Janssen, P. A. (1985) Pharmacology of potent and selective S_2-serotonergic antagonists. J. Cardiovasc. Pharmacol. 7: S2–11

Juan, H., Sametz, W. (1980) Histamine-induced release of arachidonic acid and of prostaglandins in the peripheral vascular bed: mode of action. Naunyn Schmiedebergs Arch. Pharmacol. 314: 183–190

Kang, Y. H., Wei, H. M., Fisher, H., Merill, G. F. (1987) Histamine induced changes in coronary circulation and myocardial oxygen consumption: influences of histamine receptor antagonists. FASEB J. 1: 483–489

Kantor, P. F., Coetzee, W. A., Carmeliet, E. E., Dennis, S. E., Opie, L. H. (1990) Reduction of ischemic K^+ loss and arrhythmias in rat hearts. Effect of glibenclamide, a sulfonylurea. Circ. Res. 66: 478–485

Ksoll, E., Parsons, A. A., Mackert, J. R., Schilling, L., Wahl, M. (1991) Analysis of cromakalim, pinacidil and nicorandil induced relaxation of the 5-hydroxytryptamine precontracted rat isolated basilar artery. Naunyn Schmiedebergs Arch. Pharmacol. 343: 377–383

Mitani, A., Knoshita, K., Fukamachi, K., Sakamoto, M., Kurisu, K., Tsuruhara, Y., Fukumura, F., Nakashima, A., Tokunaga, K. (1991) Effects of glibenclamide and nicorandil on cardiac function during ischemia and reperfusion in isolated perfused rat hearts. Am. J. Physiol. 261: H1864–H1871

Plant, T. D., Henquin, J. C. (1990) Phentolamine and yohimbine inhibit ATP sensitive channels in mouse pancreatic beta cells. Br. J. Pharmacol. 101: 115–120

Satoh, K., Yamada, H., Taira, N. (1991) Differential antagonism by glibenclamide of the relaxant effects of cromakalim, pinacidil and nicorandil on canine large coronary arteries. Naunyn Schmiedebergs Arch. Pharmacol. 343: 76–82

Wiest, E., Trach, V., Dammgen, J. (1989) Removal of endothelial function in coronary resistance vessels by saponin. Basic Res. Cardiol. 84: 469–478

Wilde, A. A. M., Escande, D., Schumacher, C. A., Thuringer, D., Mestre, M., Fiolet, J. W. T., Janse, M. J. (1990) Potassium accumulation in the globally ischemic mammalian heart. Circ. Res. 67: 835–843

Winquist, R. J., Heaney, L. A., Wallace, A. A., Baskin, E. P., Stein, R. B., Gracia, M. L., Kaczorowski, G. J. (1989) Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulphate and diazoxide in vascular smooth muscle. J. Pharmacol. Exp. Ther. 248: 149–156

Wolleben, C. D., Sanguinetti, M. C., Siegl, K. S. (1989) Influence of ATP-sensitive potassium channel modulators on ischaemia induced fibrillation in isolated rat hearts. J. Mol. Cell. Cardiol. 21: 783–788

Zhang, H., Stockbridge, N., Weir, B., Krueger, C., Cook, D. (1991) Glibenclamide reduces vascular smooth muscle constriction produced by prostaglandin $F_{2\alpha}$. Eur. J. Pharmacol. 195: 27–35

J. Pharm. Pharmacol. 1993, 45: 674–677
Communicated July 13, 1992

© 1993 J. Pharm. Pharmacol.

Sequential changes of [^{3}H]cyclic AMP binding in the gerbil brain following transient cerebral ischaemia

TSUTOMU ARAKI*, HIROYUKI KATO, YASUO KANAI†, KYUYA KOGURE, *Department of Neurology, Institute of Brain Diseases, Tohoku University School of Medicine, Sendai, Japan, and* † *Pharmacological Research Laboratory, Research Laboratories, Tokyo Tanabe Co. Ltd, Kita-ku, Tokyo, Japan*

