ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol Exp 2004, 64: 153-162

Fluoro-Jade and TUNEL staining as useful tools to identify ischemic brain damage following moderate extradural compression of sensorimotor cortex

Jurgita Kundrotienė[1], Anna Wägner[2] and **Sture Liljequist[1]**

[1]Department of Clinical Neuroscience, Division of Drug Dependence Research, SE-171 76 Stockholm, Sweden; [2]Department of Neurology, Karolinska Institutet, SE-182 88 Danderyd, Sweden

Abstract. Cerebral ischemia was produced by moderate compression for 30 min of a specific brain area in the sensorimotor cortex of Sprague-Dawley rats. On day 1, that is 24 h after the transient sensorimotor compression, ischemia-exposed animals displayed a marked focal neurological deficit documented as impaired beam walking performance. This functional disturbance was mainly due to contralateral fore- and hind-limb paresis. As assessed by daily beam walking tests it was shown that there was a spontaneous recovery of motor functions over a period of five to seven days after the ischemic event. Using histopathological analysis (Nissl staining) we have previously reported that the present experimental paradigm does not produce pannecrosis (tissue cavitation) despite the highly reproducible focal neurological deficit. We now show how staining with fluorescent markers for neuronal death, that is Fluoro-Jade and TUNEL, respectively, identifies regional patterns of selective neuronal death. These observations add further support to the working hypothesis that the brain damage caused by cortical compression-induced ischemia consists of scattered, degenerating neurons in specific brain regions. Postsurgical administration of the AMPA receptor specific antagonist, LY326325 (30 mg/kg; i.p, 70 min after compression), not only improved beam walking performance on day 1 to 3, respectively but also significantly reduced the number of Fluoro-Jade stained neurons on day 5. These results suggest that enhanced AMPA/glutamate receptor activity is at least partially responsible for the ischemia-produced brain damage detected by the fluorescent marker Fluoro-Jade.

The correspondence should be addressed to S. Liljequist, Email: Sture.Liljequist@ks.se

Key words: glutamate, AMPA receptors, LY326325, beam walking

INTRODUCTION

Over the years numerous animal models have been developed to facilitate the investigation of morphological, biochemical, and functional disturbances produced by cerebral ischemia (for references see Kermer et al. 1999, Lipton 1999) in attempts to mirror the conditions seen after stroke, arachnoidal haemorrhage, and head trauma in man. Occlusion of the middle cerebral artery (MCA) has by far been the most popular animal model for examination of focal brain ischemia, that is stroke, whereas paradigms of global ischemia, based upon transient occlusion of all arteries, have been used to reflect the effects of cardiac arrest in humans. In addition, animal models of experimentally induced contusion have been generated for the simulation of cerebral ischemia-induced events associated with human traumatic brain injuries (for an excellent overview see Lipton 1999).

In most studies of experimentally produced focal brain ischemia, pannecrosis (infarction) in specific brain regions concomitant with a focal neurological deficit are considered typical features for this type of brain injury. Furthermore, it is largely accepted that the focal brain damage consists not only of a core of necrotic neurons, usually represented by a cavitation in the brain tissue, but also of a surrounding area containing both necrotic and apoptotic brain neurons, the so called "penumbra" (for references see Heiss 2000, Obrenovitch 1995). Although many authors have noted and discussed the fact that mild cerebral ischemia, usually induced by transient and short-lasting (30-60 min) occlusion of MCA in some situations may cause "incomplete infarction" or "selective neuronal necrosis" (Du et al. 1996), this phenomenon, and in particular its functional consequences, has been much less investigated. However, using transient MCA occlusion, Garcia and collaborators examined in a series of excellent studies the time course and the histopathological changes observed after various degrees of incomplete infarction (Garcia et al. 1995a,b, 1996, 1997). In at least one of their studies they noted signs of subsequent motor disturbances after short-lasting arterial occlusion but described difficulties to obtain consistent findings due to large interindividual variations and the relatively diffuse pattern of motor impairments (Garcia et al. 1995b). The phenomenon of incomplete infarction and its consequences for the histopathological and neurological outcome has recently gained renewed attention with the increasing applicability of *in vivo* brain imaging techniques to investigate the effects of cerebral ischemia and to correlate the neurological disturbance with the severity of the brain injury, not only in patients (Brott et al. 1989a,b, Chua et al. 1995, Pineiro et al. 2000, Saver et al.1999) but also in various animal models of ischemic brain injury (Albensi et al. 2000, Bederson et al. 1986, Grabowski et al. 1993, Li et al. 2000, Nakagawara et al. 1997, Rogers et al. 1997, Virley et al. 2000). However, the relationship between more subtile brain injuries and functional disturbances has, with some exceptions (Allen et al. 2000, Alexis et al. 1996, Du et al. 1996, Garcia et al. 1996, 1997), received rather limited attention.

Recently we described a novel animal model of cerebral ischemia in rats (Kundrotienė et al. 2002). This model was developed as an attempt to examine molecular biology, biochemical, morphological and behavioural aspects of experimentally induced moderate cerebral ischemia following compression of a specific brain area in the sensorimotor cortex. We found that short-lasting (30 min) mild compression of this specific brain area caused a well-defined functional deficit, that is transient (5-7 days) contralateral paresis of the fore- and hindlimbs in Sprague-Dawley rats. Histopathological examination using Nissl-staining indicated that the compressive event caused selective neuronal necrosis, that is pattern of scattered dead and/or dying neurons which were particularly concentrated to pyramidal neurons in the deep cortical layer V, a brain area known to be of particular importance for limb placing reactions in the rat (De Ryck et al. 1992). On day one after the cerebral ischemic event there was a positive correlation between the number of, by Nissl staining identified dead neurons and the level of motor performance. However, more long-term (that is up to 9 days) parallel examination of the magnitude of the brain damage and the functional recovery indicated that there was a poor, and even negative correlation between these two measures.

