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Preconditioning-specific reduction of *c-fos* expression in hippocampal granule and pyramidal but not other forebrain neurons of ischemic brain: a quantitative immunohistochemical study

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Abstract

To specify targets for an ischemic preconditioning paradigm (ischemic tolerance), *c-fos* expressions in ischemic (induced by 10 min bilateral carotid-occlusions subsequent to coagulation of vertebral arteries) and preconditioned rats (treated for 4 min carotid-occlusions 72 h before ischemia) were compared in 12 forebrain areas/nuclei. Fos immunostaining was applied to serial sections of the forebrain and the density (cell number/area measured) of Fos-immunopositive (Fos+) neurons, as well as their percentile changes were determined in five hippocampal and seven extrahippocampal areas/nuclei of ischemic and preconditioned rats. The ratio of counts found in ischemic over control animals showed several fold increase of Fos+ cells in the three layers (granule cell, molecular and polymorphic) of the dentate gyrus, CA3 and CA1 pyramidal neurons, as well as in thalamic and hypothalamic nuclei and limbic cortical areas. In contrast, preconditioning did not alter *c-fos* expressions significantly in the extrahippocampal brain areas investigated. These results strengthen the hypothesis that the hippocampal and dentate neurons are more susceptible to ischemic tolerance than cells in other brain regions. In fact stress-response and induction of ischemic tolerance in different forebrain areas can be distinguished.

Keywords: Brain ischemia; Ischemic preconditioning; *c-fos* expression; Hippocampus; Limbic cortex; Thalamic nuclei; Hypothalamic nuclei

Expression of immediate early genes (IEGs), including *c-fos* has long been proposed to function as mediators of neuronal adaptive responses [19,24], and in the response of the neuron to ischemia- and seizure-induced pathological alterations [12,18,21,31]. Strong depolarizing stimuli that result in seizures induce immediate increases in *c-fos* mRNA expression in the central nervous system [7,20]. The rapid and transient signal-induced expression of *c-fos* protein serves an excellent marker of neuronal activity in early time-window [8,13,18,19,26]. Brief exposure to cerebral ischemia (ischemic preconditioning, PC) in animal models of stroke provides robust brain protection to subsequent ischemic events [3,6,11,13–17,24,27,28,30]. It has been suggested, that PC is associated with the induction of an endogenous neuroprotective state based on the activation of a genetic program involving the post-ischemic expression of regulatory transcriptional factors, IEGs [4,28,31]. The hypothesis is substantiated by several lines of evidence including a PC-induced (i) specific expression of *c-jun* proto-oncogene in the hippocampal CA1 neurons of gerbils [28]; (ii) prolonged binding potentiation of activator protein-1 that is composed of Fos and Jun proteins in the CA1 region of the gerbils [33]. It is appropriate to recognize, however, that such overlap on the functional role for IEGs in neuroprotection depends mainly on the degree

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of the PC [1,4,12,13,21,29,31,32]. Controversial results may arise from different animal models as well as PC paradigms used and brain areas investigated.

Severe conditions, like 10 min of transient global cerebral ischemia induced by four vessels occlusion [22] cause selective and delayed neuronal death in the hippocampal CA1 region of the rat [23]. Under conditions, *c-fos* expression can be detected during an early re-perfusion period (1–6 h, [4]) without significant alteration between 1 and 3 h, and with a reduction by 6 h. Brief (3–5 min) ischemia can protect hippocampal neurons against this severe ischemic insult [14]. In the present study, we provide a systematic quantitative analysis about the effect of 10 min global cerebral ischemia [23] with and without 4 min PC three days before [14] on *c-fos* protein expression in five hippocampal and seven extrahippocampal (limbic cortical, thalamic and hypothalamic) areas/nuclei. The four-vessel occlusion model of global cerebral ischemia [22] was applied in combination with immunostaining of the Fos protein. The density (cell number/brain areas) of Fos-immunoreactive (Fos+) neurons was determined 1 h after 10 min global cerebral ischemia [4]. Results are compared with published results using a similar approach.

