INCREASED BEHAVIOURAL AND HISTOLOGICAL VARIABILITY ARISING FROM CHANGES IN CEREBROVASCULAR ANATOMY OF THE MONGOLIAN GERBIL

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By

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Abstract

The bilateral common carotid artery occlusion (BCCAO) in the Mongolian gerbil has been used as a simple and highly reproducible model of forebrain ischemia due to an incomplete circle of Willis. However, increased behavioral and histological variability to a typical 5 min period of ischemia in this model led us to conclude that the vasculature was changing. To test this hypothesis I compared gerbils from two Canadian suppliers. Gerbils from Charles River (CR) exhibited a greater incidence of complete or partial circle of Willis compared to their High Oak (HO) counterparts. This altered vasculature pattern in the CR versus HO gerbils was associated with reductions in behavioral activation that characteristically accompany conditions of milder ischemia resulting in less severe hippocampal CA1 cell loss (e.g. ~70% CA1 cell loss vs. 95% CA1 loss).

Gerbils from CR, the main supplier in North America, no longer represent a reliable model for use in forebrain ischemia studies. Gerbils from HO while superior to those from HO are also more variable in their response to BCCAO than those used as recently as 5 years ago. Thus the gerbil model of forebrain ischemia, at least using CR animals, no longer produces consistent injury and behavioral alterations. Investigators are urged to consider adopting other models in future neuroprotection studies or ensure that their gerbil population lacks communicating arteries.

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ABSTRACT

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Abbreviations

2-VO, two vessel occlusion; 4-VO, four vessel occlusion; ATP, adenosine triphosphate; BCCAO, bilateral common carotid artery occlusion; CA1, Cornus of Ammon area of hippocampus 1; CA3, Cornus of Ammon area of hippocampus 3; CBF, cerebral blood flow; CPR, cardiopulmonary resuscitation; CR, Charles River; HO, High Oak; PCA, posterior cerebral artery; PcomA, posterior communicating artery; SCA, superior cerebellar artery.

Introduction

The human brain requires a continuous supply of glucose and oxygen to meet its energy requirements. Cerebral oxygen consumption is about 4.0 ml/100 g brain/min. Although the human brain represents only 2 percent of total body weight, it accounts for twenty percent of the resting total body oxygen consumption (Frackowiak et al., 1980). Due to the brain's high demand for energy any interruption can have deleterious consequences. When the circulation in the brain has ceased and available oxygen is depleted a patient will become unconscious within 5-10 sec (Rosenberg, 1990). Within 7 min brain glucose and adenosine triphosphate (ATP) levels are exhausted (Siesjo, 1984). With no energy to maintain ionic gradients there are changing levels of sodium, potassium, and calcium within and surrounding brain cells. The abnormal efflux of potassium and influx of sodium results in neuronal and interstitial swelling (Hacke, 1991). Moreover, the influx of calcium further complicates the situation by initiating numerous cellular cascades that lead to neuronal death (Hass, 1983). In addition, there is an accumulation of neurotransmitters in the synaptic cleft, most notably glutamate, which promotes excitotoxicity (Rothman and Olney, 1986). If blood flow is not restored within minutes, permanent brain damage and, ultimately, death occurs.

In cases where cardiopulmonary resuscitation (CPR) and other medical interventions are initiated early and restore blood flow, patients can be left with cognitive impairments. A landmark paper by Zola-Morgan et al. (1986) described how patient R.B. who had an ischemic episode, had developed severe anterograde amnesia with little or no retrograde amnesia. R.B. showed no signs of cognitive impairment other than memory.

Following his death, thorough histological studies revealed his memory impairments were associated with bilateral damage to the Cornus of Ammon area 1 (CA1) of the hippocampus. In subsequent investigations it was found that this type of cell death in CA1 was directly related to the duration of cardiac arrest and it occurred in a delayed fashion (Petito et al., 1987).

Fortunately, this delay may provide a window of therapeutic opportunity that may continue for several hours after ischemia where interventions can protect or possibly restore "normal" brain functioning. With this in mind we must systematically study the pathophysiology and treatment efficacy of forebrain ischemia through well-controlled and reproducible animal models if we are to reduce the memory deficits people sustain following a prolonged reduction in cerebral blood flow (CBF).

Models of Forebrain Ischemia

One of the easiest ways to produce a lack of perfusion to the brain is by decapitation. This method instantly induces a state of irreversible global brain ischemia. The decapitated head can be maintained for various lengths of time at 37°C and then freeze-trapped with liquid nitrogen or homogenized for analysis of biochemical processes. This model has been useful in studying various ischemia induced changes in many metabolic pathways (Lowry et al., 1964). The clear advantage of this model is its simplicity. However, long-term studies and behavioral assessments are impossible.

Another method to induce forebrain ischemia is the application of pressure on the cranial arteries and veins. With an anesthetized rat, a pressure cuff is inflated within one

second to approximately 600 - 700 mm Hg with the mean blood pressure kept constant at 50 mm Hg by withdrawing or re-infusing blood (Siemkowicz and Hansen, 1978). The method has been reported to reduce CBF to less than 1% of the normal rate in all brain regions (Siemkowicz and Gjedde, 1980). Studies of this nature have not reported histological outcomes and were mainly used to study the effects of glucose levels on post-ischemic recovery. A similar method has been used for larger species, such as the monkey (Nemoto et al., 1977; Scheller et al., 1992). In this model, a cuff is inflated to higher pressures (1500 mm Hg) with blood pressure control throughout the ischemic insult, as described previously. In this monkey model, injury occurred in cortical and brainstem regions (Nemoto et al., 1977), whereas others reported mainly CA1 damage (Scheller et al., 1992). Initial mortality rates were extremely high (above 50%) (Nemoto et al., 1977), but with improved postischemic supportive care no deaths were reported (Scheller et al., 1992). This model has also occasionally been used to study forebrain ischemia in cats (Chopp et al., 1987), using techniques comparable to the ones described for rats and monkeys.

The brachiocephalic artery occlusion model of forebrain ischemia has been utilized most extensively in both cats (Hossmann and Sato, 1970; Hossmann, 1971) and monkeys (Hossmann and Zimmermann, 1974; Kleihues et al., 1975; Zimmermann and Hossmann, 1975; Bodsch et al., 1986; Hossmann and Grosse Ophoff, 1986). This model involves occluding both the brachiocephalic artery and left subclavian arteries near their origin at the aortic arch. The left internal mammilary artery is ligated and blood pressure is lowered pharmacologically. Completeness of ischemia is confirmed by microscopic

observations of the pial blood vessels (Hossmann and Sato, 1970) or by monitoring the clearance of a radioactive tracer from the brain (Hossmann and Zimmermann, 1974). The earlier cat studies examined sensorimotor motor cortex and showed that complete cerebral ischemia produced no severe pathological alterations of cellular components after 1 hr (Hossmann and Sato, 1970) and electrophysiological sparing after 1.5 hr (Hossmann, 1971) following restoration of blood flow. This model has also been extended to monkeys, in which ischemic injury was produced and partial recovery of cortical function, metabolism and protein synthesis was demonstrated (Hossmann and Zimmermann, 1974; Kleihues et al., 1975; Zimmermann and Hossmann, 1975; Bodsch et al., 1986; Hossmann and Grosse Ophoff, 1986). In later studies, while it was demonstrated that there was electrophysiological sparing (Hossmann and Grosse Ophoff, 1986) after 1 hr of complete ischemia, histologically not all regions of the brain were without injury. Selective regions of the brain, particularly CA1, were damaged (Bodsch et al., 1986).