Given the fact that results from an increasing number of publication indicate that both necrotic and apoptotic cell death is involved in the development of cerebral ischemic brain damage (Chen et al. 1997, Kuschinsky and Gillardon 2000, Lee et al. 2000, Li et al. 1997, Lipton 1999, Mattson et al. 2000, Portera-Cailliau et al. 1997a,b, Snider et al. 1999) we considered it of interest to use other, more specific, markers than Nissl-staining for the detection of neuronal cell death to characterize further the ischemic brain injury produced by extradural compression in the sensorimotor cortex of

Sprague-Dawley rats. Another goal of this study was to correlate the extent of the selective neuronal cell death to the severity of the compression-induced focal neurological deficit, that is contralateral fore- and hind-limb paresis documented as impaired beam walking performance. Thus, we have in the present series of investigations identified the degeneration of brain neurons using a novel fluorescent marker for neuronal cell death, that is Fluoro-Jade (Allen et al. 2000, Schmued et al. 1997), together with a more classical protocol for the identification of presumed apoptotic cell death by *in situ* detection of fragmented DNA, that is TUNEL staining (Gavrieli et al. 1992).

METHODS

Subjects

All the current experiments were approved by the Ethical Committee for use of Animal Subjects at the Karolinska Institutet in Stockholm, and carried out in compliance with the local guidelines for care and use of experimental animals. Male Sprague-Dawley rats (weighing about 320 g) were purchased from B&K Universal (Sollentuna, Sweden) and housed in plastic cages at the animal facilities of the Karolinska Hospital with free access to food and water. The animals were kept under conditions of constant temperature (+24^{o}C) and humidity (about 50%) with a 12 h light/dark cycle with the light turned on at 7.00 a.m. There was an adaptation period of at least 7 days prior to the start of the experiments.

Beam walking and presurgical training procedure

All animals were subjected to beam walking test. The beam walking test was carried out as described by Feeney et al. (1982) with some modifications (Kundrotienė et al. 2002) in order to identify neurological deficits due to damage of cortical areas involved in the regulation of sensorimotor functions measured as disturbances of fore- and hindlimb movements. All beam walking training and test sessions were carried out in the morning between 9 a.m. and noon. Briefly, the beam walking performance was rated according to a 7 point rating scale based upon a modified version (Kundrotienė et al. 2002) of the testing paradigm presented by Feeney et al. (1982): (i) the rat falls down; (ii) the animal is unable to walk but remains sitting/lying on the beam; (iii) the rat walks along the beam dragging the parethic hindleg (without a firm grip around the beam); (iv) the animal transverses the beam and places the parethic hindleg on the horizontal surface of the beam but is unable to retain the leg on it; (v) the rat crosses the beam and places the affected limb on the horizontal surface of the beam to support its forward locomotion during less than half of its total steps; (vi) the rat crosses the beam and places the affected limb on the horizontal surface of the beam to support its forward locomotion during *more* than half of its total steps; (vii) the animal walks the beam with no more than two footslips during the testing distance. The first postsurgical beam-walking test was performed 24 hr after the sensorimotor compression, that is on day 1. After day 1, the test was repeated with 24 h intervals for up to seven days. For more details, see Kundrotienė et al. (2002).

Surgery

Prior to surgery, the animals were weighed and anesthetized with a 3% isoflurane/air mixture whereafter they were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The flat-skull position was achieved when the incisor bar was lowered 3.3 ± 0.4 mm below horizontal zero (according to Paxinos and Watson 1998). Anesthesia was maintained using a specially designed ventilation chamber (originally developed by Freedman et al. 1992) through which a constant flow of the isoflurane/air mixture could be delivered. A longitudinal incision of the skin exposed the underlying fascia and the skull. The right maseter (temporalis) muscle was detached from the bone and its uppermost part was held away by traction. A Plexiglass piston (8 × 6 mm with the medial side shortened 2 mm) was positioned over the outlined area of compression at an angle of 20^{o}. The surface of the piston was made slightly curvilinear to fit the shape of the brain and the edges of the piston were smoothed in order to avoid tissue damage of the brain surface. The compression area was identified over the right sensorimotor cortex according to the following coordinates relative to bregma and midline on the brain surface: the anterior edge 3.5 mm laterally of bregma, the posterior edge 4.5 mm posterior of bregma, laterally from 0.5 mm to 6.5 mm on the right side of midline. After the brain area of interest had been identified and marked, the piston was temporarily withdrawn. Using a fine drill the defined area of the

skull was cut out, gently removed from the dura mater and placed into a solution of sterile saline during the time of the remaining surgery. During this procedure particular care was taken to avoid any mechanical damage to surrounding blood vessels, especially the superior sagittal sinus. Whenever subdural bleeding or hematoma occurred (in approximately one out of 30 rats) the animal was excluded from further investigations. At this point the piston was repositioned to its original coordinates and lowered to touch the surface of the dura mater. Thereafter the piston was slowly lowered an additional 3.0 mm at the rate of 1 mm/min. Following this step the applied compression was kept constant for 30 min whereafter the piston was retracted, again at the speed of 1 mm/min. The brain surface was inspected for the occurrence of subdural hematoma and/or bleeding before the skull lid was returned to its original position and the skin was sutured. The core body temperature was monitored throughout the surgery and maintained constant using a heating pad (Temperature Control Unit HB 101/2; Letica Scientific Instruments) connected to a rectal probe. Since we have previously reported (Kundrotienė et al. 2002) that already small fluctuations in the body temperature (>-0.5^{o}C) significantly altered the magnitude of the ischemic brain lesion, only animals showing a constant body temperature during surgery were included in the study.