Unless otherwise stated below, all chemicals were purchased from Sigma-Aldrich (Budapest, Hungary). Animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), Animal Act 1998, Hungary and local ethical rules. Adult Sprague–Dawley rats of both sexes weighing 200–260 g (Animal House, Eötvös Loránd University, Budapest, Hungary) were anesthetized by ketamine (100 mg/kg), placed in a stereotaxic frame, and both vertebral arteries were electro-cauterized (day 1). On the next day rats were anesthetized with halothane (1.5–2.0%) in air for 4–5 min and surgically prepared for four-vessel occlusion ischemia [22]. Separated from the vagus nerve, both common carotid arteries were looped around with silk-sutures. Then, halothane anesthesia was turned off, the silk-sutures around carotid arteries were tightened by hand to occlude the circulation for 4 min (PC group) or 10 min (ischemic groups). Silk-sutures were then cut and removed to restore the blood flow. The re-perfusion was checked visually before closing the wound. During the surgical procedure, the body temperature of the animals was maintained at 37 °C by heating lamps. In the PC group, 72 h after the first occlusion, the common carotid arteries were occluded for 10 min. The animals became totally unresponsive and lost their righting reflex within 2 min after occlusion of carotid arteries and remained unconscious during the entire ischemic period without anesthesia. During the first 15–20 min of re-perfusion the animals were passive, immobilized, and 1 h after the occlusion they were killed by perfusion (see the following).

Experimental groups: (1) Ischemic rats (four-vessel occlusion group, $n = 10$): Both vertebral arteries were electrocauterized and the common carotid arteries were occluded for 10 min. The animals were killed 1 h later. (2) Preconditioned group ($n = 6$): Four-vessel occlusion for 4 min, was followed by a 10 min occlusion 72 h later. Animals were killed 1 h after the second occlusion. (3) Operated control group ($n = 5$): Rats were subjected to the same surgical procedure as rats in the ischemic group without the occlusion of common carotid arteries. (4) Intact, non-operated control group ($n = 6$).

One hour after ischemia, animals were anaesthetized by ketamine (100 mg/kg) and perfused intracardially with 4% paraformaldehyde. Brains were removed, post-fixed in the same fixative solution overnight and cryo-protected by immersing the tissues in 20% sucrose. Thereafter, 60 μm free-floating sections were cut. Immunohistochemistry was performed using an avidin–biotin peroxidase (ABC, Vectastain: Vector Labs. Inc., Burlingame, CA, USA) method with some modification. Sections were washed in 0.1 M phosphate-buffered saline (PBS), pH 7.26, incubated in PBS containing 0.5% Triton-X-100 and 10% normal goat serum for 60 min, and subsequently with specific *c-fos* antibody: c-Fos (4) sc-52 (lot# D1503 rabbit policlonal IgG Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 1:10,000 dilution for 48 h. The antibody reacts with c-Fos p62 of mouse, rat and human origin by Western blotting, immunoprecipitation and immunohistochemistry, and is the preferred reagent for immunohistochemical analysis of formaline fixed tissue sections: non-cross-reactive with Fos B, Fra-1 or Fra-2. Thereafter, the sections were incubated with secondary anti-rabbit antibody (1:1000 dilution) for 1 h and in avidin–biotin peroxidase also for 1 h. Between all steps of the procedure, the sections were washed several times with 0.1 M PBS. The antigen–antibody complexes were visualized for 5–8 min by nickel enhanced 3,3′-diaminobenzidine (NiDAB, Sigma, St. Louis, MO, USA) chromogene reaction. Light counter-staining with Kernechtrot dye (Chroma, Köngen/N, Germany) was applied. The sections were covered by synthetic resin mounting media (DePeX, Boehringer Ingelheim, Germany). Fos+ cells were counted by using rectangular grids placed randomly on the investigated area (four times/section profiles) under a projection microscope. Data from four sections of each investigated area or nuclei from both sides ($4 \times 4 \times 2 = 32$, in each area or nucleus) were averaged for each animal (mean ± S.E.M.). The surface area of the grid was 0.02 mm^2, except for the CA1 area (0.01 mm^2). To determine the cell density in intact rats, brain sections from six control rats (group no. 4) were stained with Luxol fast blue cresylviolet. To evaluate the possible cell loss in response to the first (4 min) ischemia by the time of the second ischemia (72 h), the total cell numbers (both Fos+ and Fos− cells) were also counted in preconditioned animals (group no. 2). All of the neuronal cells were counted throughout the section profiles of the 12 investigated areas/nuclei. Results for total Fos+ cell counts are presented as mean ± S.E.M. For comparisons Student's *t*-test with Bonferroni correction, employing the Welch approximation to the degrees of freedom was performed.