Abstract—Sequential alterations in the binding of [^{3}H]cyclic AMP (cAMP) as an indicator of cAMP-dependent protein kinase (cAMP-DPK) binding activity following transient cerebral ischaemia were studied in the gerbil brain using receptor autoradiography. Transient ischaemia was induced for 10 min. [^{3}H]cAMP binding in the stratum oriens and pyramidale of the hippocampal CA1 sector significantly decreased in the early post-ischaemic stage and showed severe reduction 7 days and 1 month after recirculation. By contrast, [^{3}H]cAMP binding showed no significant alterations in the stratum radiatum of the hippocampal CA1 sector and the stratum pyramidale of the hippocampal CA3 sector up to 48 h after ischaemia. However, the binding in these areas significantly decreased 7 days and 1 month after ischaemia. The stratum lacunosum-moleculare of the hippocampal CA1 sector and dentate gyrus showed no significant changes in [^{3}H]cAMP binding throughout the recirculation period. However, in the dorsolateral part of the striatum, where severe neuronal damage was seen morphologically, [^{3}H]cAMP binding was significantly reduced only one month after ischaemia. These results indicate that marked alteration of intracellular signal transduction precedes neuronal damage in the hippocampal CA1 sector, but not in the striatum. Furthermore, our autoradiographic data suggest that post-ischaemic alteration in [^{3}H]cAMP binding between the hippocampal CA1 sector and striatum may be produced by different mechanisms.

Transient cerebral ischaemia leads to neuronal damage in selectively vulnerable areas. The hippocampal CA1 pyramidal neurons and hilar neurons of dentate gyrus are most vulnerable to brief ischaemia, followed by the striatal neurons, cortical neurons, and thalamic neurons (Pulsinelli et al 1982; Johansen et al 1987; Benveniste & Diemer 1988; Araki et al 1989). Several studies have demonstrated that intracellular second messengers are involved in the pathogenesis of ischaemic brain damage (Jorgensen et al 1989; Onodera et al 1989; Cardell et al 1990) and in neurotransmitter release (Malenka et al 1986; Nishizuka 1986). We have reported that transient ischaemia caused post-ischaemic alteration in the binding sites of protein kinase C, inositol 1,4,5-triphosphate, and forskolin in selectively vulnerable areas (Araki et al 1992a). Furthermore, we have demonstrated that transient ischaemia produced marked reduction in [^{3}H]cAMP binding in the gerbil hippocampus 7 days after recirculation (Araki et al 1992b). Therefore, alterations in binding of cyclic (c) AMP as well as protein kinase C, inositol triphosphate and forskolin are considered to play a vital role in the development of ischaemic neuronal damage. The purpose of the present study was to investigate regional alterations in [^{3}H]cAMP binding after transient ischaemia in the gerbil using receptor autoradiography.

* Present address and correspondence: T. Araki, Pharmacological Research Laboratory, Research Laboratories, Tokyo Tanabe Co. Ltd, 33-3 Akabanekita 2-chome, Kita-ku, Tokyo 115, Japan.

Materials and methods

Animals and operative procedures. Male Mongolian gerbils (Seiwa Experimental Animals, Fukuoka, Japan), 65–95 g, were anaesthetized with 2% halothane in a mixture of 70% N_2O and 30% O_2. The bilateral common carotid arteries were exposed, anaesthesia was discontinued, and the arteries were clamped with aneurysmal clips for 10 min. The animals adopted a squatting posture without moving their limbs for at least 1 h, enabling the following procedures to be carried out without causing pain. After occlusion, the clips were removed and the gerbils were allowed to survive for 1, 5, 24 and 48 h, 7 days and 1

month after ischaemia. Sham-operated animals were treated in the same manner, except for the clipping of the bilateral common carotid arteries. Body temperature was maintained at 37–39°C with a heating pad equipped with a thermostat until the animals began to move. The animals were killed by decapitation. The brains were removed quickly and frozen in powdered dry-ice; coronal sections (12 μm) were cut on a cryostat at -20°C and thaw-mounted onto gelatin-coated slides. The sections were stored at -80°C until assay.

Receptor autoradiography. Autoradiographic localization of the binding of cAMP was determined according to the method of Gundlach & Urosevic (1989), with minor modifications (Araki et al 1992b). Brain sections were preincubated for 20 min at room temperature (21°C) in Krebs-HEPES buffer (in mM): NaCl 118, KCl 5, $CaCl_2$ 2·5, KH_2PO_4 1·18, $MgSO_4$ 1·18, glucose 11, HEPES 25, pH 7·4. Sections were then incubated for 90 min at room temperature in buffer containing 10 nM [^{3}H]cAMP (sp. act. 51 Ci $mmol^{-1}$, Amersham Corporation, Arlington Heights, IL, USA) and 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemicals, St Louis, MO, USA). Sections were washed for 1 min at 4°C. Nonspecific binding was determined using 10 μM cAMP (Sigma Chemicals).