Immunohistochemistry

Separate animal groups for behavioural test and histology have been used. All animals were tested for beam-walking task and euthanized at 3 and 5 days after compression, respectively. Each animal was anesthetized with sodium pentobarbital 60 mg/ml and transcardially perfused with 350 ml 0.1 M phosphate-buffered saline (PBS) followed the same amount of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brain was removed and placed in fixative at 4°C for 1 h, and subsequently cryoprotected in 10%, 20% and 30% sucrose in 0.1 M PBS. Brains were rapidly frozen using carbon dioxide. Ten coronal sections (10 μm thick) over the compression site were cut randomly between bregma +1.45 mm to bregma –3.05 mm according Paxinos and Watson (1998) on a freezing microtome, mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) air-dried for 24 h and stored at -20°C until processed. There were three sections placed on each slide. Distance between the collected sections (10 μm thick) was 25μm.

FLUORO-JADE STAINING

Degenerating neuronal somata and their processes were detected with Fluoro-Jade (Histochem, Jefferson, AK) as originally described by Schmued et al. (1997). Brain sections were incubated in each of the following solutions for the time indicated: 100% alcohol, 3 min; 70% alcohol, 1 min; distilled water (H_2O), 1 min; 0.06% potassium permanganate, 15 min; H_2O, 1 min; 0.001% Fluoro-Jade in 0.09% acetic acid, 30 min; H_2O, 2 × 1 min. Stained sections were allowed to dry at room temperature protected from light. Sections were not coverslipped in order to minimize the background fluorescence of Fluoro-Jade. Sections were examined using an Olympus BX61TRF microscope (Olympus Optical AB, Solna, Sweden). Measurements were performed using × 5 objective in three adjacent sections twice from each of 3-5 different brains per experimental group and coronal plane.

TUNEL STAINING

Pre-staining procedures were the same as with Fluoro-Jade. Alternating sections (n = 5 animals) were taken for comparative Fluoro-Jade and TUNEL studies. By alternating sections we imply that one section goes for TUNEL and the next for Fluoro-Jade staining, etc. There were three sections placed on each slide. 10 μm brain sections were cut in the middle of the injured cortical brain area (that is at bregma -1.30 mm according to Paxinos and Watson 1998) on the cryostat whereafter the TUNEL staining was carried out using the Apoptosis Detection Kit, Fluorescein (G3250, Promega Corp. Madison, MI, USA) in accordance to the protocol provided by the manufacturer. In short, slides were immersed in xylene after which they were hydrated through alcohols of decreasing strength to 0.85% NaCl, and finally rinsed with PBS. The sections were fixed in a 4% methanol-free formaldehyde solution for 15 min and washed in PBS at room temperature. After this the slices were exposed to 20 μg/ml Proteinase K for 8-10 min at ambient temperature, rinsed once more with PBS, fixed in 4% methanol-free formaldehyde solution and incubated with Equilibration Buffer (200 mM potassium cacodylate, 25 mM Tris HCL, 0.2 mM dithiotheitol (DTT), 0.25 mg/ml bovine albumin serum (BSA), 2.5 mM CoCl; pH 6.6) for an additional 10 min

at room temperature. Finally there was an incubation for 1 h in a dark humidity chamber at 37°C in 100 µl of TdT incubation buffer whereafter the reaction was stopped using 2 × standard sodium citrate and rinsed with PBS. The fluorescein-12-dUTP-label DNA was visualized using a fluorescence microscope.

Drugs and chemicals

The specific AMPA receptor antagonist, LY326325 (30 mg/kg; IP) was given 70 min after completion of the cortical compression. LY326325 was a generous gift from Eli Lilly & Co. (Indianapolis, IN) and was dissolved in prewarmed (about 37°C) 0.9% NaCl in a volume of 2 ml/kg.

Statistical analysis

The differences between the groups of animals were tested using one-way analysis of variance (ANOVA) with a Bonferroni correction. All data are presented as means ± SEM for *n* animals. **P*<0.05, ***P*<0.001 were considered to be statistically different.

RESULTS

We have previously described how the body weight, temperature, and age of the animals modulate the functional and histopathological outcome (using Nissl staining) produced in the current model for brain ischemia (Kundrotienė et al. 2002).