The cell density (cell number/0.02 mm^2) in group no. 2 did not differ significantly from the cell numbers determined in group no. 4: dentate gyrus (DG) granular layer: 178.7 ± 16.8 versus 185.6 ± 23.0, $p = 0.64$; DG molecular layer: 5.9 ± 1.7

versus 6.1 ± 0.8, $p = 0.80$; DG polymorphic layer: 15.3 ± 3.8 versus 12.8 ± 3.2, $p = 0.25$; CA3 pyramidal cells: 57.3 ± 13.1 versus 61.6 ± 9.2, $p = 0.52$; CA1 pyramidal cells: 60.8 ± 14.9 versus 52.9 ± 10.5, $p = 0.32$.

The percentage of Fos+ cells in the CA1 and CA3 pyramidal and the DG granule cells was negligible (0.3–1.1% of the neurons) in operated control rats (group no. 3), and very low in the DG molecular and polymorphic layers 3.9 and 4.6%, respectively. In contrast, the percentage of Fos+ cells varied between 10.7 and 20.1% of neurons in limbic cortical areas, 11.9% in the hypothalamic paraventricular, 10.9% in the arcuate, and 20.4% in the thalamic periventricular nucleus (Table 1). Except one rat in the control group, the medial habenular nucleus was free of Fos+ cells.

The percentage of Fos+ cells in ischemic rats (group no. 1) increased strongly but unevenly 1 h after 10 min occlusions. The vast majority of neurons in the DG-established Fos immunopositivity: 82.7% of the granule cells, 86.3% of neurons in the polymorphic and 69.5% in the molecular layer were Fos+. Strong, but less pronounced increase of Fos activity occurred in the pyramidal cells: practically the half of the neurons (49.2% in the CA1, 48.9% in the CA3 area) expressed Fos 1 h after occlusions (Table 1). In the extrahippocampal areas, the most pronounced increase in the number of Fos+ neurons was seen in the hypothalamic paraventricular nucleus: 94% of the parvicellular neurons became Fos+. Moderate increase was counted in the thalamic periventricular nucleus (44%), while the number of Fos+ cells in the medial habenular (14.6%) and the arcuate nuclei (17.7) were relatively low (Table 1).

Marked differences were observed in the hippocampal *c-fos* expression between preconditioned (group no. 2) and ischemic animals (group no. 1), specifically in the granule and pyramidal cells (Fig. 1 and Table 1). The 4 min occlusion 72 h before ischemia markedly reduced the effect of 10 min ischemia on *c-fos* expression: only 12.1% of the granule cells, 13.0% of CA1 pyramidal cells and 12.0% of CA3 pyramidal cells showed Fos+ reactivity (Table 1). Significantly lower percentage of neurons in the molecular (42.4%) and the polymorphic layer (30.1%) established Fos+ reactivity comparing to those in the ischemic group. In contrast to the hippocampus, most of the neurons in the extrahippocampal areas express Fos, just like in rats without PC. In this group of animals, the density of Fos+ neurons in the cingulate and piriform cortex, as well as in the hypothalamic paraventricular and arcuate nuclei and the thalamic periventricular nuclei was comparable to that in the ischemic group (Table 1). A non-significant reduction in Fos+ cell density was seen in the entorhinal cortex neurons projecting to the hippocampus. The percentile changes in Fos+ cell densities in different experimental groups are summarized on Fig. 2.

It has been demonstrated by a fairly high number of immunohistochemical studies that neurons in the hippocampus, especially the DG neurons respond with a rapid and very strong expression of *c-fos* to short-term (4–20 min) global ischemia (see relevant references that follows). The present study is one of the numerous reports on *c-fos* expression in response to cerebral ischemia, however, the first systematic quantitative comparison of the effect of 10 min ischemia with

