ELSEVIER

European Journal of Pharmacology 395 (2000) 137–141

ejp

www.elsevier.nl/locate/ejphar

Neuroprotective effect of 8-OH-DPAT in global cerebral ischemia assessed by stereological cell counting

Lars Torup [a,c,*], Arne Møller [b], Thomas N. Sager [b], Nils H. Diemer [c]

[a] *Department of Neurodegenerative Disorders, H. Lundbeck A/S, Valby, Denmark*
[b] *Department of Pharmacology, NeuroSearch A/S, Pederstrup, Denmark*
[c] *Laboratory of Neuropathology, Institute of Molecular Pathology, University of Copenhagen, and Neuroscience Pharmabiotech Center, Frederik V's Vej 11, 2100-Denmark*

Received 30 September 1999; received in revised form 17 February 2000; accepted 25 February 2000

Abstract

The neuroprotective effect of the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) was tested in a 2-vessel occlusion model in rats. The post-ischemic core temperature was carefully monitored for 24 h. After 7 days of survival, the viable CA1 neurons were counted in an 8-OH-DPAT (125 μg/kg/h) and vehicle-treated group using the optical fractionator method. The vehicle-treated ischemic rats had a median number of dorsal CA1 neurons of 49,900 whereas the 8-OH-DPAT-treated ischemic rats had a significant lower median number of dorsal CA1 neurons 105,200 ($P = 0.018$). 8-OH-DPAT significantly lowered the core temperature compared to the vehicle-treated group during the 24-h post-ischemic period. Hypothermia is proposed as a possible explanation of the neuroprotective effect of 8-OH-DPAT.

Keywords: Cerebral ischemia; 5-HT receptor; 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin); Stereology; (Rat)

1. Introduction

Global cerebral ischemia as for example seen during cardiac arrest is known to produce delayed irreversible brain damage. Induction of global cerebral ischemia by a transient occlusion of the carotid arteries in rats causes delayed neuronal death in specific areas of the brain. Cell loss develops in the CA1 sector of hippocampus 2–4 days after transient global ischemia. Many different pharmacological approaches have been used to prevent the delayed neuronal death. Previously, 5-HT_{1A} receptor agonists such as buspirone, urapidil, *R*-(−)-2-4-[(chroman-2-ylmethyl)-amino]-butyl-1,1-dioxo-benzo[*d*] isothiazolone HCl (Bay X 3702) and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) have been shown to have neuroprotective properties in both global and focal models of cerebral ischemia (Bielenberg and Burkhardt, 1990; Prehn et al., 1991, 1993; Shibata et al., 1992; Peruche et al., 1994; Nakata et al., 1997; Semkova et al., 1998). 5-HT_{1A} receptors are located in CA1, CA3 and dentate gyrus in hippocampus, septum, amygdala, cortex and raphe nuclei. In the raphe nuclei, the 5-HT_{1A} receptor acts as an autoreceptor regulating the firing of the serotonergic neurons. In all other areas, the 5-HT_{1A} receptor is situated post-synaptically. Stimulation of 5-HT_{1A} receptors decreases neuronal activation by opening of potassium channels. The proposed neuroprotective mechanism by 5-HT_{1A} receptor agonists is that a stimulation of 5-HT_{1A} receptors in hippocampus leads to hyperpolarization and thus inhibits the excitotoxic stimulation by glutamate after cerebral ischemia. A down-regulation of the 5-HT_{1A} receptor density in dentate gyrus, cortex and lateral reticular thalamic nucleus has been seen 24 h after global cerebral ischemia in rats (Torup et al., 1999). The 5-HT_{1A} receptor response to cerebral ischemia seems to be selective for this receptor as no change is seen for the 5-HT_{2A} receptor in the same areas. Although 5-HT_{1A} receptor agonists have been tested in various models of cerebral ischemia the cell damage has not previously been estimated in an unbiased manner. The purpose of this study was to assess the neuroprotective effect of a 5-HT_{1A} receptor agonist after 2-vessel occlusion using the newly developed optical fractionator technique.