Data depicted in Fig. 1 show the beam walking performance in animals with and without post-treatment with the specific AMPA/glutamat receptor antagonist, LY326325 (Kundrotienė et al. 2002, Schoepp et al. 1995) and demonstrate that LY326325 (30 mg/kg, IP), given 70 min after completion of the extradural compression produced a significant (one-way ANOVA with Bonferroni correction **P*<0.05 control-d1 vs. LY-d1, control-d3 vs LY-d3, ***P*<0.001 control-d2 vs, LY-d2; $n_{control}$ = 14, n_{LY} = 8 animals) improvement of the functional recovery on day 1 to 3, whereas both groups of animals had regained their beam walking performance to the same extent on day 5 postsurgery. Animals subjected to the sham operation procedure displayed no difficulties in transversing the wooden beam, evidenced by the highest score in the beam-walking performance. These data are in excellent agreement with our previously reported findings (Kundrotienė et al. 2002).

Figure 2 shows two typical representations of neuronal cells stained with the fluorescent markers Fluoro-Jade (panel A, D), or the TUNEL techniques (panel B) and illustrates the localization of the damage (panel C). Analyzing and comparing alternating slices marked with Fluoro-Jade and TUNEL staining, respectively, we found that both staining matched the same cells in 80% of cases. As it was shown in our previous studies no evidence of neuronal or glial Fluoro-Jade or TUNEL labeling was observed in brains from intact (*n* = 3 animals) control animals or from sham operated (*n* = 3 animals) rat brains (Kundrotienė et al. 2004). As can be seen, labeling cells with these two different markers, respectively, gave an almost identical, scattered pattern of stained neurons when the staining was performed in two consecutively cut brain slices obtained at the bregma –1.30 mm (according to Paxinos and Watson 1998) from the same animal. In this context it should again be pointed out that we found no signs of necrosis, that is tissue cavitation, as identified by TTC staining in these animals (data not shown).

Cells identified with the fluorescent marker, Fluoro-Jade, were counted under a confocal microscope in a series of ten consecutive slices from the bregma +1.45 mm to bregma –3.05 mm (see Methods). Data

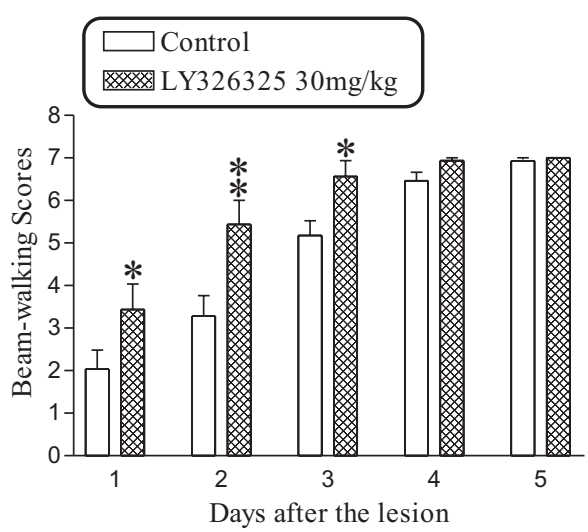

Fig. 1. Effects of the AMPA receptor antagonist LY326325 on neurological recovery after sensorimotor cortex compression in rats. Postlesion treatment with LY326325 significantly improved the neurological recovery. LY326325 was given 30 mg/kg IP 70 min after the ischemic insult. Shown are the means ± SEM; n_1 = 14 (control group), n_2 = 8 (LY326325 30 mg/kg) animals per group and time point, respectively. **P*<0.05, ***P*<0.001 (one-way ANOVA with Bonferroni levels).