Table 1
c-fos expression 1 h post-ischemia in 12 selected brain areas

Brain region	Number of cells/0.02 mm^2 section profile[a] (number of rats)				I/CO[b]	PC/I[c]
	[d]Luxol+	Fos+				
	CNO[e] (6)	CO[f] (5)	I[g] (10)	PC[h] (6)		
Hippocampus DG						
Molecular layer	5.9 ± 1.7	0.2 ± 0.12	4.1 ± 1.2	1.6 ± 0.86	20.5	0.39
Granule cell layer	178.7 ± 26.8	2.0 ± 0.62	147.7 ± 22.6	15.9 ± 7.64	73.9	0.11
Polymorphic layer	15.3 ± 3.8	0.7 ± 0.25	13.2 ± 3.8	4.7 ± 3.07	18.9	0.36
Hippocampus CA1 pyramidal cell layer	60.8 ± 14.9	0.2 ± 0.14	29.9 ± 10.1	7.1 ± 6.09	150	0.24
Hippocampus CA3 pyramidal cell layer	57.3 ± 13.1	0.6 ± 0.43	28.0 ± 11.3	6.4 ± 4.84	46.7	0.23
Cortex						
Cingulate	42.2 ± 12.9	6.7 ± 1.58	14.8 ± 6.9	12.4 ± 5.04	2.21	0.84
Piriform	53.3 ± 5.5	10.7 ± 2.62	20.2 ± 7.7	18.8 ± 10.24	1.89	0.93
Entorhinal	45.9 ± 6.6	4.9 ± 1.47	25.6 ± 10.6	16.4 ± 7.13	5.22	0.64
Paraventicular n.	68.7 ± 15.0	8.2 ± 1.85	64.3 ± 14.5	60.5 ± 13.55	7.84	0.94
Thalamic periventicular n.	54.4 ± 17.9	11.1 ± 2.92	23.7 ± 6.4	24.4 ± 5.95	2.14	1.03
Medial habenular n.	75.9 ± 10.7	0.9 ± 0.33	11.1 ± 5.7	7.3 ± 2.22	12.3	0.66
Arcuate n.	58.6 ± 9.1	6.4 ± 1.52	10.4 ± 4.6	11.7 ± 5.20	1.63	1.13

[a] Mean values from measurements on 12 section profiles/brain regions/animals.
[b] I/CO: ischemic over operated control.
[c] PC/I: preconditioned over ischemic.
[d] Luxol fast blue cresylviolet staining.
[e] CNO: control, non-operated (group no. 4).
[f] CO: control, operated (group no. 3).
[g] PC: preconditioned (group no. 2).
[h] I: ischemic (group no. 1).