* Corresponding author. Department of Neurodegenerative Disorders, H. Laundback A/S, Ottiliavej 9, 2500-Denmark. Tel.: +45-36-44-24-25/2504, fax: +45-36-30-10-79.

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00175-8

2. Materials and methods

2.1. Surgical procedure

Male wistar rats weighing 300–320 g (Moellegaard Breeding Center, Denmark) were anaesthetized with mebumal (pentobarbital 50 mg/ml NYCOMED DAK, DK) and a mini-osmotic pump model 2001 (Alza, USA) delivering either 125 μg/kg/h 8-OH-DPAT, Tocris Cookson, Bristol, UK) or vehicle was inserted subcoutaneously. Vehicle consisted of 50% ethanol. Furthermore, a radio-thermometer PhysioTel® implant (DSI, St. Paul, MN, USA) was implanted i.p. The ischemia was performed according to the method developed by Smith et al. (1984). The rats were fasted overnight with access to water. On the following day, they were anesthetized with halothane in a mixture of N_2O:O_2 and orally intubated and respirated during the surgical procedure. The left femoral artery was cannulated to monitor the blood gasses and blood glucose and to withdraw blood (0–10 ml). Arterial pCO_2 was maintained between 40 and 42 mm Hg and pO_2 between 100 and 120 mm Hg. The rectal temperature was kept at 37.5°C with a heating lamp. After discontinuation of halothane, the carotid arteries were exposed and clamped for 11 min during which the mean systemic blood pressure was maintained at 60 mm Hg by blood redrawal. After the surgery, the animals were caged individually and the core temperature was sampled on a computer every 5 min for 24 h.

All animal experiments were carried out in accordance with the European Communities Council Resolves of 24th November 1986 (86/609/EEC) and approved by the Danish State Research Inspectorate. Effort was made to minimize any suffering of the animal used.

2.2. Tissue preparation

Seven days after the ischemia, the rats were anaesthetized in halothane and the brains were perfusion-fixed by transcardiac perfusion with phosphate buffered formaldehyde. The brains were placed in phosphate buffered formaldehyde solution for at least a week after which 6-mm blocks containing the hippocampus were cut. The blocks were dehydrated in ascending concentrations of ethanol solutions (70–96–99%) for 24 h after which the blocks were infiltrated with hydroxy ethyl methacrylate (Technovit 7100 added hardner I, Kulzer Histo-Technic, Wehrheim, Germany). After 9 days, the brain blocks were embedded in hydroxy ethyl methacrylate added hardener II. After at least 24 h, the plastic blocks were coronally cut on a microtome (Leica RM 2165, Nussloch, Germany) in 40 μm section and every 10th section was sampled from bregma −1.8 mm to bregma −6.8 mm (Paxinos and Watson, 1997). The slides were placed in water and mounted on object glass (Menzel, Superfrost Plus, Struers, Copenhagen, Denmark) and immediately placed in an oven at 60°C for 24 h. The slides were stained for 70 min in a solution of 50 ml. Giemsa solution (Azur–eosin–methylene blue solution, Merck Diagnostica, Darmstadt, Germany) and 200 ml 1/15 m potassium dihydrogenphosphate followed by 20 dips in 1% acetic acid and differentiated in ethanol (Iniguez et al., 1985).

2.3. Cell counting

The number of viable ca1 neurons in dorsal hippocampus was counted by use of the optical fractionator method (Gundersen et al., 1988; West, 1999). A viable neuron was identified by an intact nucleolus. The counting was performed on either the left or right part of the hippocampus. Every 10th section was sampled ($f_1 = 10$) and a fraction of the sampling area was counted (counting frame area: $a_{\text{frame}} = 606\ \mu m^2$, sampling area: $a_{\text{sampling}} = 22{,}500\ \mu m^2$). Each starting frame was randomly selected and the size of the step from frame to frame was determined by $x = 150$ μm and $y = 150$ μm. The counted fraction was thus fraction factor $f_2 = a_{\text{sampling}}/a_{\text{frame}}$. A fraction of the depth (height of the dissector, $h_{\text{dis}} = 15$ μm) was counted (fraction factor $f_3 =$ section depth$/h_{\text{dis}}$). The estimated total number of neurons in CA1 was thus the sum of the counted nucleoli ΣQ^- multiplied by the fraction factors f_1, f_2, and f_3:

$N_{\text{total}} = \Sigma Q^- f_1 f_2 f_3$.