All procedures were performed under subdued lighting conditions. The sections were dried under a cold air stream and were exposed to Hyperfilm-^{3}H (Amersham) for 4 weeks in X-ray cassettes with a set of ^{3}H microscales (Amersham). The optical density of the brain regions at $\times$ 10 magnification was measured by a computer-assisted image analyser without the examiner knowing the experimental protocol, as described previously (Araki et al 1991a, b). The relationship between optical density and radioactivity was obtained with reference to the ^{3}H microscale co-exposed with the tissue sections, using a third-order polynomial function. Specific binding was determined by subtracting the value of nonspecific binding from that of total binding. Since there was no specific difference in nonspecific binding, the difference in specific binding was probably not an artifact of the difference in the tritium quenching level (Onodera et al 1987). Anatomical structures were verified by examination of cresyl violet-stained sections, using the gerbil brain atlas of Loskota et al (1974). Binding assays were performed in duplicate.

Adjacent sections prepared for receptor autoradiography were also stained with cresyl violet and haematoxylin-eosin. The sections were examined with a light microscope.

Results

Representative autoradiograms of [^{3}H]cAMP binding sites are shown in Fig. 1. Post-ischaemic changes of [^{3}H]cAMP binding are summarized in Table 1. In sham-operated gerbils, [^{3}H]cAMP binding was greatest in the granule layer of the dentate gyrus and the stratum pyramidale of the hippocampus. Other regions also had a relatively high density of [^{3}H]cAMP binding. In gerbils subjected to 10-min ischaemia, [^{3}H]cAMP binding was significantly reduced in the stratum oriens and pyramidale of the hippocampal CA1 sector, 5 and 48 h after recirculation. Seven days and one month after ischaemia, [^{3}H]cAMP binding markedly decreased in the hippocampal CA1 and CA3 sectors except for the stratum lacunosum-moleculare. Other regions, in contrast, showed no significant alteration in [^{3}H]cAMP binding throughout the recirculation period, except that a significant reduction was seen in the dorsolateral part of striatum, one month after recirculation.

Representative photographs are shown in Fig. 2. Sham-operated gerbils showed no neuronal damage throughout the brain. Gerbils subjected to 10-min ischaemia also showed no conspicuous neuronal damage in the brain 5 h after recirculation. At 48 h after ischaemia, severe neuronal damage was noted in the dorsolateral part of the striatum and the thalamus, and mild damage was seen in the hippocampal CA1 sector. At 7 days and 1 month after ischaemia, the most frequently affected regions were the hippocampal CA1 sector and the dorsolateral part of the striatum, followed by the hippocampal CA3 sector. However, the dentate gyrus and ventromedial part of striatum were morphologically intact. These results are consistent with our previous reports (Araki et al 1991b, 1992a).

Discussion

The present study indicated that transient cerebral ischaemia produced severe reduction in [^{3}H]cAMP binding in selectively vulnerable areas. This was particularly so in the hippocampal CA1 sector, which was most vulnerable to ischaemia. Furthermore, our results suggested that marked reduction in [^{3}H]cAMP binding preceded neuronal damage in the hippocampal CA1 sector, but not in the dorsolateral part of the striatum. These results demonstrated that post-ischaemic alteration in

