Behavioral evaluation of ischemic damage to CA1 hippocampal neurons: Effects of preconditioning

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Abstract. In Mongolian gerbils, global forebrain ischemia induces enhanced locomotor activity and the disruption of nest building immediately after the insult, followed by damage to hippocampal neurons developing 3 days later. Preconditioning by a brief episode of sublethal ischemia induces the protection of CA1 hippocampal neurons against a lethal ischemic insult. We examined how preconditioning with 2-min ischemia affects disturbances in the nest building behavior and locomotor activity induced by the injurious 3-min ischemia. Morphological examination confirmed that preconditioning significantly reduced neuronal damage in CA1 evoked by injurious ischemia. Behavioral studies demonstrated that preconditioning reduced the locomotor hyperactivity and latency in nest building after test ischemia, in comparison to sham or naive animals. The results indicate that the nest building test and measurement of locomotor activity may be used for an early in vivo prediction of the extent of ischemic brain damage and tolerance induced by ischemic preconditioning.

Key words: behavior, gerbil, global ischemia, hyperactivity, ischemic tolerance, nest-building

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INTRODUCTION

Even brief periods of global forebrain ischemia can induce neuronal lesions, particularly in vulnerable brain regions such as the hippocampal CA1. Mongolian gerbils (Meriones unguiculatus) have a unique architecture of cerebral arteries, with almost complete separation of the carotid from the vertebral systems. Therefore, bilateral carotid artery occlusion in these animals lasting for 3 or more minutes after 3 days results in the selective neurodegeneration of the pyramidal neurons in the hippocampal CA1 (Brown et al. 1979, Kirino 1982). The histological damage in this model is reproducible and easy to quantify by counting the number of lost pyramidal cells, e.g., in order to evaluate the effects of neuroprotective strategies (see Green and Cross 1997 for review, Strosznajder and Gajkowska 2006, Strosznajder et al. 2005). This delayed morphological damage is preceded by early behavioral abnormalities. Glickman and others (1970) and later Green and Cross (1997) showed that hippocampal lesions evoked by ischemic insult can disrupt nest building by the gerbils. Also, there are many reports demonstrating that an increase in locomotor activity develops early after global ischemia in gerbils (Andersen et al. 1997, Colbourne et al. 1998, Katsuta et al. 2003).

Decreased susceptibility to lethal forebrain ischemia, called ischemic tolerance, may be induced by preconditioning with preceding sublethal ischemia (for review see Kirino 2002). Studies on tolerance to ischemia may have a pathophysiological meaning in the clinic, as it probably reflects an adaptive mechanism to recurrent reversible brain ischemia or hypoxia, which may develop during episodes of transient ischemic attack (TIA) or obstructive sleep apnea syndrome (Brzecka 2005, Dirnagl et al. 2003). Several studies demonstrated that ischemic tolerance in gerbils resulting in the reduction of morphological damage may be induced by preconditioning using 2-min ischemia (Bond et al. 1999, Kato et al. 1992, Kitagawa et al. 1990, Ohtsuki et al. 1996). However, it is not clear if morphological neuroprotection after preconditioning in these animals is accompanied by an adequate functional protection (Corbett and Crooks 1997, Dooley and Corbett 1998). Behavioral tests such as nest building as well as locomotor activity have been used to predict the extent of delayed damage to the hippocampal CA1 (Baldwin et al. 1993, Katsuta et al. 2003, Kuroiwa et al. 1991). The aim of this study was to test the utility of the selected behavioral markers focusing on consequences of preconditioned ischemia.

METHODS

Animals

Male Mongolian gerbils (Meriones unguiculatus) were bred in the Animal Colony of the Medical Research Centre, Polish Academy of Sciences in Warsaw. The animals were fed ad libitum and kept at room temperature. A total of 80 gerbils was used in this study. Gerbils at the age of 12–13 weeks, weighing about 60 grams were randomly assigned into experimental groups. The animals were either submitted to a 3-min forebrain ischemia without preconditioning, or to a 3-min ischemia induced 2 days following a 2-min preconditioning ischemia. Moreover naive and sham operated gerbils served as controls. The animals from these groups were treated in vivo as described below. Animal experiments were carried out according to the Polish and the European Community Council regulations concerning experiments on animals. The First Local Ethical Committee in Warsaw approved the protocols.