Ventricular fibrillation is another technique to produce complete forebrain ischemia. This technique mimics the clinical situation of cardiac arrest and has the added benefit of being paired with CPR following cardiac arrest. It should be noted that this model produces complete ischemia followed by incomplete ischemia due to the CPR component. Ventricular fibrillation has predominately been used to study ischemia in dogs (Safar et al., 1976; Koehler et al., 1983; Michael et al., 1984; Brown et al., 1986; Schleien et al., 1986; Brown et al., 1987; Berkowitz et al., 1991; Radovsky et al., 1995) and to a lesser extent in pigs (Berkowitz et al. 1991; Brown et al. 1986; Brown et al.

1987; Schleien et al. 1986). Ventricular fibrillation was often induced by electric shocks via subcutaneous electrodes on both sides of the thorax or by passing an alternating current through a bipolar electrode directly to the right ventricle. In both types of animals it was found that ventricular fibrillation and defibrillation was most successful when CPR was used in combination with epinephrine. Epinephrine, through its action of reversing and preventing arterial collapse, increasing cerebral and myocardial perfusion pressures by vasoconstricting peripheral vascular beds, increased CBF by 75% over controls (Michael et al., 1984).

Histopathologic data were gathered in a study by Radovsky et al. (1995) where animals were subjected to varying durations of cardiac arrest from 0 to 20 min. While longer durations of ischemia produced a marginally increased prevalence of necrotic neurons, they clearly showed that 10 min of ischemia selectively damaged CA1 neurons and spared other sectors even after 20 min of arrest. This type of neuronal selectivity is reminiscent of damage seen in humans. While selective CA1 loss has been observed in the previous models discussed it is the hallmark of the next few animal models.

Other than the advantages already pointed out for each of the previous three models (neck cuff, brachiocephalic artery occlusion, and ventricular defibrillation), clearly the ease with which physiological measurements can be taken and the similarities that exist between the brains of larger animals (such as the canine and primate) to humans make each of these models appealing. However, the sheer cost of purchasing, maintaining, and caring for larger animals, along with the concern for animal welfare in

today's society make the rodent models of forebrain ischemia more practical and ethically acceptable.

Knowledge of the anatomy of the blood vessels arising from the aorta and reaching the brain is critical for the production of successful ischemic models. In general, blood reaches the brain through two main pairs of arteries, the internal carotid and the vertebral. The internal carotid originates from the common carotid and gives rise to the posterior, middle and anterior cerebral arteries. The vertebral arteries arise from a branch of the subclavian artery and merge to form the basilar artery. The basilar artery later divides into the superior cerebellar arteries (SCA). The connections between the carotid and basilar circulations in the form of communicating arteries form the circle of Willis. Although this arterial circuit occurs in the rat its presence is questioned in the gerbil.

The 2-vessel occlusion (2-VO) model is a reversible form of forebrain ischemia which is produced by occluding the carotid arteries, coupled with systemic hypotension (usually 50 mm Hg) by blood withdrawal and reinfusion (Eklof and Siesjo, 1972b, 1972a) or in combination with pharmacological agents (Smith et al., 1984b). In this method the vertebrobasilar artery system remains perfused during the ischemic insult and thus structures in the hindbrain, which are responsible for regulating breathing and heart rate, remain undamaged. CBF values during 2-VO have been reported to be less than 5% of normal values in many forebrain structures, including the cerebral cortices, caudoputamen, and hippocampus (Kagstrom et al., 1983; Smith et al., 1984b).

With this model damage can be seen in CA1 and subiculum after 2 min of ischemia, in the neocortex after 4 min, in Cornus of Ammon area of hippocampus 3

(CA3) after 6 min, and in the caudoputamen after 8 – 10 min of ischemia (Smith et al., 1984a). Modifications to the model by reducing the blood pressure to 30 mm Hg during the 2-VO yielded more consistent histological results, while maintaining the same temporal vulnerability profile of neuronal populations previously discussed.

More recently the 2-VO model was modified to become the four-vessel occlusion (4-VO) model of reversible forebrain ischemia in the rat (Pulsinelli and Brierley, 1979). Later the procedure was updated and a more detailed methodology was published to eliminate ambiguity between laboratories (Pulsinelli and Buchan, 1988). The procedure is performed in a two-day process. On the first day atraumatic arterial clasps are placed around each common carotid artery without interrupting carotid blood flow and exteriorized through the closed incision. In addition, surgical silk is placed through the cervical region of the rat so that the suture lies posterior to the trachea, esophagus, external jugular vein, and common carotid arteries. The vertebral arteries are electrocauterized via the alar foramen before they enter the posterior fossa. A similar method of ischemia has been developed, but involves cauterizing the basilar artery (Kameyama et al., 1985; Shirane et al., 1991).

On the second day, both carotid clasps are tightened while the animal is awake. However, some investigators have adapted the model for use in anesthetized rats (Yoshida et al., 1982; Globus et al., 1988). Following the carotid occlusions the cervical suture is slowly tightened to stop collateral blood vessels in the neck muscles from supplying the head with blood flow. This second occlusion is done in a gradual fashion depending on the animals' level of consciousness and its ability to breath. The 4-VO

must result in the animal losing both consciousness and righting reflex to be considered adequately ischemic. The success rate in the original model was about 75% (Pulsinelli and Brierley, 1979; Pulsinelli and Buchan, 1988). However, with the 4-VO modifications which accounted for collateral blood flow in cervical and paravertebral arteries the success rate was closer to 90% (Pulsinelli and Buchan, 1988). It should be noted that while a 90% success rate was observed, only two-thirds of those animals survived 3 days free of convulsions. A success rate of between 50 – 75% is most likely to occur with this model, but varies with the strain of rat used (Traystman, 2003).

CBF in the 4-VO model is dramatically reduced in all areas of the brain, with reductions less than 3% of control values in cortex, striatum, and hippocampus during ischemia, whereas diencephalic blood flow was reduced to 10%, cerebellar flow to 15% and brainstem flow was approximately 30% of controls (Pulsinelli et al., 1982b). While the reductions in CBF in the cortex, hippocampus and striatum were equivalent, the damage exhibited in these regions varied dramatically (Pulsinelli et al., 1982a). Following 10 min of ischemia, vulnerable regions of the hippocampus showed severe ischemic damage in about 40% of the cerebral hemispheres following a 3-day survival time. The striatum and cortex were not damaged at this duration. With 20 min of ischemia, 85% of CA1 and 35% of cortex were damaged. If ischemia extended out to 30 min, virtually all of the hippocampus, particularly CA1 was damaged. Striatal injury occurred in 90% of the cerebral hemispheres with minimal changes seen in cortical damage. It is important to note that to produce consistent histological damage (with this and all other reversible global ischemic models) brain temperature must be carefully

regulated. Subsequent studies have found that by lowering the brain temperature a few degrees during ischemia, CA1 neurons were protected (Busto et al., 1987). This may account for some of the discrepancies between the 2-VO and 4-VO models when comparing ischemic durations and histopathological outcomes.

While not the first to describe the selective vulnerability of CA1, Pulsinelli et al. (1982a) were the first to report (in the rat) that it occurred in a delayed fashion. This phenomenon was further verified and elaborated on by Kirino et al. (1984) and was deemed identical to the delayed cell death observed in the gerbil model of forebrain ischemia (discussed later) (Kirino, 1982). Significantly, this kind of selective damage to CA1, as found with other global models, produced permanent impairments in working memory (Volpe et al., 1984).