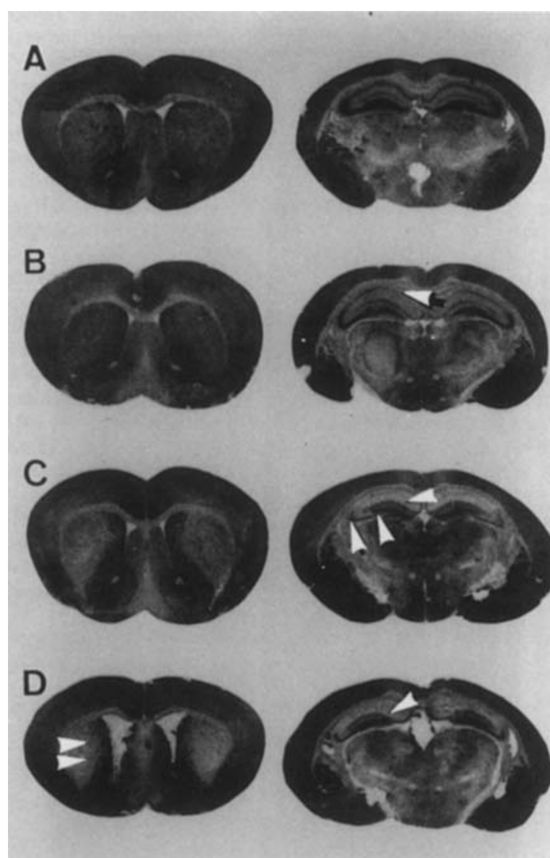

FIG. 1. Representative autoradiograms of [^{3}H]cAMP binding in the brain of sham-operated gerbils (A), and in animals 48 h (B), 7 days (C) and 1 month (D) after transient ischaemia. A significant reduction in [^{3}H]cAMP binding was noted in the hippocampal CA1 pyramidal cell layer (B, arrow). Severe reduction in [^{3}H]cAMP binding was found in the hippocampal CA1 and CA3 pyramidal cell layers (C, arrow), but the binding was well preserved in the stratum lacunosum-moleculare of hippocampal CA1 sector (C, arrows). A significant reduction in [^{3}H]cAMP binding was observed in the dorsolateral part of striatum (D, arrows). The striatum lacunosum-moleculare of hippocampal CA1 sector showed no significant alteration in [^{3}H]cAMP binding even 1 month after ischaemia (D, arrowhead).

Table 1. Time course of [^{3}H]cAMP binding in the gerbil brain following transient cerebral ischaemia.

Regions	Sham-operated	Recirculation time 1 h	5 h	24 h	48 h	7 days	1 month
Striatum							
Lateral	197±15	195±14	202±14	195±18	226±9	157±10	136±14**
Medial	243±17	220±22	235±20	247±40	290±30	260±37	242±34
Hippocampus							
CA1 sector							
Stratum oriens	191±18	147±21	145±7*	189±20	146±10*	125±20*	107±12**
Stratum pyramidale	268±22	229±21	200±10*	234±32	196±25*	107±15**	92±11**
Stratum radiatum	177±15	155±23	143±6	180±23	151±14	96±15**	92±12**
Stratum lacunosum-moleculare	206±19	179±25	181±10	249±24	225±9	198±28	182±44
CA3 sector							
Stratum pyramidale	466±5	404±12	399±13	425±25	370±36	205±59**	301±58**
Average	369±15	326±16	308±14	340±25	316±30	172±37**	242±38**
Dentate gyrus (average)	277±25	250±20	222±10	288±23	253±18	230±30	278±29

Optical density was converted to fmol (mg tissue)$^{-1}$ using ^{3}H microscales. Values are expressed as means±s.e. * $P<0.05$, ** $P<0.01$ vs sham-operated group (Duncan's multiple range test). n=4–7. Striatum (lateral), the dorsolateral part of striatum; striatum (medial), the ventromedial part of striatum.

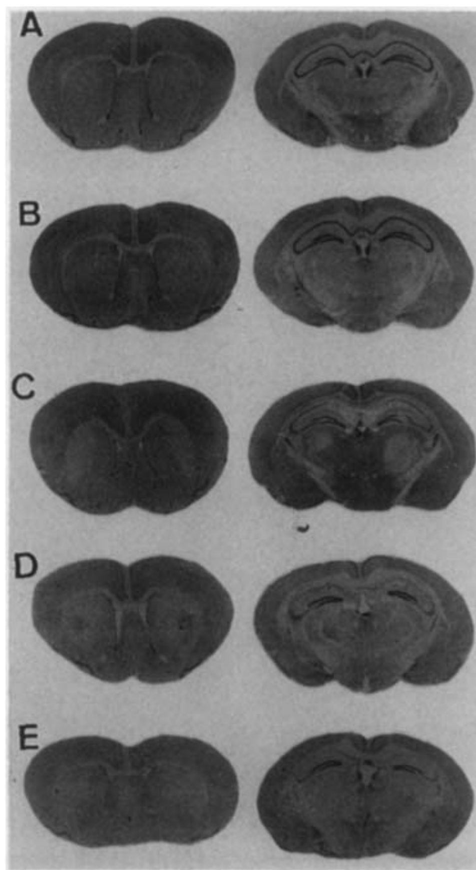

FIG. 2. Representative photographs in the brain of sham-operated gerbils (A), and in animals 5 h (B), 48 h (C), 7 days (D) and 1 month (E) after transient ischaemia. Sham-operated gerbil brain was intact (A). At 5 h after ischaemia, neuronal damage was not seen in the brain (B). Severe neuronal damage was noted in the dorsolateral part of the striatum and mild damage was found in the hippocampal CA1 sector 48 h after ischaemia (C). At 7 days and 1 month after ischaemia, severe neuronal damage was seen in the dorsolateral part of the striatum and the hippocampal CA1 sector. The hippocampal CA3 sector also showed severe neuronal damage, whereas the dentate gyrus was intact (D, E).