2.4. Statistics

The median cell counts for the two groups were compared by Mann–Whitney U test. The temperature was analyzed by repeated measure of analysis of variance.

3. Results

Five out of 13 operated rats in the 8-OH-DPAT-treated group died of pulmonary edema immediately after the operation. All animals appeared healthy before the surgery. No animals died in the vehicle-treated group. During the tissue preparation, two of the brain blocks were distorted in such a way that it was impossible to sample from the CA1 region of hippocampus. The vehicle-treated ischemia group ($n = 7$) had a median number of 49,900 neurons in

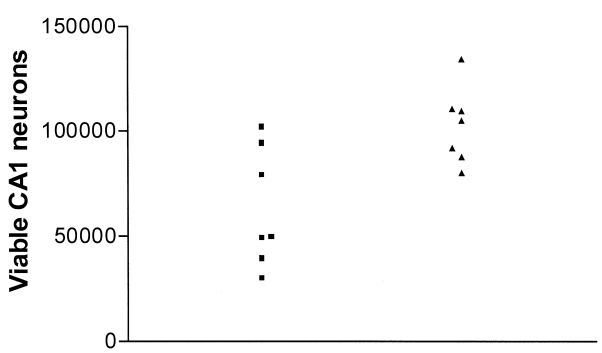

Fig. 1. Number of viable CA1 neurons in one hemisphere dorsal hippocampus. Vehicle-treated ischemic rats are indicated by solid squares and 8-OH-DPAT-treated ischemic rats are indicated by solid triangles. Medians are compared by Mann–Whitney U test, $P = 0.018$.

