 1 DPL. Abbreviations: a: alveus; so: stratum oriens; sp: stratum pyramidale; sl: stratum lucidum; sr: stratum radiatum; sml: stratum moleculare-lacunosum; h: hilus; gl: granule cell layer; iml: inner molecular layer; oml: outer molecular layer; DG: dentate gyrus. Scale bar: 500 μ m (A, B), 20 μ m (C), and 100 μ m (D–F).

Fig.6. NPY IR in the hippocampus of lesioned nonepileptic (A, B) and lesioned epileptic (C, D) animals at 180 DPL. NPY IR was markedly increased in epileptic brain in the hilus (white arrows) and dentate gyrus (black arrows) (C), and in CA3 (black arrows) (D). Scale bar: 100 μ m (A–D).

Fig.7.

A representative photomicrograph of an ischemic lesion in the frontoparietal cortex following photothrombosis; the border zone between a preserved and apparently intact neocortex and the ischemic core (ic) can be seen (A). Higher power photomicrographs of the region indicated by a box insert (A) are presented in the panels (B–H) at 1 (B, C, E, G), and 7 DPL (D, F, H). Nissl staining (C, D) revealed cellular injury and loss following photothrombosis. NeuN staining (E, F) highlighted the lesioned area more extensively than that estimated with Nissl staining. Neuronal changes were accompanied by activated astrocytes (G, H). Astrocytes exhibited hyperplasia and hypertrophy within the rim of the ischemic core at 7 DPL (H). Hoechst 33258 staining demonstrated that the majority of cells in the lesioned hemisphere were present at the 1 DPL time point (B). Scale bar: 1000 μm (A) and 100 μm (B–H).

Fig.8.

A representative photomicrograph of NeuN expression in the hippocampus of lesioned (A, B, C) and contralateral (D, E, F) hemispheres at 1 DPL. Marked decrease of NeuN IR was present in CA1, and CA3 subfields of the lesioned vs. the contralateral hemisphere. Scale bar: 100 μm (A–F).

Fig.9.

Representative photomicrographs of FJB-stained tissue sections of lesioned animals. FJB-positive staining appeared in degenerating neurons and dendrites. FJB-positive somata were maximal in the ischemic core at 1 DPL (A), decreased at 3 DPL (B), and diminished to nearly zero at 7 DPL (C). Scale bar: 100 μ m (A–C).