[^{3}H]cAMP binding between the striatum and the hippocampal CA1 sector may be produced by different mechanisms.

Several lines of biochemical evidence support the concept that cAMP binding protein is identical to the regulatory subunit of cAMP-dependent protein kinase (cAMP-DPK) (Hofmann et al 1977; Walter et al 1977). For two major categories of cAMP binding proteins, the regulatory subunits of the type I and type II cAMP-DPK exist (Nairn et al 1985). The brain contains higher levels of the type II cAMP-DPK than that of the type I cAMP-DPK (Corbin et al 1975; Walter et al 1977). Therefore, type II cAMP-DPK seems to be a major factor for mediating the effects of cAMP in the central nervous system. However, sequential alterations in cAMP after transient cerebral ischaemia have not been examined in selectively vulnerable areas. Furthermore, the changes of particulate cAMP-DPK activity after transient ischaemia are not fully known.

Previous studies suggested that severe neuronal damage occurred quickly in the dorsolateral part of striatum, whereas the hippocampal CA1 pyramidal cell death occurred after an interval of approximately 2 days following transient ischaemia, during which time no energy crisis or histological changes were observed (Kirino 1982; Pulsinelli & Duffy 1983). Thus, the neuronal damage between the striatum and hippocampal CA1 sector proceeds at different rates. However, whether a similar mechanism is implicated in the pathogenesis of ischaemic neuronal damage between the striatum and hippocampus is unclear.

The present study indicated that the stratum oriens and pyramidale of the hippocampal CA1 sector showed a significant decrease early in the recirculation period without neuronal cell loss, and these changes were then reversed. [^{3}H]cAMP binding was again reduced in the above sites 48 h after ischaemia, and this alteration may reflect neuronal damage in the hippocampal CA1 sector. By contrast, [^{3}H]cAMP binding showed no significant alteration in stratum radiatum and lacunosum-moleculare of the hippocampal CA1 sector at an early stage after ischaemia. Seven days after ischaemia, severe reduction in [^{3}H]cAMP binding was noted in the hippocampal CA1 sector. However, the stratum lacunosum-moleculare of the hippocampal CA1 sector showed no significant alteration in [^{3}H]cAMP binding during the recirculation period. Thus, in the hippocampal CA1 sector, [^{3}H]cAMP binding sites in the stratum oriens and pyramidale

were more vulnerable to ischaemic insult than those in the stratum *radiatum* and lacunosum-moleculare. However, [^{3}H]cAMP binding significantly decreased in the hippocampal CA3 sector 7 days and 1 month after ischaemia, when neuronal damage was observed. The dorsolateral part of striatum exhibited a significant reduction in [^{3}H]cAMP binding only 1 month after ischaemia, although severe neuronal damage was noted in this region 48 h after recirculation, as shown in Fig. 2. This finding seems to suggest that an alteration in [^{3}H]cAMP binding cannot precede histological neuronal damage in the dorsolateral part of striatum, and that [^{3}H]cAMP binding in this region is relatively resistant to degenerative processes, as shown in Fig. 1. Furthermore, this phenomenon indicates that post-ischaemic alteration in [^{3}H]cAMP binding in the hippocampus was more pronounced than that in the striatum. Thus, our data suggest that marked alteration of intracellular signal transduction may precede neuronal damage in the hippocampal CA1 sector, but not in the striatum. Therefore, these results demonstrate that post-ischaemic alteration in [^{3}H]cAMP binding between the hippocampal CA1 sector and striatum may be produced by different mechanisms. However, the present study could not determine whether the observed variations in the binding are due to variations in the affinity constant, K_d, or the number of binding sites, B_{max}. Further studies are needed to investigate the precise biochemical mechanism for our findings.