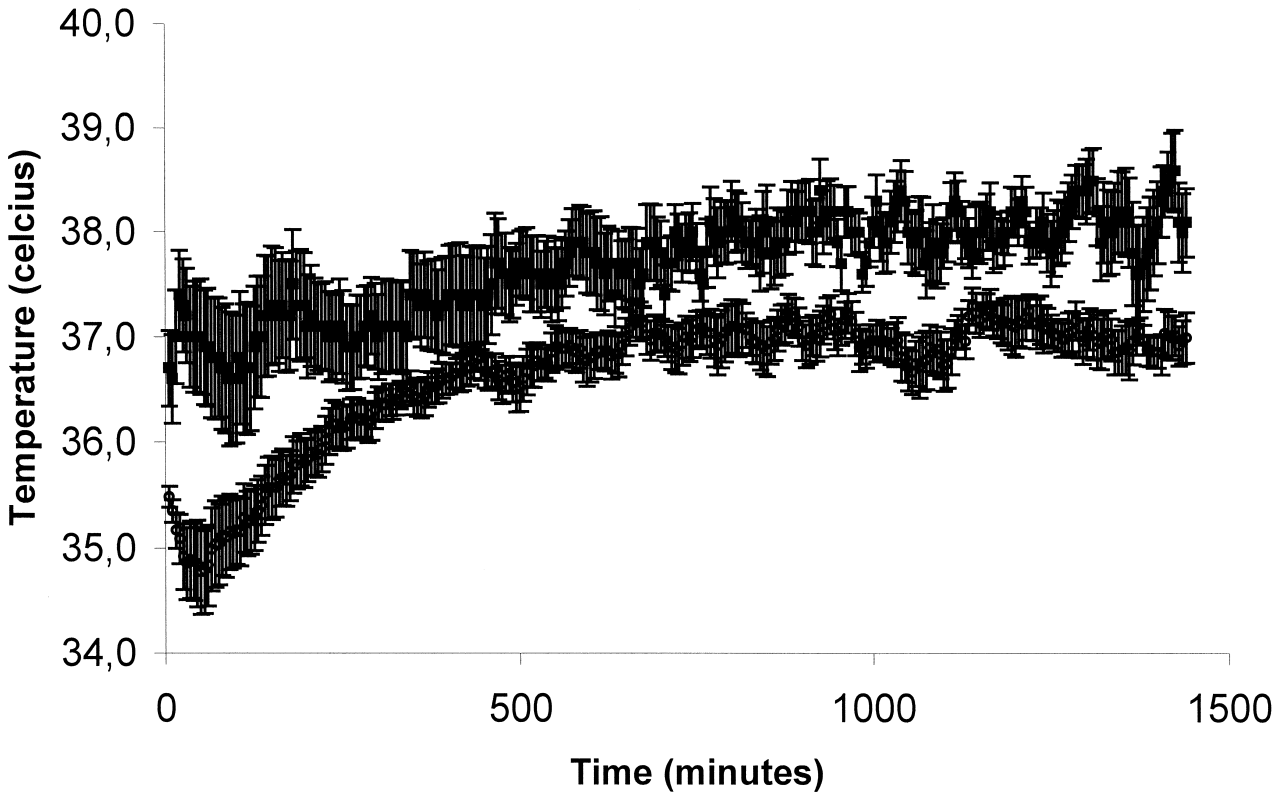

Fig. 2. Core temperature was recorded every 5 min following ischemia. Data are presented as means of 8 ± S.E.M. The lower temperature curve represents the 8-OH-DPAT-treated ischemic rats and the upper curve represents the vehicle-treated ischemic rats. There was a significant effect of time of measurement and group ($P = 0.020$) without a significant interaction of those two parameters.

dorsal CA1 ranging from 30,300 to 101,900 and the 8-OH-DPAT-treated ischemia group ($n = 7$) had a median number of 105,200 neurons in dorsal CA1 ranging from 80,100 to 134,500 ($P = 0.018$), see Fig. 1. A group of age matched, untreated control rats had a median number of 186,000 neurons in dorsal CA1 ranging from 177,000 to 196,000 neurons ($n = 4$). Core temperature was recorded every 5 min for 24 h, see Fig. 2. Any difference in temperature between the 8-OH-DPAT-treated and vehicle-treated groups was tested by repeated measure of analysis of variance. There was a significant effect of time of measurement and group ($P = 0.020$) without a significant interaction of those two parameters. The mean temperature for the 8-OH-DPAT-treated group was between 0.2°C and 1.2°C lower than the mean temperature of the vehicle-treated group with a mean difference of 0.6°C over 24 h.

4. Discussion

No previous studies have estimated the neuroprotective effect of 5-HT_{1A} receptor agonists by stereological cell counting. The stereological method using the optical fractionator estimates the total number of neurons in a brain region of interest without assumption of neuronal size and shape. The exact volume need not to be known and the estimate is unaffected by shrinkage or expansion of the tissue. The method is based on systematic random sampling of a small fraction of the total volume. Therefore, the method is simple and fast to use.

The high mortality in the drug-treated group is a problem when the drug-treated group is compared to the vehicle-treated group. The surviving eight individuals could constitute a subpopulation of the thirteen operated animals in such a way that it makes a selection of the total population that is not made in the vehicle-treated group. To allow comparison between the to groups an assumption must be made that the to groups are alike except for the drug treatment. This assumption may be violated by the high mortality in one of the groups. Due to interindividual differences in collateral blood supply or differences in surgery it could be that the eight animals survived the ischemia because they received a less severe ischemia compared to the five animals that died. The degree of ischemia in the surviving eight drug-treated animals could be less than the vehicle-treated group. However, no parameters (pCO_2, pO_2, pH, mean blood pressure, blood glucose, time before surgery, total time in surgery) differed between the animals that died, the surviving animals and the vehicle-treated animals (data not shown). The over-mortality seems to be a drug effect as all the rats died in the same way and no rats died in the vehicle-treated group. The pulmonary edema was triggered by the ischemia as the animals had already been in drug treatment for 24 h

before the ischemia. The drug effect could be a systemic effect local in the lungs or it could be a centrally activated lung edema. A pulmonary edema induced by the serotonin system has previously been described. Serotonin acts as a vasoconstrictor in the pulmonary system and 5-HT_{1A} receptors has been implicated in the development of pulmonary edema (Mais and Bosin, 1984; Kamiya et al., 1987; Sumita et al., 1989).