Induction of forebrain ischemia

To induce brain ischemia, the gerbils were anesthetized with 4% halothane in a gas mixture containing 30% O2 and 70% N2O. Two minutes before the operation, halothane concentration was reduced to 2%, and was kept at this level during ischemia. The carotid arteries were isolated through an anterior midline cervical incision, made after the injection of local anesthetics. Cerebral ischemia was induced by the occlusion of both common carotid arteries with miniature aneurismal clips for 2 min (preconditioning), or 3 min (test ischemia). Sham-operated animals were exposed to the surgery without carotid occlusion. During the surgery animals were kept on the heating pad set at 38°C. After the wound closure, animals were kept at an ambient temperature of 21–24°C. Then the gerbils were moved to the animal house for 14 days. In this study there was no mortality that could be associated with the experimental protocol.
Measurements of brain temperature

For continuous recording and analysis of brain temperature in control and preconditioned conscious and freely moving gerbils, a telemetric system to measure the brain temperature (Mini Mitter VitalView hardware and software system, Mini Mitter Co. Inc. Oregon, USA) was utilized. For these measurements separate sub-groups of gerbils were created (n=4) submitted to a 3-min forebrain ischemia without preconditioning, or to a 3-min ischemia induced 2 days following a 2-min preconditioning ischemia. The implantation procedure of the brain temperature probes (probe type: Mini Mitter XM-FH-BP), adapted from Corbett and coauthors (1997), was previously described in detail (Duszczyk et al. 2005, 2006). Briefly, brain temperature probe holders were implanted unilaterally, tips into the striatum approximately the same depth as the hippocampus, in gerbils submitted to halothane anesthesia. Two days later the probes were inserted into the probe holders, and the gerbils were placed in a Plexiglas box resting on the telemetry receiver. Temperature signals from the telemetry probe were sampled every 30 s, to ensure strict control of the temperature. The mean temperatures for individual time points in each group (usually every 1 h) were calculated.

Histology

Fourteen days after ischemia the animals were anaesthetized with halothane and subjected to intracardiac perfusion fixation with 4% neutralized formalin. The brains were removed, immersed in 4% formalin for 1 week, transferred to absolute ethanol and embedded in paraffin. Cross sections from the dorsal part of the hippocampus at the level of between 2.2 and 3.5 mm posterior to bregma, 10 µm thick, were stained with cresyl violet.

Pathology

For each animal at least 5 sections of the central part of CA1 in both hippocampi were analyzed using a light microscope set at ×400 magnification. The density of viable CA1 pyramidal neurons was quantified in ten 0.1-mm portions per section, and an average number of neurons was expressed in percentage of the separately estimated mean neuronal density in the CA1 region of the sham-operated animals, i.e., 320 neurons per 1 mm.
system as an indication that the probe had moved and was scored as an activity count. With the probe implanted subcutaneously between the scapulae, the behavior involving movements such as feeding or grooming occurrences were not registered as activity (Harkin et al. 2002). Signals from the telemetry probe were sampled every 30 s, to ensure strict control of activity. Counts were summed every hour. In each group the mean of measurements was then calculated.

**STATISTICAL METHODS**

Apart from nest building scores, presented as medians and tested by the Mann–Whitney U test, in other groups the means of measurements were calculated ± SEM. Statistical significance of differences between means was tested using analysis of variance (ANOVA) followed by Dunnett or Turkey tests. Significance was taken at $P<0.05$.

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![Graph showing effects of ischemic preconditioning on postischemic neuronal damage in Mongolian gerbils.](image1)

**Fig. 2.** Effects of ischemic preconditioning on postischemic neuronal damage in Mongolian gerbils. Ischemic preconditioning was induced by 2-min ischemia 48 h before test 3-min ischemia. Histological damage in CA1 area of gerbil hippocampus was evaluated 14 days after a 3-min forebrain ischemia. The results are expressed as percentage of pyramidal cell loss from control level of 320 cell/mm (means ± SEM, $n=10$). Asterisks mark results significantly different from 3-min test ischemia ($P<0.05$, one-way ANOVA followed by Tuckey test).