Between the two models discussed, the 2-VO model has the advantage of being a one-stage surgical procedure and less technically challenging. However, the requirement for anesthesia and drugs during the ischemic insult may introduce extraneous variables that the 4-VO model avoids. Both models lend themselves to the use of sophisticated neurosensory and motor behavior measurements as outcome measures of injury from ischemia.

As mentioned in the previous rodent models of forebrain ischemia (2-VO and 4-VO), steps have to be taken to prevent collateral blood flow from the posterior circulation from reaching the forebrain. With the gerbil model of bilateral common carotid artery occlusion (BCCAO), researchers were able to use a one-step process to produce reliable ischemia in gerbils that lack a complete circle of Willis. This surgery involves making a

midline incision under anesthesia, as in the other rodent models. The carotid arteries are isolated and looped with surgical silk. When it is time to induce ischemia, microaneurysm clips are placed on the arteries for a desired length of time (usually 5 min). The clips are then removed and blood flow is restored. CBF during BCCAO may range anywhere from 0 – 10 ml/100 g/min (Osburne and Halsey, 1975; Crockard et al., 1980) with regional hippocampal blood flow being approximately 6% of sham values (115 ml/100 g/min) (Osburne and Halsey, 1975; Crockard et al., 1980; Suzuki et al., 1983; Tomida et al., 1987).

Typically, the 5 min occlusion restricts histopathological changes to the dorsal hippocampus. As discussed previously, this restrictive cell loss is of a delayed nature (Kirino, 1982). Cell death becomes apparent after 2 days and is almost complete by 4 days following ischemia. If the ischemic insult is prolonged to 10 min, changes are reminiscent of 5 min occlusions. Longer periods of ischemia (20 – 30 min) produce changes similar to shorter insults, but are seen in a more rapid and severe form (Kirino and Sano, 1984). Due to the delayed nature of cell death in CA1, researchers have attempted to elucidate the mechanism(s) surrounding this form of cell death and have attempted to use various neuroprotective strategies, such as employing pharmacological agents (Hewitt and Corbett, 1992), enrichment (Farrell et al., 2001) and hypothermia (Corbett et al., 1997). Overall, the gerbil model of forebrain ischemia has been extensively used because of its relative simplicity in terms of the surgical procedure and the reproducibility of CA1 injury. However, the usefulness of this model is in jeopardy.

The susceptibility of the Mongolian gerbil (*Meriones unguiculatus*) to ischemia from either unilateral or bilateral occlusions was first described in the mid 1960's (Levine and Payan, 1966). Based on behavioral outcomes it was suggested that the animal's vulnerability to ischemia induced by BCCAO was due to the presence of an incomplete or functionally inadequate circle of Willis. Detailed anatomical studies examining the posterior circulation of the gerbil corroborated the original hypothesis. Levine and Sohn (1969) found that "None of the gerbil brains revealed any anteriorly directed branch of the basilar or superior cerebellar arteries that might have reached the posterior cerebral or other part of the carotid circulation." They did acknowledge the existence of small "posterior communicating arterioles", but they suggested that their small size would make their contribution "insignificant". Later work published by Kahn (1972) found that the "gerbil brain did not form an anastomotic circle." Harrison et al. (1973) came to the same conclusion regarding a lack of PcomAs and agreed that the gerbils' circle of Willis was incomplete. However, they confirmed the existence of small (less than 50 μm) arterioles that linked the posterior and anterior circulation. In a detailed study, Levy and Brierley (1974) concluded that variability was due to connections between the carotid and vertebrobasilar circulation. They demonstrated the presence of dye in territories only supplied by carotid arteries, even after they had been ligated during perfusions. Upon macroscopic examination, numerous arterioles (30 – 60 μm) were found to connect the SCA to the posterior cerebral arteries (PCA). With few exceptions (Schonfeld and Glick, 1979), all published material concerning gerbil ischemia after Berry et al. (1975) assumed

that BCCAO led to complete forebrain ischemia. In that study they unequivocally stated the absence of communication between anterior and posterior cerebral circulations.

The assumption that the BCCAO creates complete ischemia in all gerbils was finally addressed in a series of studies in the early 1990's comparing the Mongolian and Israeli gerbil cerebral circulation (Breuer and Mayevsky, 1992; Mayevsky and Breuer, 1992). They made detailed correlations between anatomical and metabolic responses and concluded that a unilateral or bilateral posterior communication existed respectively in 20% or 10% of the Mongolian gerbils tested. This represented a three-fold increase in anterior-vertebrobasilar communication noted ten years prior in unreported work. They attributed this change to unknown genetic factors. Recent studies have found a direct relationship between ischemic injury and cerebral vasculature in the mouse model of forebrain ischemia (Murakami et al., 1998). Could this genetic shift become more of a problem and be explained, like in the mouse model, by an increased presence of PCAs connecting the carotid and vertebral circulations?

In recent experiments in our laboratory and those of our colleagues (Dr. Colbourne, University of Alberta) the gerbil BCCAO has become extremely variable (unpublished observations). While typical CA1 cell loss is observed following ischemia, in an alarming number of cases there is only unilateral damage, whereas in other animals both hippocampi appear intact. We suspect that the gerbils from Charles River (CR) (our current supplier) may have been subject to the same changes as Mayevsky's lab, but perhaps to a greater degree. To test this hypothesis I decided to compare CR gerbils to a supply from the University of Alberta (originally High Oak). In the past, High Oak (HO)

gerbils, in our hands, yielded an extremely high success rate of animals with > 95% CA1 cell loss (Colbourne and Corbett, 1994; Nurse and Corbett, 1994; Colbourne and Corbett, 1995). The goal of this experiment was to determine the overall state of the two available gerbil colonies for use in forebrain ischemia studies, and to relate biological, behavioral and histopathological outcomes with postulated changes in cerebrovasculature.

Experimental Methods

Subjects

A total of 88 Mongolian gerbils weighing 53 – 85 g were used for this study. There were 45 (24 male and 21 female) from Charles River Laboratories (Montreal, QC) and 43 (20 male and 23 female) from the University of Alberta breeding colony, which were originally obtained from High Oak Ranch (Baden, ON). There were a total of 29 and 28 ischemic animals in the CR and HO groups respectively. The remaining gerbils within the groups underwent sham surgery. All experiments met the guidelines established by the Canadian Council of Animal Care and were approved by the Memorial University of Newfoundland Animal Care Committee.

Temperature and Activity

Core body temperature and activity were closely monitored in all animals, except sham group, before and after the ischemic insult. All animals were implanted with body probes (XM-FH, Mini-Mitter Co., Sunriver, OR) to monitor temperature and activity levels. Implant surgeries were performed under 2.0% isoflurane (3.0% induction, 30% O_2/70% N_2O). An incision was made in the midline of the abdominal wall and using a pair of scissors, a small cut was made in the muscle wall. The scissors were inserted into the opening and were used to separate the muscle fibers. The probes were inserted into the abdominal cavity and the wound was closed with sutures. Animals were placed back in their cages and allowed to recover for 7 days. Temperature and activity levels were measured every 30 sec for a period of 24 hr before and after the ischemic insult. Both

temperature and activity data were averaged over a 24 hr period prior to ischemia to give baseline values and following ischemia data were averaged into 8 hr segments.