The findings in the present study that 8-OH-DPAT reduced the neuronal damage after global cerebral ischemia are in agreement with previous findings. 1 mg/kg 8-OH-DPAT decreased the infarct area after permanent middle cerebral artery occlusion in rats and 8-OH-DPAT at doses of 1, 3 and 10 mg/kg reduced the infarct area after permanent middle cerebral artery occlusion in mice (Bielenberg and Burkhardt, 1990). 8-OH-DPAT also reduced the CA1 damage after 2-vessel occlusion in gerbils at doses of 3 and 10 mg/kg (Piera et al., 1995). However, another study failed to show anti-ischemic effects of 8-OH-DPAT after 2-vessel occlusion in gerbils (Bode-Greuel et al., 1990). One explanation for the discrepancy could be the difference in model. In order to be able to show neuroprotection in the 2-vessel gerbil model a large number of animals is needed because of the large interindividual variability seen in this model (Clifton et al., 1989). Lack of neuroprotection could thus be explained by a relatively low number of animals (5–9) in the Bode–Greuel study compared to the Piera study (16–22). The administration of 8-OH-DPAT is another difference between the present and previous studies. In this study, there is a continuous administration of 8-OH-DPAT and a steady level is already established when the ischemia is induced. The half-life of 8-OH-DPAT has previously been determined between 15 and 45 min (Yu and Lewander, 1997). In previous studies 8-OH-DPAT has been administered once or twice a day. It is reasonable to assume that the brain levels of 8-OH-DPAT must drop to a point below therapeutic levels in a regime with injection once or twice a day.

The importance of intraischemic and post-ischemic temperature has become very clear in the recent years. Even a mild hypothermia after ischemia provides long-lasting protection against the CA1 damage. Nurse and Corbett showed that 1–1.5°C decline in brain temperature induced by NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline) treatment after ischemia is sufficient to protect against neuronal damage. When the NBQX-treated group was temperature adjusted to the same levels as the vehicle-treated group no beneficial effect was seen of the NBQX treatment (Nurse and Corbett, 1996). Therefore, it is of outmost importance to regulate the intraischemic temperature and monitor the post-ischemic temperature. A very appropriate question when drugs are being tested for neuroprotection is whether the neuroprotection is due to hypothermia. The central serotonin system is known to be associated with thermoregulatory mechanisms. 5-HT_{1A} receptor activation by 8-OH-DPAT challenge has been shown to induce hypothermia in rats (Goodwin et al., 1987; Bill et al., 1991) A dose of 0.1 mg/kg s.c. 8-OH-DPAT induced an immediate hypothermia lasting for 90 min, whereas 0.03 mg/kg did not produce hypothermia (Hjorth, 1985). In the present study, 8-OH-DPAT was administered as a slow infusion 125 μg/kg/h (3 mg/kg/24 h). This was, however, sufficient to induce hypothermia lasting for at least 24 h. Although the 8-OH-DPAT treatment only lowered the core temperature with 0.5–1°C, it is likely that the neuroprotective properties of 8-OH-DPAT in part could be explained by hypothermia. Some studies reporting neuroprotection by 5-HT_{1A} receptor agonist have monitored the temperature (Prehn et al., 1991, 1993; Piera et al., 1995), others regulated the temperature (Peruche et al., 1994), while others have neither monitored or regulated the temperature after the ischemia (Bielenberg and Burkhardt, 1990; Bode-Greuel et al., 1990; Wagener et al., 1990; Backhauss et al., 1992; Semkova et al., 1998). Post-ischemic hypothermia after 8-OH-DPAT treatment has not previously been reported. Species difference in 5-HT_{1A} receptor agonist induced hypothermia could account for some of the discrepancy (Bill et al., 1991). Even within the same species, the sensitivity to 8-OH-DPAT induced hypothermia has been shown to vary (Knapp et al., 1998). Whether the hypothermia induced by 8-OH-DPAT is the only mechanism of action remains to be established. It would thus be interesting to test the neuroprotective properties of 8-OH-DPAT in ischemic rats while maintaining the core temperature at a physiological level. It should be noted that the decrease in post-ischemic temperature for the 8-OH-DPAT-treated group is small (0.5–1°C) compared to decrease seen after NBQX treatment ($\sim$2°C) (Nurse and Corbett, 1996).