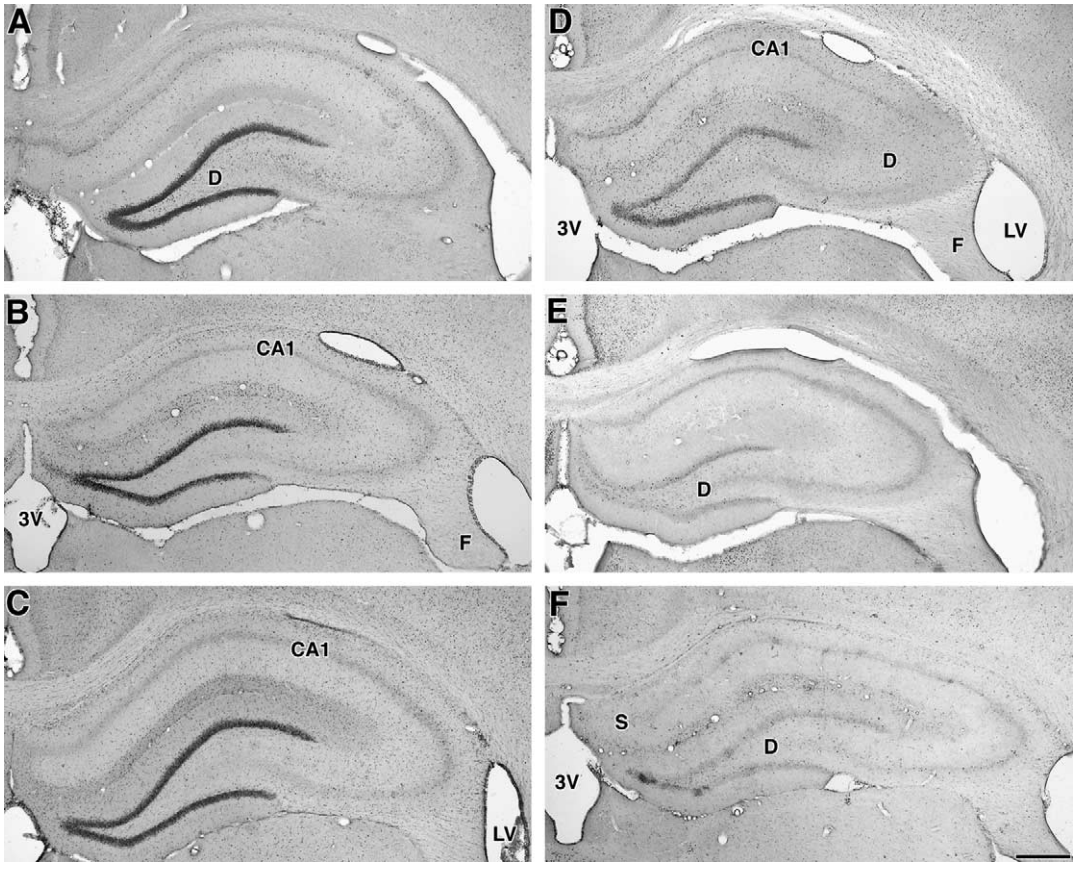

Fig. 1. Strong Fos-immunostaining in the dentate gyrus of ischemic rats (A–C) vs. weak or moderate immunostaining in tolerant animals (D–F) after four-vessel occlusions. Abbreviations: CA1: CA1 hippocampal area, CA3: CA3 hippocampal area, D: dentate gyrus, F: fimbria hippocampi, LV: lateral ventricle, S: subiculum, 3V: third ventricle. Scale bar: 200 μm.

and without PC on *c-fos* expression in five hippocampal and seven extrahippocampal brain areas.

Although, under normal conditions, *c-fos* expression is low in the central nervous system, numerous areas show a number of scattered neurons with moderate activity. This Fos immunostaining is confined to neurons in the piriform, cingulate and entorhinal cortex, the hypothalamic arcuate and paraventricular nuclei, the thalamic periventricular and habenular nuclei, the DG and pyramidal neurons in the CA1 and CA3 hippocampal areas [9,10]. All of these brain areas and nuclei have been investigated in the present study. Our procedure, the most commonly used ischemic model (four-vessel occlusion) represents one of the most severe stressful stimuli. Our results are consistent with previous reports [5,9], showing that brain regions with a relatively high basal expression of *c-fos* establish the strongest *c-fos* expression in response to stress. For instance, the highest percentage of Fos+ cells 1 h after 10 min ischemia are found in the paraventricular nucleus. PC failed to influence this effect.

In the current approach, *c-fos* protein expression was detected during a 1-h re-perfusion period. Consistent with the findings by Cho et al. [4], the *c-fos* expression was strongest among the DG granule cells but moderate among the CA3 and CA1 pyramidal neurons. The effect of ischemic preconditioning on *c-fos* expression was the most pronounced in the DG granular layer. In contrast, the PC-induced short-term decrease of *c-fos* protein expression did not occur in other cortical areas (cingulate and piriform cortex) and stress-sensitive nuclei (paraventicular, thalamic periventicular and arcuate), whereby *c-fos* protein expression remained high and relatively unchanged after PC. These findings are in accordance with the known stress-sensitivity of neurons in these areas, suggesting that the *c-fos* expression seen there in naïve animals can be considered as a sign of the stress-response. These observations strengthen the hypothesis that the hippocampal granule and pyramidal cells are more susceptible to ischemic tolerance than cells in other brain regions.

Injurious ischemia alone caused up-regulation of *c-fos* expression in several forebrain areas. The most striking difference among the brain areas was in the response to ischemic injury after PC. In this situation, the down-regulation of *c-fos* expression by PC preceding injurious ischemia seems to be a component of the complex cellular response that reprogrammes gene expression thereby inducing protein synthesis inhibition and “cellular arrest” during brain re-perfusion [25,29] within the hippocampal, but not the stress-sensitive areas. Thus, triggers inducing ischemic tolerance may be specific for brain areas. In fact stress-response and induction of