Surgery

Both sham and ischemic surgeries were performed under isoflurane anesthesia (3.0% induction, 2.0% maintenance, 30% O_2/70% N_2O). In both surgical groups a ventral midline incision was made in the neck and the carotid arteries were carefully isolated from the surrounding tissue. In the ischemic group a piece of 4-0 silk suture was threaded underneath the arteries allowing them to be lifted away from the underlying tissue and to be occluded for 5 min by microaneurysm clips (Fine Science Tools, Vancouver, BC). Blockage of blood flow was visually confirmed. Brain temperature was carefully monitored by a tympanic temperature probe (Model HH23, Omega, Stamford, CT) throughout the entire procedure and was maintained at approximately 36.5°C using a heated water blanket (Model TP-3E, Gaymar Industries, Orchard Park, NY) wrapped around the head and a homeothermic blanket (Harvard Apparatus, South Natick, MA) that enveloped the animals' body.

Following occlusion, clips were removed and the arteries checked for reflow. The incision was sutured and anesthesia discontinued. The animals were placed back in their cages and placed under computer controlled heating lamps that ensured their core temperature never fell below 36.5°C in the first 30 min following ischemia (Colbourne et al., 1993).

Behavioral Testing

An open field apparatus was used to assess habituation behavior of the gerbils following ischemia (Wang and Corbett, 1990). Testing was performed on days 3, 7, and 10 after ischemia in a soundproof room and all external environmental cues in the room remained constant. The floor of the open field (72 X 76 X 57 cm) was electronically divided into 25 equal squares, and a visual tracking system (HVS Systems, Kingston, UK) recorded the number of squares entered per min over a 10-min test session.

Evaluation of Posterior Circulation

Following the last behavioral testing session (10 days post-ischemia), gerbils were given an overdose of pentobarbital and perfused transcardially with heparinized saline followed by 10% formalin. Thereafter, 0.5 ml of Indian Ink (Product Number 1005754, Windsor and Newton, UK) in an equal volume of 7.0% gelatin was injected into the circulation. The brains were left *in situ* overnight at 4°C allowing the gelatin/ink mixture to solidify. The brains were then removed and post-fixed in 10% formalin.

The posterior communication was evaluated using a dissecting microscope (Leica MZ6) and digital camera (Sony, SSC-DC50A). Communication between the PCA and SCA was documented, photographed and later quantified using image analysis software (MicroBrightfield, Williston, VT). The patency of the PcomAs was used to divide the subjects into two groups: vessels less than 50 μm were considered to have insignificant anastomoses between the PCA and SCA and were considered PcomA(-). Vessels larger

than 50 μm were considered to be significant anastomoses between the posterior and anterior circulation and were deemed PcomA(+). The vasculature data were collapsed into those that had either significant unilateral or bilateral PcomAs (PcomA(+)) and those without patent PcomAs (PcomA(-)). These procedures were adapted from a study used to evaluate cerebral vasculature in mice (Murakami et al., 1998). It should be noted that six gerbils (five HO and one CR ischemic gerbil and one HO sham) could not be included in the vasculature analysis due to filling complications.

Histology

Brains were embedded in paraffin and a series of 6 μm sections were cut and subsequently stained with haematoxylin and eosin. Neurons exhibiting a distinct nucleus and lack of shrinkage or eosinphilia were counted in the medial, middle and lateral regions of CA1 in sections taken from the rostral level of hippocampus (-1.7 mm from bregma) (Loskoto et al., 1975) by placing a 200 μm long grid over these regions and counting cells that fell within the grid (Figure 1). One animal in the sham group could not be counted due to perfusion and staining artifacts.

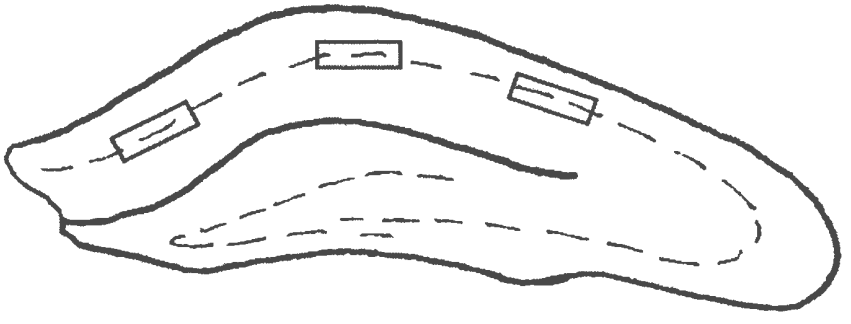

Figure 1. Hippocampal level (-1.7 mm) used for counting CA1 neurons. Each box represents the location of the 200 μm counting grid.

Statistical Analysis

The activity and open field data were analyzed using repeated-measures ANOVA. Cell count data was analyzed using one-way ANOVA. Following a significant ANOVA, Student Newman-Keuls post-hoc tests were used to assess differences in treatment means. A frequency analysis evaluating PcomA between the two groups was performed using Chi-squared analysis. In the qualitative analysis of neuronal injury as it related to vascular patency, statistically significant differences between groups were analyzed using Mann-Whitney *U* test. A multiple regression analysis was carried out between open field data (Days 3, 7, 10) and total CA1 cells. Significance between groups was assigned at the level of less than 5% probability ($P < 0.05$).

Results

Activity and Temperature Measurement

Telemetry data revealed that there were temperature differences between the two suppliers ($P < 0.05$). However, as this temperature variation between both groups were consistently < 0.5°C at all time points measured (Table 1) they are unlikely to account for experimental findings.

Table 1. Core temperature data. Values are mean ± SE expressed in °C.

Supplier	Time			
	Baseline	**0 - 8 hr**	**8 -16 hr**	**16 - 24 hr**
High Oak	37.13 ± 0.06	37.93 ± 0.08	37.37 ± 0.09	37.08 ± 0.06
Charles River	37.45 ± 0.05	38.28 ± 0.08	37.54 ± 0.09	37.13 ± 0.06

The activity data collected by telemetry are shown in Figure 2. The ANOVA revealed a significant effect of supplier ($F_{1,55} = 9.07$, $P = 0.0039$) and time ($F_{3,55} = 45.73$, $P < 0.0001$). There was also a significant interaction of time by supplier ($F_{3,165} = 3.26$, $P = 0.023$). Comparisons of both groups with their respective baseline data revealed that the HO animals were more active following ischemia for the entire 24 hr they were monitored (0 – 16 hr, $P < 0.01$; 16 – 24 hr, $P < 0.05$). In contrast the CR group only maintained heightened activity levels for 16 hr post-ischemia (0 – 8 hr, $P < 0.01$; 8 – 16 hr, $P < 0.05$). In addition, except for baseline data, comparisons between suppliers showed that the HO group were more active following ischemia then the CR group across all time points measured (0 – 16 hr, $P < 0.01$; 16 – 24 hr, $P < 0.05$).