References

Araki, T., Kato, H., Kogure, K. (1989) Selective neuronal vulnerability following transient cerebral ischemia in the gerbil: distribution and time course. Acta Neurol. Scand. 80: 548–553

Araki, T., Kato, H., Hara, H., Kogure, K. (1991a) Postischemic alteration of [^{3}H]forskolin binding sites in selectively vulnerable areas: an autoradiographic study of gerbil brain. Neurosci. Lett. 125: 129–162

Araki, T., Kato, H., Kogure, K., Saito, T. (1991b) Postischemic alteration of muscarinic acetylcholine, adenosine A_1 and calcium antagonist binding sites in selectively vulnerable areas: an autoradiographic study of gerbil brain. J. Neurol. Sci. 106: 206–212

Araki, T., Kato, H., Hara, H., Kogure, K. (1992a) Postischemic binding of [^{3}H]phorbol 12,13-dibutyrate and [^{3}H]inositol 1,4,5-trisphosphate in the gerbil brain: an autoradiographic study. Neuroscience 46: 973–980

Araki, T., Kato, H., Kogure, K. (1992b) Mapping of second messenger and rolipram receptors in mammalian brain. Brain Res. Bull. 28: 843–848

Benveniste, H., Diemer, N. H. (1988) Early postischemic ^{45}Ca accumulation in rat dentate hilus. J. Cereb. Blood Flow Metab. 8: 713–719

Cardell, M., Bingren, H., Wieloch, T., Zivin, J., Saitoh, T. (1990) Protein kinase C is translocated to cell membranes during cerebral ischemia. Neurosci. Lett. 119: 228–232

Corbin, J. D., Keely, S. L., Park, C. R. (1975) The distribution and dissociation of cyclic adenosine 3,5-monophosphate-dependent protein kinase in adipose, cardiac and other tissues. J. Biol. Chem. 250: 218–225

Gundlach, A. L., Urosevic, A. (1989) Autoradiographic localization of particulate cyclic-AMP-dependent protein kinase in mammalian brain using [^{3}H]cyclic-AMP: implications for organization of second messenger systems. Neuroscience 29: 695–714

Hofmann, F., Bechtel, P. J., Krebs, E. G. (1977) Concentration of cyclic AMP-dependent protein kinase subunits in various tissues. J. Biol. Chem. 252: 1441–1447

Johansen, F. F., Zimner, J., Diemer, N. H. (1987) Early loss of somatostatin neurons in dentate titres after cerebral ischemia in the rat precedes Ca-1 pyramidal cell loss. Acta Neuropathol. 73: 110–114

Jorgensen, M. B., Deckert, J., Wright, D. C. (1989) Binding of [^{3}H]inositoltrisphosphate and [^{3}H]phorbol 12,13-dibutyrate in the rat hippocampus following transient global ischemia: a quantitative autoradiographic study. Neurosci. Lett. 103: 219–224

Kirino, T. (1982) Delayed neuronal death in the gerbil hippocampus following transient ischemia. Brain Res. 239: 57–69

Loskota, W. J., Lomax, P., Verity, M. A. (1974) A Stereotaxic Atlas of the Mongolian Gerbil Brain. Ann Arbor Science, Michigan

Malenka, R. C., Madison, D. V., Nicoll, R. A. (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. Nature 321: 175–177

Nairn, A. C., Hemmings, H. C., Greengard, P. (1985) Protein kinases in the brain. Annu. Rev. Biochem. 54: 931–976

Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. Science 233: 305–321

Onodera, H., Araki, T., Kogure, K. (1989) Protein kinase C activity in the rat hippocampus after forebrain ischemia: autoradiographic analysis by [^{3}H]phorbol 12,13-dibutyrate. Brain Res. 481: 1–7

Onodera, H., Sato, G., Kogure, K. (1987) Quantitative autoradiographic analysis of muscarinic cholinergic and adenosine A_1 binding sites after transient forebrain ischemia in the gerbil. Brain Res. 415: 309–322

Pulsinelli, W. A., Duffy, T. E. (1983) Regional energy balance in rat brain after transient forebrain ischemia. J. Neurochem. 40: 1500–1503

Pulsinelli, W. A., Brierley, J. B., Plum, F. (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann. Neurol. 11: 491–498

Walter, U., Kanof, P., Schulman, H., Greengard, P. (1977) Identification, characterization and quantitative measurement of cyclic AMP receptor proteins in cytosol of various tissues using a photoaffinity ligand. J. Biol. Chem. 252: 6494–6500