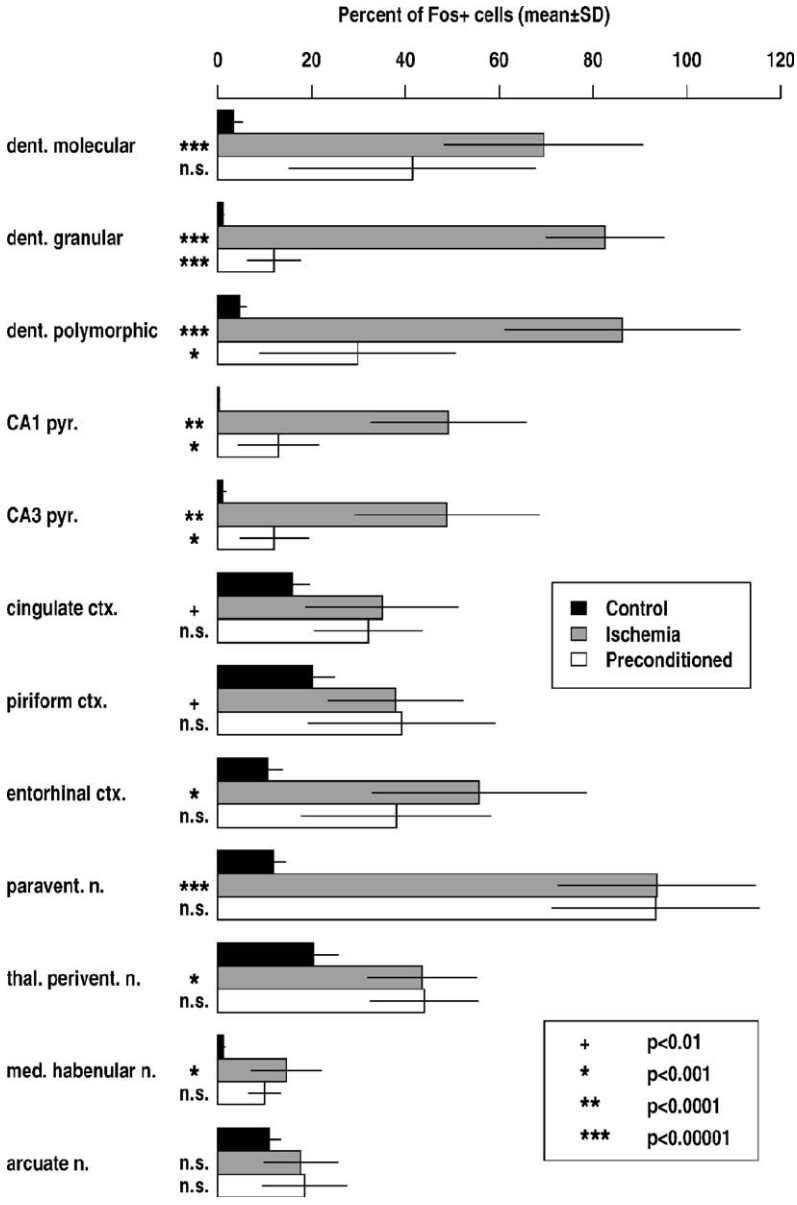

Fig. 2. Effects of 10 min ischemia on intact and preconditioned (4 min ischemia) rats on Fos expression 1 h following occlusions in 12 brain regions investigated. Numbers of Fos+ cells are given in percentage of the total cell number of the investigated area. Significance marks indicate that ischemic group was compared to preconditioned group (*t*-test).

ischemic tolerance in different forebrain areas can be distinguished.

Although sensible inferences can be made in relating the PC-specific dynamic changes of *c-fos* protein expression within the hippocampal areas, there is no firm basis for the interpretation of the PC-specific short-term reduction of *c-fos* protein as a neuroprotective effect or sign. At present, we have to rely rather heavily on convergent lines of evidence from different approaches, including pharmacological ones. Using a neuroprotective drug application strategy, Bokesch et al. [2] showed that dextromethorphan, an antitussive drug with anticonvulsant and neuroprotective properties, reduced the ischemia-induced *c-fos* protein expression at 1 h after ischemia, and also protected against delayed neuronal degeneration in the CA1 region of the hippocampus. This observation suggests that the reduction of *c-fos* expression at 1 h after ischemia may be positively correlated with surviving neurons.

The present study clearly shows that forebrain neurons do not respond universally to acute ischemia or PC. While PC seems to be ineffective in blocking acute ischemia-induced cell (*c-fos*) activation in certain hypothalamic, thalamic and limbic cortical neurons, most of the hippocampal pyramidal and dentate granule cells avoided establishing such activation in preconditioned animals. However: (i) neurons in the hippocampal granule cell layer reacted more strongly to ischemia than the pyramidal cells; (ii) and the pyramidal cells showed almost equal responses (in term of the percentage of Fos+ neurons) both to ischemia and

PC, although their fate is completely different. The CA3 pyramidal cells typically survive the ischemic insults with or without PC while a high percentage of CA1 pyramidal cells die in both conditions [8]. The present study has not determined the cellular mechanisms of either ischemia or ischemic tolerance [6,13,14,22,24,25], but the quantitative data provide solid evidence on the existence of specific target areas and target neurons in the forebrain, sensitive to acute ischemia as well as to ischemic preconditioning.

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