The degree of CA1 damage in the vehicle and 8-OH-DPAT-treated groups was quite severe compared to non-ischemic control rats. In the vehicle-treated ischemic group, only 27% of the CA1 neurons survived, whereas the 8-OH-DPAT treatment increased the survival to 56%. Other studies using the same technique have obtained a similar total number of 200,000 neurons in one hemisphere dorsal CA1 (Olsen et al., 1994) and 380,000 in one hemisphere total CA1 (West and Gundersen, 1991). In spite of the severe damage in CA1 induced by the ischemia, the 8-OH-DPAT treatment is still capable of saving a significant part of the neurons in CA1. The 8-OH-DPAT induced hypothermia, however, needs further investigation.

Acknowledgements

This study was supported by a grant from the Faculty of Health Sciences, University of Copenhagen.

References

Backhauss, C., Karkoutly, C., Welsch, M., Krieglstein, J., 1992. A mouse model of focal cerebral ischemia for screening neuroprotective drug effects. J. Pharmacol. Toxicol. Methods 27, 27–32.

Bielenberg, G.W., Burkhardt, M., 1990. 5-hydroxytryptamine$_{1A}$ agonists. A new therapeutic principle for stroke treatment. Stroke 21, 161–163.

Bill, D.J., Knight, M., Forster, E.A., Fletcher, A., 1991. Direct evidence for an important species difference in the mechanism of 8-OH-DPAT-induced hypothermia. Br. J. Pharmacol. 103, 1857–1864.

Bode-Greuel, K., Klisch, M.J., Hováth, E., Glaser, T., Traber, J., 1990. Effects of 5-hydroxytryptamine1A receptor agonists on hippocampal damage after transient forebrain ischemia in the mongolian gerbil. Stroke 21, 164–166.

Clifton, G.L., Taft, W.C., Blair, R.E., Choi, S.C., DeLorenzo, R.J., 1989. Conditions for pharmacologic evaluation in the gerbil model of forebrain ischemia. Stroke 20, 1545–1552.

Goodwin, G.M., De Souza, R.J., Green, A.R., Heal, D.J., 1987. The pharmacology of the behavioural and hypothermic responses of rats to 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). Psychopharmacology (Berlin) 91, 506–511.

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sorensen, F.B., Vesterby, A., West, M.J., 1988. The new stereological tools. Dissector, frationator, nucleator and point sampled intercepts and their use in pathological research and diagnostics. APMIS 96, 857–881.

Hjorth, S., 1985. Hypothermia in the rat induced by the potent serotonergic agent 8-OH-DPAT. J. Neural Transm. 61, 131–135.

Iniguez, C., Gayoso, M.J., Carreres, J., 1985. A versatile and simple method for staining nervous tissue using Giemsa dye. J. Neurosci. Methods 13, 77–86.

Kamiya, I., Sumita, T., Ishikawa, N., 1987. S1-receptor participation in serotonin-induced pulmonary edema in the dog. Jpn. J. Pharmacol. 45, 121–124.

Knapp, D.J., Overstreet, D.H., Crews, F.T., 1998. Brain 5-HT1A receptor autoradiography and hypothermic responses in rats bred for differences in 8-OH-DPAT sensitivity. Brain Res. 782, 1–10.

Mais, D.E., Bosin, T.R., 1984. A role for serotonin in alpha-naphthylthiourea-induced pulmonary edema. Toxicol. Appl. Pharmacol. 74, 185–194.

Nakata, N., Suda, H., Izumi, J., Takaka, Y., Ikeda, Y., Kato, H., Itoyama, Y., Kogure, K., 1997. Role of hippocampal serotonergic neurons in ischemic neuronal death. Behav. Brain Res. 83, 217–220.