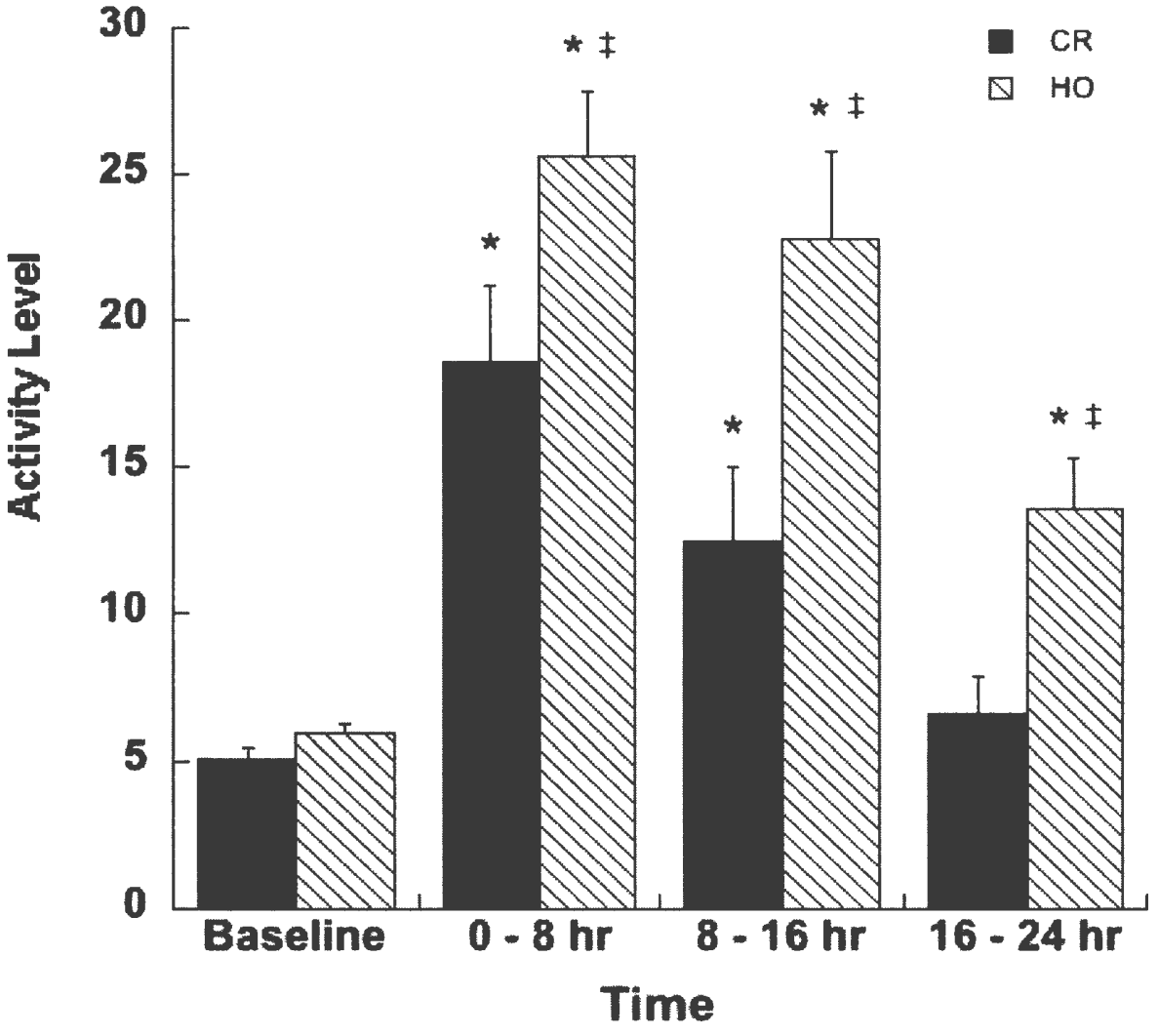

Figure 2. Activity level of animals monitored by telemetry (mean ± SE). At each time point following ischemia the groups were compared to their respective baseline data (∗). CR animals were more active following ischemia for 16 hr (0 – 8 hr, $P < 0.01$, 8 – 16 hr, $P < 0.05$) and the HO animals were still significantly more active up until telemetry ceased at 24 hr (0 – 16 hr, $P < 0.01$, 16 – 24 hr, $P < 0.05$). Also, except for baseline data, comparison between suppliers (‡) revealed that the HO group was more active than the CR group across all time points following ischemia (0 – 16 hr, $P < 0.01$, 16 – 24 hr, $P < 0.05$).

Evaluation of PcomA Plasticity

PcomAs were evaluated in each hemisphere and measured to determine whether the anastomoses were PcomA(-) and PcomA(+) (Figure 3). Table 2 shows the hemispherical frequency and patency of PcomA of animals from CR and HO.

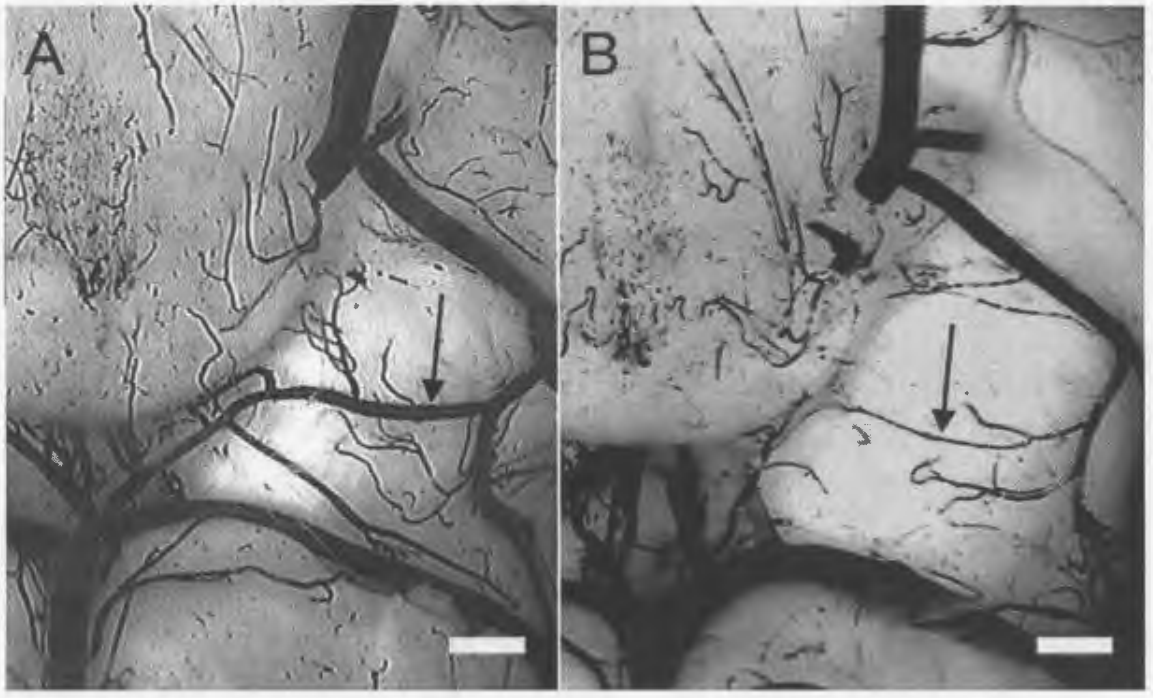

Figure 3. Photomicrographs of gerbils with varying sizes of PcomAs. A: shows a PcomA(+) (83 μm). B: shows a PcomA(-) (28 μm). Scale bar: 500 μm.

Table 2. Values are of animals that had unilateral, bilateral or absent PcomA communication following visual inspection. Measurements of PcomA diameter subdivided the unilateral and bilateral animals depending on whether the vessels were > 50 μm or < 50 μm. For each supplier, sham and ischemic animals are included.

Supplier	Distribution of PcomAs (By Inspection)	n	Number of Patent PcomAs (> 50 μm)		
			One	Two	Zero
High Oak (HO) (n=38)	Unilateral	13	1	0	12
	Bilateral	13	4	1	8
	Absent	12	0	0	12
Charles River (CR) (n=44)	Unilateral	20	13	0	7
	Bilateral	18	4	10	4
	Absent	6	0	0	6

The HO group had 2.6% (1/38) of animals with significant anastomoses on both sides and 13.2% (5/38) with only significant connections unilaterally. Thus 84.2% (32/38) of gerbils from HO lacked patent PcomA on both sides. In contrast, 22.7% (10/44) of CR gerbils had patent PcomAs bilaterally and 38.6% (17/44) had significant unilateral connections. The remaining 38.6% (17/44) had insignificant, or a lack of, PcomAs. These data show that the HO group had a significantly fewer number of gerbils with patent PcomAs as compared to the CR animals (Table 3) ($\chi^2 = 17.61$, $P < 0.0001$).