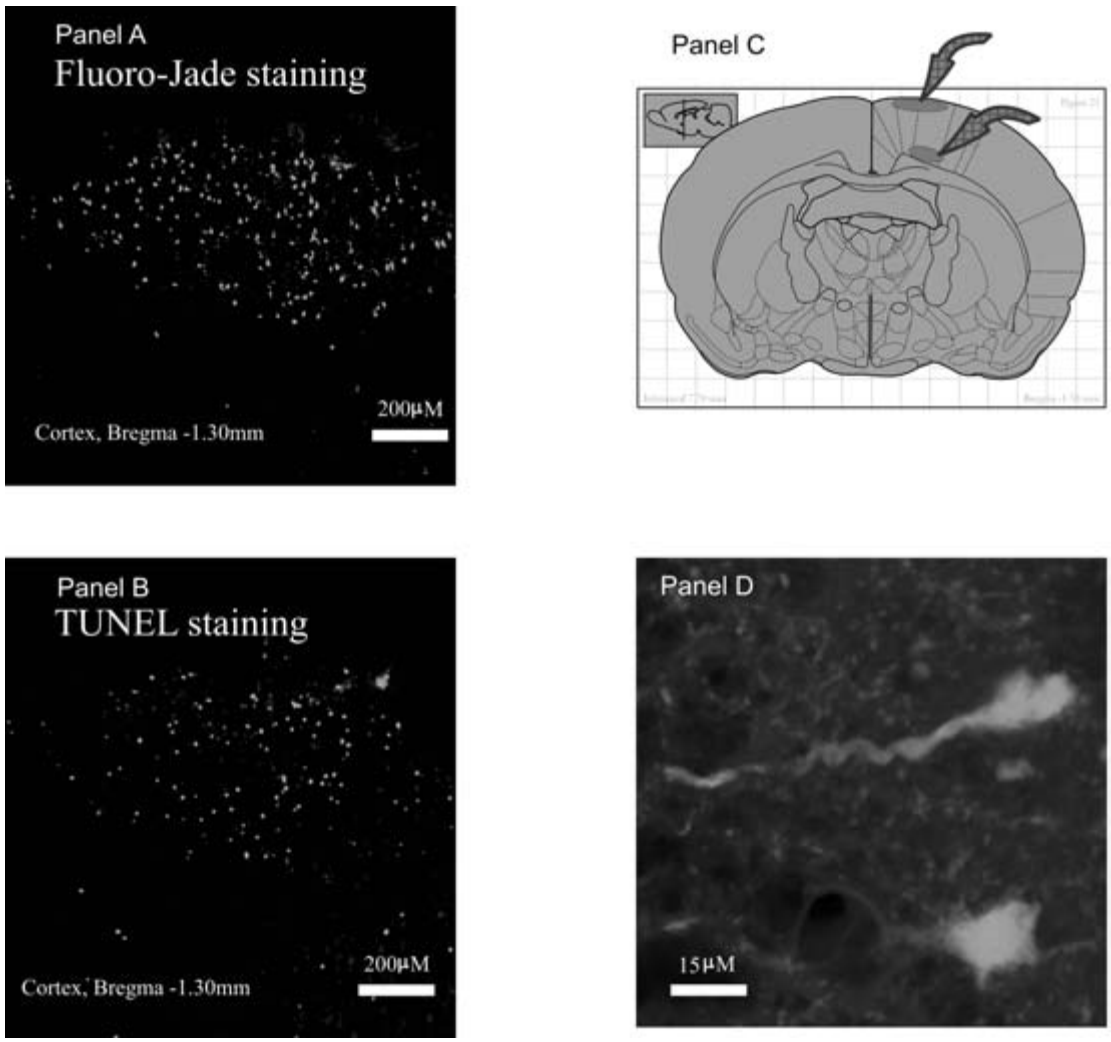

Fig. 2. Characterization of the ischemic brain damage using Fluoro-Jade (panel A) and TUNEL (panel B) staining, respectively. Note the similarity between the patterns of scattered neurons marked with Fluoro-Jade and with TUNEL staining in corresponding brain areas. Schematic drawing of the injured neurons area (panel C); degenerating neurons marked with Fluoro-Jade (panel D), magnification × 100.

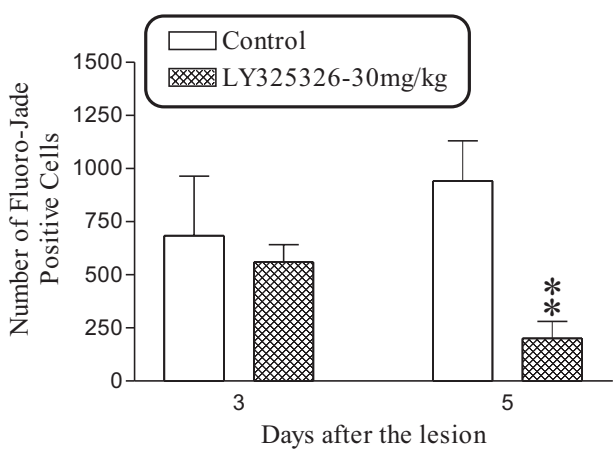

Fig. 3. Effect of the AMPA receptor antagonist LY326325 on the number of degenerating neurons identified by Fluoro-Jade after brain ischemia induced by compression of a specific brain area in the sensorimotor cortex. Postlesion administration of LY326325 (30 mg/kg, IP, 70 min after the compression) significantly reduced the amount of Fluoro-Jade positive cells. Shown are the means ± SEM, n_1 = 3 (control group day 3), n_2 = 4 (treated group day 3), $n_{3,4}$ = 5 (control and treated groups day 5, respectively). (**P<0.001 control group vs. treated group, one-way ANOVA with Bonferroni levels)

(representing means ± SEM of three to five animals per group) depicted in Fig. 3 shows the number of Fluoro-Jade-positive cells obtained on day 3 and day 5 in control and treated groups, respectively, after the ischemic event. As demonstrated, treatment with the specific AMPA receptor antagonist caused a significant reduction in the number of damaged cells on day 5 (**P<0.001 when control group vs. treated group; one-way ANOVA with Bonferroni levels) postsurgery.

DISCUSSION

An important observation in the present series of investigations is that transient compression of a specific brain area in the sensorimotor cortex produced a focal neurological disturbance (impaired beam walking), which was not accompanied by a major infarct (that is tissue cavitation). Instead there was, as detected by traditional Nissl-staining, a well-defined, scattered pattern of "selective neuronal necrosis" in specific brain structures, that is in cortical layer V, a brain area known to be of particular importance for limb placing reactions in the rat (De Ryck et al. 1992). These findings provide support for the idea that a specific pattern of selective (critically localized) neuronal cell death shows a better correlation to the functional neurological outcome than the previously often used assessment of infarct volume, that is the area of necrotic brain tissue (see Introduction). Another relevant issue in this context is whether the ischemia-produced scattered cell death gives rise to, not only necrotic, but also apoptotic neuronal and/or non-neuronal cell death. As a first step to approach this question we employed two different fluorescent markers to identify ischemia-produced selective cell death in the brain, that is staining with the relatively novel marker of neuronal cell death, Fluoro-Jade (Schmued et al. 1997), and the well-established TUNEL technique (Gavrieli et al. 1992). The TUNEL protocol has previously been regarded as a useful method for the detection of apoptotic cell death but today there is a large understanding that positive staining using this assay cannot be regarded as sufficient evidence to prove or disprove the existence of apoptotic cell death (see also below).