Nurse, S., Corbett, D., 1996. Neuroprotection after several days of mild, drug-induced hypothermia. J Cereb. Blood Flow Metab. 16, 474–480.

Olsen, G.M., Scheel-Krüger, J., Jensen, L.H., 1994. Relation of spatial learning of rats in the Morris water maze task to the number of viable CA1 neurons following four-vessel occlusion. Behav. Neurosci. 108, 681–690.

Paxinos, G., Watson, C., 1997. The Brain in Stereotaxic Coordinates. 3rd edn. Academic Press, London.

Peruche, B., Backhauss, C., Prehn, J.H., Krieglstein, J., 1994. Protective effects of 5-HT1A receptor agonists against neuronal damage demonstrated in vivo and in vitro. J. Neural Transm.: Parkinson's Dis. Dementia Sect. 8, 73–83.

Piera, M.J., Beaughard, M., Michelin, M.T., Massingham, R., 1995. Effects of the 5-hydroxytryptamine1A receptor agonists, 8-OH-DPAT, buspirone and flesinoxan, upon brain damage induced by transient global cerebral ischemia in gerbils. Arch. Int. Pharmacodyn. 329, 347–359.

Prehn, J.H.M., Backhauss, C., Karoutly, C., Nuglisch, J., Peruche, B., Rosberg, C., Kriegelstein, J., 1991. Neuroprotective properties of 5-HT1A receptor agonists in rodent models of focal and global cerebral ischemia. Eur. J. Pharmacol. 203, 213–222.

Prehn, J.H.M., Welch, M., Backhauss, C., Nuglisch, J., Ausmeier, F., Karoutly, C., Kriegelstein, J., 1993. Effects of serotonergic drugs i eperimental brain ischemia: evidence for a protective role of serotonin in cerebral ischemia. Brain Res. 630, 10–20.

Semkova, I., Wolz, P., Krieglstein, J., 1998. Neuroprotective effect of 5-HT1A receptor agonist, Bay X 3702, demonstrated in vitro and in vivo. Eur. J. Pharmacol. 359, 251–260.

Shibata, S., Kagami-Ishi, Y., Tominaga, K., Kodama, K., Ueki, S., Watanabe, S., 1992. Ischemia-induced impairment of 2-deoxyglucose uptake and CA1 field potentials in rat hippocampal slices:protection by 5-HT1A receptor agonists and 5-HT2 receptor antagonists. Eur. J. Pharmacol. 229, 21–29.

Smith, M.L., Bendek, G., Dahlgren, N., Rosén, I., Wieloch, T., Siesjö, B.K., 1984. Models for studying long-term recovery following forebrain ischemia in the rat: 2. A 2-vessel occlusion model. Acta Neurol. Scand. 69, 385–401.

Sumita, T., Ishikawa, N., Hashiba, Y., Takagi, K., Satake, T., 1989. 5-Hydroxytryptamine receptor subtypes participating in pulmonary edema in dogs. Eur. J. Pharmacol. 164, 69–75.

Torup, L., Mellerup, E.T., Diemer, N.H., 1999. Density of serotonin receptors in rat brain following global cerebral ischemia: an autoradiographic study. Neurosci. Res. Commun. 24, 33–40.

Wagener, G., Hielscher, H., Rossberg, C., Wagner, H.J., Bielenberg, G.W., 1990. Effects of ipsapirone on spatial and temporal changes in somatosensory evoked potentials after middle cerebral artery occlusion in the rat. Stroke 21, IV167–IV170.

West, M.J., 1999. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. TINS 22, 51–61.

West, M.J., Gundersen, H.J.G., 1991. Unbiased stereological estimation of the total number of neurons in the subdivision of the rat hippocampus using the optical fractionator. Anat. Rec. 231, 482–497.

Yu, H., Lewander, T., 1997. Pharmacokinetic and pharmacodynamic studies of (*R*)-8-hydroxy-2-(di-*n*-propylamino)tetralin in the rat. Eur. Neuropsychopharmacol. 7, 165–172.