Table 3. Values show the frequency of animals with patent (> 50 μm) or non-patent (<50 μm) PcomAs. The PcomA(+) group includes both the PcomA(+/+) and PcomA(+/-) subgroups. The PcomA(-) groups includes only the PcomA(-/-) group.

Supplier	PcomA(-)	PcomA(+)
High Oak	32	6
Charles River	17	27

Histological Data

The total number of CA1 neurons in the rostral hippocampus are depicted in Figure 4. The 5-min occlusion produced a profound loss of CA1 neurons ($F_{2, 84} = 255.64$, $P < 0.0001$). The ischemic groups from CR and HO showed a significant loss of CA1 neurons as compared to shams ($P < 0.0001$). Moreover, between suppliers, the cell loss in the CR ischemic group was less severe than the HO ischemic group ($P < 0.0001$). Overall, the HO ischemic group lost 95% of CA1 neurons while the CR ischemic group showed only a 71% loss of neurons.

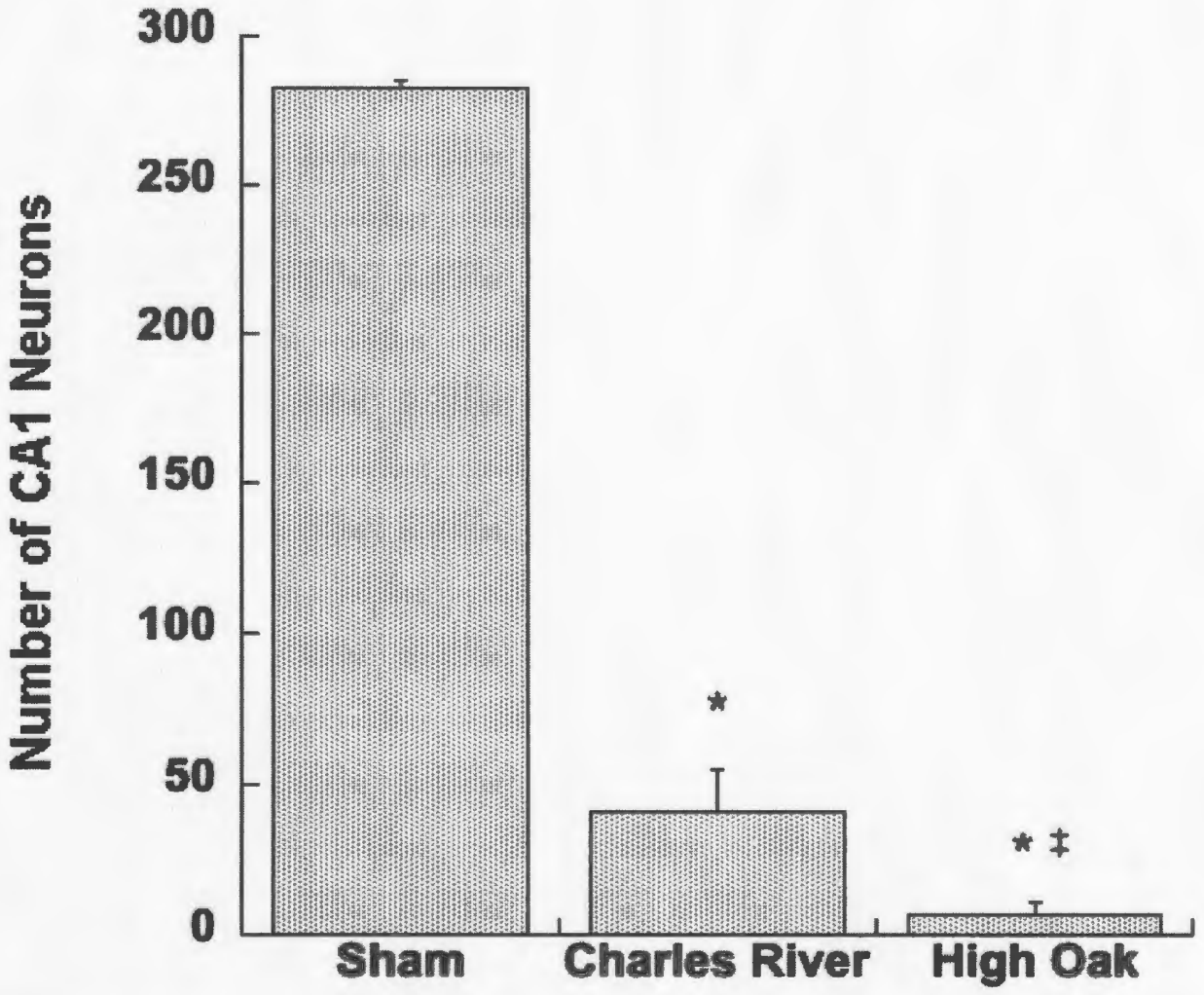

Figure 4. Surviving bilateral neuronal counts (mean ± SE). Both the CR and HO ischemic animals showed a significant reduction in CA1 cells following a 5-min occlusion as compared to shams (*$P < 0.01$). HO animals had significantly more CA1 cell loss (‡$P < 0.01$). The sham data above includes both CR and HO sham animals pooled, which were not statistically different.

In converting the neuronal cell count data into a rating scale (0-4, with 0 being most severely injured and 4 being normal) and comparing this with the patency of the PcomA the data shows that the presence of a PcomA(+) is more indicative of neuronal sparing ($U = 481.00$, $P < 0.0001$) (Figure 5). Illustrative of this phenomenon, a photomicrograph of a gerbil with both types of PcomA [PcomA(+) and PcomA(-)] is shown (Figure 6). Neuronal damage following a 5-min ischemic insult is near complete in the absence of a PcomA and absent when the PcomA is present.