![Graph showing effects of ischemic preconditioning and test ischemia on brain temperature of Mongolian gerbils.](image2)

**Fig. 3.** Effects of ischemic preconditioning and test ischemia on brain temperature of Mongolian gerbils. Experimental conditions as described for Fig. 2. Brain temperature in gerbils was measured for 72 h, beginning 2 h before 2-min preconditioning ischemia, which was followed after 48 h by 3-min test ischemia. Brain temperature of control animals submitted only to test ischemia is compared with temperature of the gerbils submitted to ischemic preconditioning. The data, which were sampled every 30 s for 1 h, represent means ± SEM ($n=4$). Asterisks mark results significantly different from 3-min test ischemia ($P<0.05$, one-way ANOVA followed by Dunnett test).
RESULTS

As presented in Fig. 2, 3-min ischemia induces a significant – about 90 percent – neuronal loss in the hippocampal CA1. A nominally sublethal 2-min preconditioning ischemia in our experiments was slightly injurious to neurons, inducing the death of approximately 20% of the pyramids. Preconditioning significantly (up to about 50%) reduced neuronal damage induced by test ischemia.

The measurements of brain temperature after preconditioning and test ischemia (Fig. 3) demonstrated that both these episodes were followed by brief periods of hyperthermia. Preconditioning did not change the brain temperature after test 3-min ischemia.

Measurement of locomotor activity of the gerbils indicates that animals submitted to 3-min test ischemia developed hyperactivity. This behavior returned to normal after 24 hours. In the group of gerbils preconditioned with 2-min ischemia, significant attenuation of hyperactivity was observed (Fig. 4).

Figure 5 shows the nest building behavior score. Animals were scored for 7 days following ischemic insult. Our studies demonstrated that naive and sham operated animals started nest building immediately after putting them into the experimental cage. The gerbils submitted to 3-min common carotid occlusion exhibited a 2-day delay, in comparison to sham-operated animals. In the group of preconditioned gerbils, delay in the nest building was reduced to only 1 day.

DISCUSSION

The results of this study demonstrate that preconditioning with sublethal brain ischemia in gerbils induces not only the morphological neuroprotection of the CA1 pyramidal neurons challenged by subsequent lethal ischemia, but also reduces their early dysfunction, which precedes a delayed neuronal death and causes behavioral deficit. Moreover, our results show the validity of some parameters of home cage behavior as reliable in vivo indicators of the tolerance to ischemia and CA1 protection in the hippocampus of Mongolian gerbils, induced by ischemic preconditioning. Thus, a reduction in the posts ischemic locomotor hyperactivity and preservation of nest building behavior in preconditioned animals appear to have equal value for predicting diminution in delayed neuronal death in CA1.
Due to the anomaly of their Circle of Willis, Mongolian gerbils are particularly susceptible to bilateral carotid occlusion, which results in forebrain global ischemia in that species. In the present study we utilized a model of 3-min forebrain ischemia as a standard injurious test ischemia. It induced the death of over 90% of the CA1 pyramidal neurons. These results confirm our previous data and agree with the reports of other authors that 3-min ischemia induces the death of 70–90% of the CA1 neurons (Lazarewicz et al. 1997, Ohtsuki et al. 1996). According to previous reports, 2-min forebrain ischemia in gerbils was sublethal, i.e., producing no damage to hippocampal neurons (Bond et al. 1999, Dowden and Corbett 1999, Kirino 1982, Ohtsuki et al. 1996). However, in our studies 2-min preconditioning ischemia produces modest loss of neurons, i.e., about 20% of CA1 pyramidal neurons die (Duszczyk et al. 2005).