Fluoro-Jade is a relatively novel fluorescent marker for the identification of cell degeneration, preferentially brain neurons, which was originally developed by Schmued and co-workers and shown to have a similar sensitivity as suppressed silver staining techniques (Schmued et al. 1997). Fluoro-Jade has since then been successfully used for depicting neuronal death after exposure to various neurotoxic agents (Eisch et al. 1998, Freyaldenhoven et al. 1997, Hopkins et al. 2000, O'Dell and Marshall 2000, Zuch et al. 2000), after mild brain injuries (Allen et al. 2000) and cerebral ischemia induced by MCA (Pennypacker et al. 2000). Fluoro-Jade staining detects neuronal cells, as well as their dendrites, axons and axon terminals. There is also some recent evidence that Fluoro-Jade shows affinity for astrocytic elements but these observations have not been generally confirmed (Zuch et al. 2000). Quite recently it was reported that staining with Fluoro-Jade and TUNEL, respectively, shows a remarkable overlap thereby suggesting that Fluoro-Jade could be a useful marker of apoptotic cell death following exposure to the neurotoxin 6-hydroxy-dopamine (Zuch et al. 2000). In our hands Fluoro-Jade produced a very distinct pattern of brightly stained neurons which was easily recognized under the confocal microscope. The interindividual variation in cell counts were, with exception of a few brain slices, surprisingly low thereby giving highly consistent histopathological results. In the present study damaged cells were identified at 3 and 5 days after the ischemic event in control (saline injected) animals and in animals given a neuroprotective dose of the AMPA/glutamate receptor specific antagonist, LY326325. Following the administration of LY326325 there was a clear (~75%) reduction in the absolute number of Fluoro-Jade stained cells in cortex on day 5 as compared to the number of stained cells in control animals. However, and at least to our knowledge, there are at the present time no studies available which would suggest that Fluoro-Jade staining alone can be used for the identification and separation of necrotic *versus* apoptotic cell death, respectively.

Using the well-established protocol for TUNEL staining we obtained clear evidence supporting the idea that this assay identified a very similar, scattered pattern of selective cell death after cortical compression as with Fluoro-Jade staining. Together with the previous reports (for refs, see above) that there is a good correlation between the staining with silver grain (see above) and Fluoro-Jade, respectively, our current findings further confirm the view that Fluoro-Jade can be used as a reliable marker of selective neuronal death in brain tissue. TUNEL staining was originally regarded as a good indicator for the occurrence of apoptotic cell death. Although some authors strongly support this notion (Saraste and Pulkki 2000, Zuch et al. 2000), others sug-

gest that positive TUNEL staining does not necessarily provide sufficient proof for the existence of apoptotic neurons (Bicknell and Cohen 1995, Collins et al. 1992, Grasl-Kraupp et al. 1995) unless increased TUNEL staining is accompanied by ultrastructural changes typically associated with apoptosis (Krupinski et al. 2000, Sugawara et al. 1999).

CONCLUSIONS

The fact that the compression-induced ischemic brain injury was partially blocked by the specific AMPA/glutamate receptor antagonist LY326325 (Kundrotienė et al. 2002, Schoepp et al. 1995) suggest that the currently observed selective neuronal death at least to some extent could be due to increased glutamate receptor activity, perhaps due to an increased release of glutamate. Based upon the observations that high concentrations of glutamate produce pronounced neurotoxicity in cultured brain neurons and that various classes of glutamate receptor antagonists act as effective neuroprotectants both *in vitro* and *in vivo* (Doble 1999, Ikonomidou and Turski 1996, Scatton 1994), it has been suggested that glutamate plays a crucial role in the mediation of ischemia-produced brain injury, although the significance of an increased glutamate release as a causative factor has recently been questioned (Obrenovitch and Urenjak 1997). Despite the consistent reports that experimentally induced focal brain ischemia causes various degrees of brain injury, there is very little direct evidence for the assumption that apoptotic cell death is involved in ischemia-produced brain damage. In order to resolve this issue Colbourne et al. (1999a) investigated the mechanisms of neuronal death following global ischemia but found no evidence of apoptotic cell death in the hippocampus of gerbils, whereas Colbourne et al. (1999b) failed to obtain electron microscopic evidence for apoptosis, again following global ischemia in gerbils. It has also been reported that overactivation of NMDA receptors preferentially causes necrotic cell death while enhanced stimulation of AMPA receptors produces "apoptotic-like" morphological changes, which, however, cannot be regarded as "true" apoptotic (Ishimaru et al. 1999). The possibility that the brain injury, using our model for brain ischemia, would produce apoptotic cell death still remains an open question. Further experiments are needed to verify whether currently seen brain injury is accompanied by ultrastructural morphological changes which would prove or disprove the possible involvement of apoptotic cell death.

ACKNOWLEDGEMENT

This study was supported by the Swedish Medical Research Council (project no. 7688), Åke Wibergs Foundation, Sigurd och Elsa Goljes Minne Foundation, Ragnhild and Einar Lundströms Minne Foundation, Stroke-förbundets Stiftelse och fonder, Neurologiskt handikappades riksförbund (NHR), and funds from the Karolinska Institutet.

REFERENCES

Albensi BC, Knoblach SM, Chew BG, O'Reilly MP, Faden AI, Pekar J (2000) Diffusion and high resolution MRI of traumatic brain injury in rats: Time course and correlation with histology. Exp Neurol 162: 61-72.