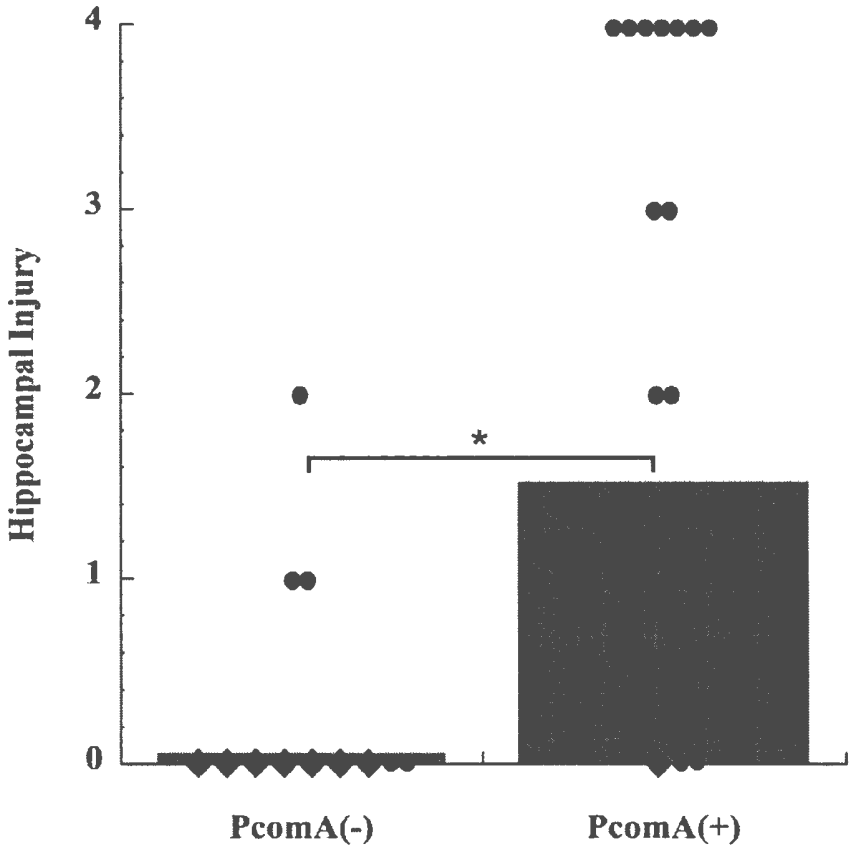

Figure 5. Qualitative analysis of neuronal injury in relation to patency of PcomA in the hippocampus. Each ◆ represents 10 hemispheres and a ● indicates one hemisphere graded with a hippocampal injury score between 0-4, with 0 being most severely injured and 4 being normal. *P < 0.0001, Mann-Whitney *U* Test. All ischemic hemispheres from both suppliers were used (57 animals, 114 hemispheres), except 5 animals (ten hemispheres) could not be included due to filling problems.

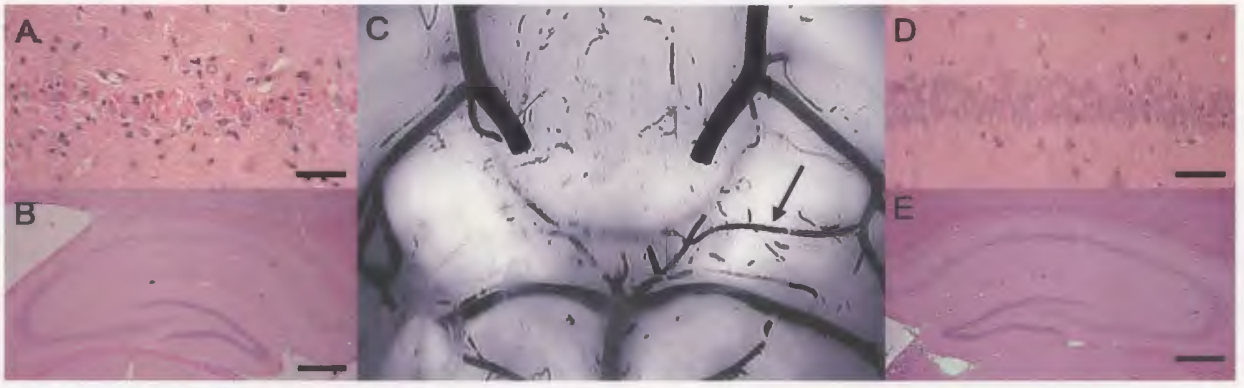

Figure 6. Posterior circulation from a gerbil (C) with a unilateral PcomA (see arrow). (A) and (D) are high magnification images of right and left CA1, respectively. (B) and (E) are lower magnification images of right and left hippocampi. The images on the left (A and B) illustrate a successful ischemic insult resulting in pyknotic and eosinophilic neurons. The images on the right (D and E) show a virtually intact CA1, with few eosinophilic neurons. Scale bar: 50 μm (A and D) 500 μm (B and E).

Behavioral Data

Data from the open field are presented in Figure 7. Repeated-measures ANOVA revealed a significant effect of supplier ($F_{1,76} = 26.54$, $P < 0.0001$) treatment ($F_{1,76} = 20.79$, $P < 0.0001$) and day ($F_{2,76} = 124.43$, $P < 0.0001$). There were also significant interactions of day by supplier ($F_{2,152} = 8.36$, $P = 0.0004$) and day by condition ($F_{2,152} = 7.30$, $P = 0.0009$). The complex interaction of day by supplier by condition was not significant ($F_{2,152} = 0.97$, $P = 0.38$).When comparing the HO and CR shams it was revealed that on Day 3 the HO group had significantly higher open field scores ($P < 0.05$). However, these effects were not seen on Days 7 and 10 ($P > 0.05$). A comparison of the HO and CR ischemic groups to their respective shams revealed that the CR ischemic group did not show any increase in activity following ischemia on all days tested ($P > 0.05$). In contrast, the HO ischemic showed hyperactivity on days 3, 7 and 10

($P < 0.01$). In addition comparisons between ischemic groups revealed that at each time point tested the HO ischemic group were consistently more active than the CR ischemic ($P < 0.01$). Open field performance (days 3, 7, 10; multiple regression) predicted histological outcome in CA1 ($R^2 = 0.479$, $P < 0.0001$). It should be noted that eight CR male animals were not included in the open field analysis due to a computer malfunction.