Preconditioning ischemia is well known as a factor which attenuates the effects of lethal ischemia. It has been shown that it preserves vulnerable neurons, such as pyramids in the hippocampal CA1 region, against ischemic damage (Kato et al. 1991, Kirino et al. 1991). Our results, in accordance with the reports of Corbett and Crooks (1997) and Wada and coauthors (1997), confirm that 2-min preconditioning ischemia significantly reduces the damage induced by 3-min test ischemia. In agreement with previous reports (Corbett and Crooks 1997) preconditioning does not change posts ischemic hyperthermia. Our unpublished data show that 5-min test ischemia is more resistant to preconditioning. Published literature indicates that the extent of ischemic neuronal damage and the effectiveness of preconditioning ischemia may vary between laboratories and even between different sets of experiments (Bond et al. 1999). This diversity in susceptibility to carotid occlusion may depend on individual differences in sensitivity to ischemia of gerbils used in particular studies, and/or on even slight differences in such experimental conditions like brain temperature (Duszczyk et al. 2005).

The death of neuronal cells after global ischemia is termed “delayed neuronal death” because pyramidal neurons of the CA1 initially retain normal appearance, but die 3–4 days after the insult (Kirino 1982, Pusinelli et al. 1982). However, during the initial posts ischemic period preceding the death of the CA1 neurons, gerbils demonstrate a behavioral deficit, which seems to be linked to hippocampal dysfunction. It is of great importance that the behavioral evaluation of neuronal damage can predict the morphological consequences (Baldwin et al. 1993).

Locomotor hyperactivity is a typical, well-known early behavioral effect of injurious ischemia, preceding the overt damage of the CA1 neurons. This effect, developing a few hours after the insult and lasting for at least 24 hours, has been interpreted as a symptom of functional impairment of injured but live pyramidal neurons of the CA1 sector (Babcock et al. 1993, Kuroiwa et al. 1991). Some authors suggested that the mechanism of this phenomenon is connected to a reduced ability of gerbils to form spatial maps and to their deficit in habituation rather than to a simple form of motor hyperactivity (Babcock et al. 1993, Wang and Corbett 1990). However, this explanation was challenged by others (Andersen et al. 1997). More recent studies point to the involvement of the dopaminergic system in the ischemia-induced hyperactivity in Mongolian gerbils (Yamamoto et al. 2001). There are data indicating that neuroprotective substances, which reduce posts ischemic damage in CA1, also attenuate hypermotility; and there is evidence for a correlation between early hyper locomotion and delayed death of the CA1 neurons after lethal ischemia (Baldwin et al. 1993, Katsuta et al. 2003). There are other data supporting the assumption that posts ischemic hyperactivity
and CA1 injury share a common mechanism. The result of studies by Mileson and Schwartz (1991) showed that hyperactivity is associated with the extent of hippocampal pyramidal cell lesion. Colbourne and others (1998) demonstrated that hypothermia, which reduces neuronal damage in CA1 after ischemia, also reduces functional impairments. In their experiments, animals spontaneously developed hyperactivity that returned to normal after 1 day. Identical behavior was observed in our experiment after 3-min ischemia (Fig. 4). An enhanced locomotor activity of gerbils observed in our study and by others is a component of disruption to normal home cage behavior (Colbourne et al. 1998, Katsuta et al. 2003, Kuroiwa et al. 1991). A similar phenomenon was also recognized in open field tests or in the other tests using novel environments (Babcock et al. 1993, Corbett and Crooks 1997, Wang and Corbett 1990). The former phenomenon typically lasts only for the first 24 hours after the insult, whereas the latter may be observed for much longer time after injurious ischemia or indefinitely, depending on the extent of the CA1 damage.

It is well documented that, in addition to locomotor hyperactivity (Andersen et al. 1997, Colbourne et al. 1998, Katsuta et al. 2003, Mileson and Schwartz 1991), global brain ischemia in gerbils results in disruptions to nest building behavior (Antonawich et al. 1997, Baldwin et al. 1993). Normally, Mongolian gerbils build a nest in a specific manner. They grasp a soft tissue such as paper towel, pull it into a pile, then proceed to chew and shred the material (Antonawich et al. 1997). The ischemic lesion of the hippocampus disrupts such behavior (Antonawich et al. 1997, Baldwin et al. 1993, Glickman et al. 1970). It was shown that the latency in the nest building process is proportional to the duration of global forebrain ischemia (Antonawich et al. 1997). A typical male gerbil builds a nest within 12–24 hours. In our studies, both naive and sham operated animals started nest building immediately after placing them in the experimental cage, while the animals submitted to 3-min common carotid occlusion demonstrated significant decrease in nesting. They started building with 2 days of latency in comparison to sham-operated and naive animals.