Allen GV, Gerami D, Esser MJ (2000) Conditioning effects of repetitive mild neurotrauma on motor function in an animal model of focal brain injury. Neuroscience 99: 93-105.

Alexis NE, Back T, Zhao W, Dietrich WD, Watson BD, Ginsberg MD (1996) Neurobehavioral consequences of induced spreading depression following photothrombotic middle cerebral artery occlusion. Brain Res 706: 273-282.

Bederson JB, Pitts L, Tsuji M, Nishimura MC, Davis RL, Bartkowski H (1986) Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurologic examination. Stroke 3: 472-476.

Bicknell GR, Cohen GM (1995) Cleavage of DNA to large kilobase pair fragments occurs in some forms of necrosis as well as apoptosis. Biochem. Biophys Res Commun 207: 40-47.

Brott T, Adams HP, Olinger CP, Marler JR, Barsan WG, Biller J, Spilker J, Holleran R, Eberle R, Hertzberg V, et al. (1989) Measurements of acute cerebral infarction: a clinical examination scale. Stroke 20: 864-867.

Brott T, Marler JR, Olinger CP, Adams HP, Tomsick T, Barsan WG, Biller J, Eberle R, Hertzberg V, Walker M (1989b) Measurements of acute cerebral infarction: lesion size by computed tomography. Stroke 20: 871-875.

Chen J, Jin K, Chen M, Pei W, Kawaguchi K, Greenberg DA, Simon RP (1997) Early detection of DNA strand breaks in the brain after transient focal ischemia: Implications for the role of DNA damage in apoptosis and neuronal cell death. J Neurochem 69: 232-245.

Chua MG, Davis SM, Infeld B, Rossiter SC, Tress BM, Hopper JL (1995) Prediction of functional outcome and tissue loss in acute cortical infarction. Arch Neurol 52: 496-500.

Colbourne F, Li H, Buchan AM, Clemens JA (1999a) Continuing postischemic neuronal death in CA1: Influence of ischemia duration and cytoprotective doses of NBQX and SNX-111 in Rats. Stroke 30: 662-668.

Colbourne F, Sutherland GR, Auer RN (1999b) Electron microscopic evidence against apoptosis as the mechanism of

neuronal death in global ischemia. J Neurosci 19: 4200-4210.

Collins RJ, Harmon BV, Gobe GC, Kerr J (1992) Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Int J Radiat Biol 61: 451-453.

De Ryck M, Reempts JV, Duytschaever H, Deuren BV, Clincke G (1992) Neocortical localization of tactile/proprioceptive limb placing reactions in the rat. Brain Res 573: 44-60.

Doble A (1999) The role of excitotoxicity in neurodegenerative disease: Implications for therapy. Pharmacol Ther 81: 163-221.

Du C, Hu R, Csernansky CA, Hsu CY, Choi DW (1996) Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? J Cereb Blood Flow Metab 16: 195-201.

Eisch AJ, Schmued LC, Marshall JF (1998) Characterizing cortical neuron injury with Fluoro-Jade labeling after a neurotoxic regimen of methamphetamine. Synapse 30: 329-333.

Feeney DM, Gonzalez A, Law WA (1982) Amphetamine, haloperidol, and experience interact to affect rate of recovery after motor cortex injury. Science 217: 855-857.

Freedman NL (1992) A sliding gas-delivery system and ventilating chamber for small animals fixed in standard stereotaxis instruments. Behav Res Methods Instrum Comput 24: 423-425.

Freyaldenhoven TE, Ali SF, Schmued LC (1997) Systemic administration of MPTP induces thalamic neuronal degeneration in mice. Brain Res 759: 9-17.

Garcia JH, Liu KF, Ho KL (1995a) Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex. Stroke 26: 636-642.

Garcia JH, Wagner S, Liu KF, Hu XJ (1995b) Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. Stroke 26: 627-634.

Garcia JH, Lassen NA, Weiller C, Sperling B, Nakagawara J (1996) Ischemic stroke and incomplete infarction. Stroke 27: 761-765.

Garcia JH, Liu K-F, Ye Z-R, Gutierrez JA (1997) Incomplete infarct and delayed neuronal death after transient middle cerebral artery occlusion in rats. Stroke 28: 2303-2310.

Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119: 493-501.

Grabowski M, Brundin P, Johansson BB (1993) Paw-reaching, sensorimotor, and rotational behavior after brain infarction in rats. Stroke 24: 889-895.

Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) In situ detection of fragmented DNA (TUNEL Assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21: 1465-1468.

Heiss W-D (2000) Ischemic penumbra: evidence from functional imaging in man. J Cereb Blood Flow Metab 20: 1276-1293.

Hopkins KJ, Wang G, Schmued LC (2000) Temporal progression of kainic acid induced neuronal and myelin degeneration in the rat forebrain. Brain Res 864: 69-80.

Ikonomidou C, Turski L (1996) Prevention of trauma-induced neurodegeneration in infant and adult rat brain: Glutamate antagonists. Metab Brain Dis 11: 125-141.

Ishimaru MJ, Ikonomidou C, Tenkova TI, Der TC, Dikranian K, Sesma MA, Olney JW (1999) Distinguishing excitotoxic from apoptotic neurodegeneration in the developing rat brain. J Comp Neurol 408: 461-476.