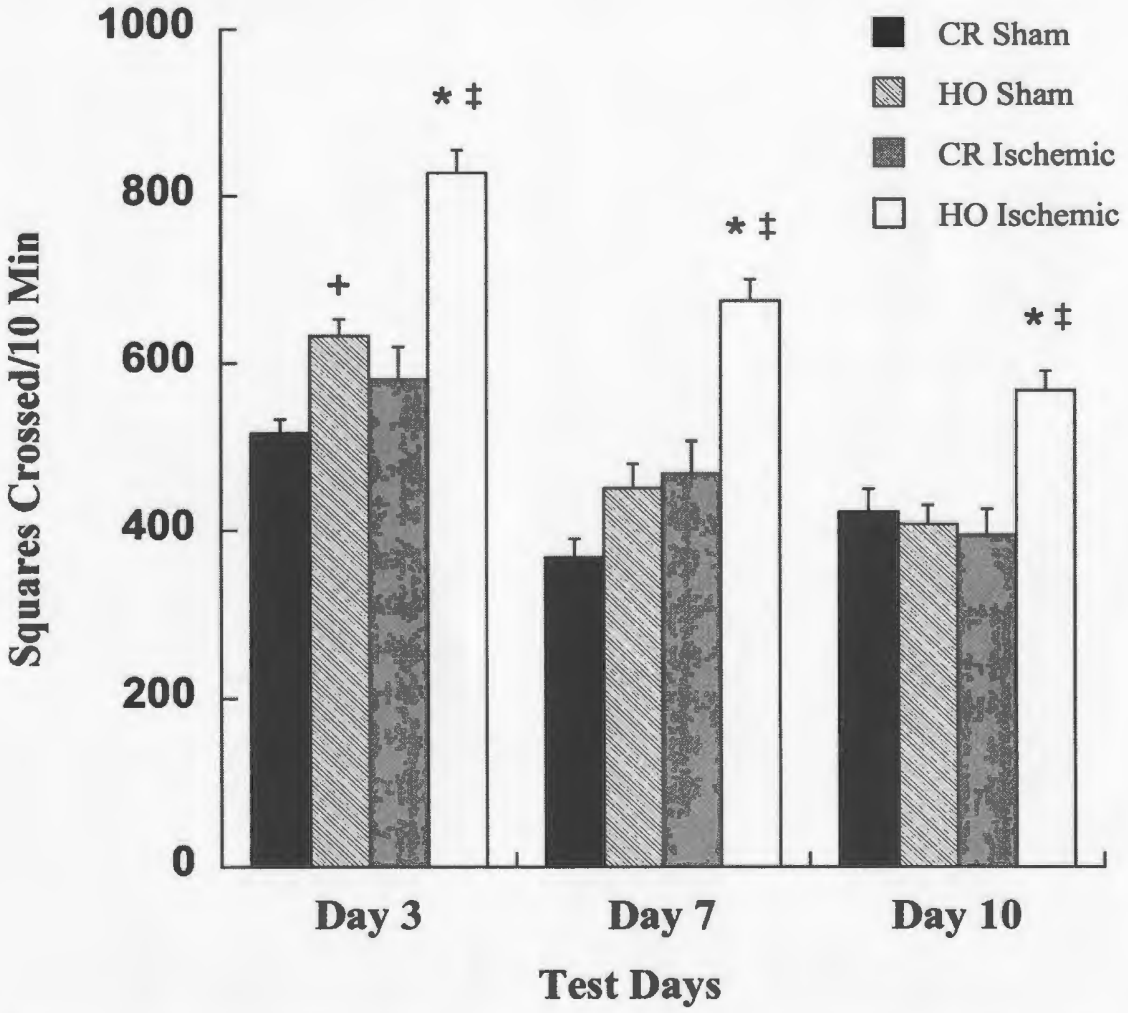

Figure 7. Open-field data (mean ± SE) 3, 7, 10 days after surgery. On Day 3 the CR sham group differed from the HO sham group ($^{+}P < 0.05$). At no other time points were the sham groups statistically different. On Days 3, 7 and 10 the HO ischemic group differs from its control ($^{*}P < 0.01$) and the CR ischemic group ($^{‡}P < 0.01$).

Discussion

The cerebrovasculature of the Mongolian gerbil was originally described as having an incomplete circle of Willis (Levine and Payan, 1966). Thirty years later more detailed experiments revealed that 15% of animals tested had some form of communication (unilateral or bilateral) between the basilar and carotid circulations (Breuer and Mayevsky, 1992). Our study clearly shows a dramatic change in the cerebrovasculature of CR gerbils, the main supplier of gerbils in North America and to a lesser extent in another supplier of gerbils (HO) in Canada. It is important to note that all gerbils used in North America were derived from breeding pairs captured in Eastern Mongolia in 1935 and introduced to the United States in 1954 (Rich, 1968). While both suppliers show a high incidence of PcomA (26/38 HO and 38/44 CR; see Table 2), direct measurement of the diameter of the vessels highlighted critical differences between the two suppliers. While 16% of HO animals had PcomAs greater than 50 μm (Figure 3A), 61% of CR animals had vessels that were considered significantly patent (> 50 μm). In light of these striking differences in cerebrovasculature between suppliers the question as to whether the PcomA anastomoses are capable of compensating for the reduced blood flow through the carotid circulation had to be addressed. Previous work suggested that despite the presence of connections the degree of ischemia would be almost complete and result in near total CA1 cell loss (Levine and Sohn, 1969; Breuer and Mayevsky, 1992). This is clearly no longer the case in CR gerbils.

One of the classical signs following forebrain ischemia in the gerbil is an acute increase in locomotion that persists for at least 1 day following a 5 min occlusion

(Kuroiwa et al., 1991; Corbett et al., 1997). Our results show that ischemic animals from both suppliers exhibited an increase in locomotion following ischemia, yet only the HO ischemic group displayed a sustained increase at 24 hr (Figure 2). Interestingly, the activity levels of the HO ischemic animals were significantly greater than the CR ischemic group at all time points measured. In tandem with the acute increase in locomotion a second type of ischemia-induced increase in locomotion is typically detected when animals are exposed to a novel open field. Previous research has shown that ischemic animals when tested on Days 3, 7 and 10 have substantially higher levels of locomotion and impairments in habituation (Colbourne and Corbett, 1994). Consistent with these data the HO ischemic animals showed increased activity levels over the three days tested whereas the CR ischemic animals did not differ from their respective sham group (Figure 7). This effect is quite startling as no previous studies have reported a failure of ischemic gerbils to increase their activity levels in response to exposure to novel open-field (Wang and Corbett, 1990; Colbourne and Corbett, 1994; Corbett and Crooks, 1997; Dooley and Corbett, 1998). Taken together the above behavioral data clearly shows that the CR gerbils, with their increased presence of patent PcomA, are not representative of gerbil populations used in past research.

Previous studies have shown extensive CA1 cell loss from ischemia after a 5 min insult (Colbourne and Corbett, 1994; Nurse and Corbett, 1994; Colbourne and Corbett, 1995; Corbett et al., 1997). In support of these findings, the HO ischemic animals had 95% CA1 neuronal loss while CR ischemic animals sustained 71% CA1 loss (Figure 4). To account for the discrepancy in neuronal numbers a relationship between patency of

PcomA and the level of hippocampal injury sustained was found (Figure 5). As the CR animals were shown to have significantly more animals with patent PcomA it is not surprising that they experienced less cell damage. Furthermore, levels of open field activity have been used to predict the extent of CA1 cell loss (Gerhardt and Boast, 1988; Mileson and Schwartz, 1991; Babcock et al., 1993; Nurse and Corbett, 1994). Our study showed a similar correlation, as the open field data were predictive of CA1 cell counts over the 3 days tested as shown by a multiple regression analysis. Therefore, the present data establishes a link between vasculature, CA1 injury and open field performance.

The mechanism(s) underlying the variation in the circle of Willis are unknown but may have a genetic component as has been suggested in a previous study (Mayevsky and Breuer, 1992). Nonetheless, given the uncertainty of the cerebrovascular pattern in the gerbil population of these two suppliers it is questionable if the gerbil can be used in future ischemia research. Data gleaned from this study suggest a way for the gerbil model to continue to be used. Based on acute activity levels between 16-24 hr post-ischemia, any animals whose activity levels have returned to baseline could be eliminated from a study. While this may reduce variability, it also increases the number of animals required, the time it takes to complete a study and the cost of the experiment. Another method to reduce variability might be to use a laser Doppler to verify that blood flow is reduced to an acceptable injury level (e.g. <10%) in forebrain regions. However, this would require extra costs and would increase the time each surgery took, negating one of the major benefits of this model. A more permanent solution might be to selectively breed gerbils that have an incomplete circle of Willis and for breeding farms to routinely

assess the incidence of partial or complete PcomA anastomoses in their population. Using one or a combination of the elimination criteria mentioned above, a number of inbred gerbil generations could be produced that might yield gerbils without significant PcomAs. However in the absence of these measures it might be advisable to abandon the gerbil 2-VO model altogether and use the rat 4-VO or 2-VO modified models of occlusion. While each have their own set of problems, described above, the gerbil BCCAO model as it currently exists does not offer any unique advantages to justify its continued use. This is particularly true in neuroprotection studies because with current variability only very robust protective effects would be detectable.

In summary, the presence of anastomoses between the carotid and basilar circulation must be recognized. We have clearly shown that the CR supply of gerbils has a significant presence of PcomA that is sufficient to markedly attenuate the effectiveness of a 5 min ischemic insult. Most importantly, this resulted in a failure of the CR animals to exhibit typical behavioural signs of global ischemic injury such as protracted increases in postischemic locomotor activity and habituation deficits in the open field. In addition CR gerbils do not exhibit extensive CA1 (> 95%) damage following ischemia. While the HO animals still display the prototypical behavioral and histological endpoints seen in previous experiments the increased presence of PcomAs in this supply is a cause of concern and likely contributes to the increased variability encountered in our and other laboratories in the last 4-5 years. Clearly the HO supply is in eminent danger while the CR supply is unusable for producing reliable global ischemic injury.

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