It seems that postischemic hyperactivity and disruption of nest building in gerbils are closely related. Previous studies demonstrated that a mechanical lesion of the gerbil hippocampus induced both locomotor hyperactivity as well as a decreased frequency of such patterns as shredding and nesting (Glickman et al. 1970). Baldwin and co-workers described a strict correlation between postischemic hippocampal lesion, locomotor hyperactivity and delayed nest building in gerbils (Baldwin et al. 1993). More recent studies demonstrated nonhabituating hyperactivity and impairment of nest building in mice carrying a targeted point mutation in the glycine binding site of the NMDA receptor (Ballard et al. 2002). It has been suggested that the disruption of nest building is not a secondary effect to hyperactivity but rather represents defects in the animals’ ability to properly sequence cues or behaviors (Murphy and Cyrilla 1986), or that both these behavioral abnormalities are related to delays in habitation and spatial mapping resulting from hippocampal damage (Antonawich et al. 1997, Babcock et al. 1993).

It was not clear if tolerance to ischemia in Mongolian gerbils induced by preconditioning comprises equal morphological and functional behavioral neuroprotection. In spite of well documented morphological neuroprotection evoked by ischemic preconditioning in this model of brain ischemia (see above), there were reports that preconditioned gerbils after test ischemia demonstrate a habituation deficit in an open field test not different from untreated ischemic gerbils (Corbett and Crooks 1997, Dooley and Corbett 1998). Previous studies concerned only locomotor activity or T-maze tests whereas, to our knowledge, the level of nest building disruption in preconditioned gerbils was not investigated. The results of our study clearly demonstrate that 3-min untreated ischemia induces severe damage of CA1 neurons, locomotor hyperactivity and a significant latency in nesting, whereas after preconditioned ischemia this damage was reduced and this effect was accompanied by an inhibition of hyperactivity and reduction of latency in nesting. Authors of previous studies showed that the time of occlusion, size of hippocampal lesion and delay in nest building are correlated (Antonawich et al. 1997, Baldwin et al. 1993). Also, the correlation between hyperactivity and disruption of nesting in postischemic gerbils has been previously reported (Glickman et al. 1970), in line with current study. More importantly, our data indicate that this relation also concerns tolerance evoked by preconditioning and demonstrate that preconditioning 2-min ischemia induces a significant morphological and functional neuroprotection to 3-min test ischemia. It is difficult to explain the differences between our data and the
results of Corbett and Crooks (1997) and Dooley and Corbett (1998). These authors reported a lack of functional protection by preconditioning within the first 10 days after ischemia. In their studies, two episodes of short 1.5-min preconditioning ischemia did not reduce hyperactivity induced by 5-min test ischemia. It seems that apparently slight distinctions in the duration of test ischemia or in protocols of testing the locomotor activity (see above) between both studies might result in these differences. An alternative explanation might be a well-known variability of the extent of ischemic neuronal damage and the effectiveness of preconditioning ischemia (Bond et al. 1999). Nevertheless, in our model of ischemic tolerance, functional protection observed in behavior strictly corresponds to morphological neuroprotection of CA1 neurons, demonstrated in histological evaluations. This study showed that nest building behavior may be equally as useful as locomotor activity in the in vivo evaluation of the extent of neuroprotection against lesions of hippocampal CA1 neurons in ischemic tolerance. Moreover, since both these behavioral alterations evolve very early after ischemia, long before any morphological changes in the CA1 neurons could be observed, they can be utilized for in vivo prediction of the size of the expected lesion, which will develop in individual gerbils.

CONCLUSIONS

The results of this study indicate that morphological neuroprotection induced by ischemic preconditioning in the gerbil model of reversible forebrain ischemia is accompanied by a corresponding functional protection against postischemic disruption of normal home cage behaviors. These data demonstrate that the evaluation of nest building and of locomotor activity is a useful behavioral indicator of the ischemic tolerance of Mongolian gerbils. These indices of cage behavior may be used for an early in vivo prediction of the extent of ischemic brain damage and the tolerance induced by ischemic preconditioning.

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