Kermer P, Klöcker N, Bähr M (1999) Neuronal death after neuronal injury. Cell Tissue Res 298: 383-395.

Krupinski J, Lopez E, Marti E, Ferrer I (2000) Expression of caspases and their substrates in the rat model of focal cerebral ischemia. Neurobiol Dis 4: 332-342.

Kundrotienė J, Wägner A, Liljequist S (2002) Extradural compression of sensorimotor cortex: a useful model for studies on ischemic brain damage and neuroprotection. J Neurotrauma 19: 69-84.

Kundrotienė J, Cebers G, Wagner A, Liljequist S (2004) The NMDA NR2B subunit-selective receptor antagonist, CP-101,606, enhances the functional recovery the NMDA NR2B subunit-selective receptor and reduces brain damage after cortical compression-induced brain ischemia. J Neurotrauma 21: 83-93.

Kuschinsky W, Gillardon F (2000) Apoptosis and cerebral Ischemia. Cerebrovasc Dis 10: 165-169.

Lee JM, Grabb MC, Choi DW (2000) Brain tissue responses to ischemia. J Clin Invest 106: 723-731.

Li Y, Chopp M, Powers C, Jiang N (1997) Apoptosis and protein expression after focal cerebral ischemia in rat. Brain Res 765: 301-312.

Li F, Liu KF, Silva MD, Omae T, SotakCH, Fenstermacher JD, Fisher M, Hsu CY, Lin W (2000) Transient and permanent resolution of ischemic lesions on diffusion-weighted imaging after brief periods of focal ischemia in rats: correlation with histopathology. Stroke 31: 946-954.

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 4: 1431-1568.

Mattson MP, Culmsee C Yu ZF (2000) Apoptotic and antiapoptotic mechanisms in stroke. Cell Tissue Res 301: 173-187.

Nakagawara J, Sperling B, Lassen NA (1997) Incomplete brain infarction of reperfused cortex may be quantitated with iomazenil. Stroke 28: 124-132.

Obrenovitch TP (1995) The ischaemic penumbra: Twenty years on. Cerebrovasc. Brain Metabol Rev 7: 297-323.

Obrenovitch TP, Urenjak J (1997) Altered glutamatergic transmission in neurological disorders: from high

extracellular glutamate to excessive synaptic efficacy. Progr Neurobiol 51: 39-87.

O'Dell SJ, Marshall JF (2000) Repeated administration of methamphetamine damages cells in the somatosensory cortex: overlap with cytochrome oxidase-rich barrels. Synapse 37: 32-37.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates (4th ed.) Academic Press, New York, 256 p.

Pennypacker KR, Eidizadeh S, Kassed CA, O'Callaghan JP, Sanberg PR, Willing AE (2000) Expression of *fos*-related antigen-2 in rat hippocampus after middle cerebral arterial occlusion. Neurosci Lett 289: 1-4.

Pineiro R, Pendlebury ST, Smith S, Flitney D, BlamireAM, Styles P, Matthews PM (2000) Relating MRI changes to motor deficit after ischemic stroke by segmentation of functional motor pathways. Stroke 31: 672-679.

Portera-Cailliau C, Price DL, Martin LJ (1997a) Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. J Comp Neurol 378: 70-87.

Portera-Cailliau C, Price DL, Martin LJ (1997b) Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in adult brain are morphologically distinct: further evidence for an apoptosis-necrosis continuum. J Comp Neurol 378: 88-104.

Rogers DC, Campbell CA, Stretton JL, Mackay KB (1997) Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 28: 2060-2065.

Saraste A, Pulkki K (2000) Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 45: 528-537.

Saver JL, Johnston KC, Homer D, Wityk R, Koroshetz W, Truskowski LL, Haley EC (1999) Infarct volume as a surrogate or auxiliary outcome measure in ischemic stroke clinical rials. The RANTTAS Investigators. Stroke 30: 293-298.

Scatton B (1994) Excitatory amino acid receptor antagonists: a novel treatment for ischemic cerebrovascular diseases. Life Sci 55: 2115-2124.

Schmued LC, Albertson C, Slikker W (1997). Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 751: 37-46.

Schoepp DD, Lodge D, Bleakman D, Leander JD, Tizzano JP, Wright RA, Palmer AJ, Salhoff CR, Ornstein PL (1995) *In vitro* and *in vivo* antagonism of AMPA receptor activation by (3S, 4aR, 6R, 8aR)-6-[2-(1(2)H-Tetrazole--5-yl) Ethyl] decahydroisoquinoline-3-carboxylic acid. Neuropharmacology 34: 1159-1168.

Snider BJ, Gottron FJ, Choi DW (1999) Apoptosis and necrosis in cerebrovascular disease. Ann N Y Acad Sci 893: 243-253.

Sugawara T, Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH (1999) Mitochondrial release of cytochrome C corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia. J Neurosci. 19: RC39.

Virley D, Beech JS, Smart SC, Williams SCR, Hodges H, Hunter AJ (2000) A temporal MRI assessment of neuropathology after transient middle cerebral artery occlusion in the rat: correlations with behavior. J Cereb Blood Flow Metab 20: 563-582.

Zuch CL, Nordstroem VK, Briedrick LA, Hoernig GR, Granholm AC, Bickford PC (2000) Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. J Comp Neurol 20: 440-454.

Received 7 July 2003, accepted 20 November 2003