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Neuroprotective effects of enriched environment housing after transient global cerebral ischaemia are associated with the upregulation of insulin-like growth factor-1 signalling

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Aims: Use of enriched environment (EE) housing has been shown to promote recovery from cerebral ischaemic injury but the underlying mechanisms of their beneficial effects remains unclear. Here we examined whether the beneficial effects of EE housing on ischaemia-induced neurodegeneration and cognitive impairment are associated with increased insulin-like growth factor-1 (IGF-1) signalling in the hippocampus. **Methods:** Forty-two adult male Wistar rats were included in the study and received either ischaemia or sham surgery. Rats in each group were further randomized to either: EE or standard laboratory cage housing (control). Rats were placed in their assigned housing condition immediately after recovery from anaesthesia. Behavioural testing in the cued learning and discrimination learning tasks were conducted 2 weeks after ischaemia. Rats were euthanized after behavioural testing and the hippocampus was analysed for IGF-1 level, IGF-1 receptor (IGF-1R) activation, protein kinase B (Akt) pathway activation, neurone loss and caspase 3 expression. **Results:** Our data showed that EE housing: (1) mitigated ischaemia-induced neuronal loss; (2) attenuated ischaemia-induced increase in caspase 3 immunoreactivity in the hippocampus; (3) ameliorated ischaemia-induced cognitive impairments; and (4) increased IGF-1R activation and signalling through the Akt pathway after ischaemic injury. **Conclusion:** Ultimately, these findings suggest the possibility that IGF-1 signalling may be one of the underlying mechanisms involved in the beneficial effects of EE in optimizing recovery following cerebral ischaemic injury.

Keywords: Akt signalling, caspase 3, complex environment, contextual learning, Fluoro-Jade, water maze

Introduction

Transient global cerebral ischaemia commonly seen in clinical conditions such as cardiac arrest leads to selective neuronal loss in the hippocampus [1,2]; and as this brain region is involved in memory processing [3], it is not surprising that memory impairment is the most common neurological dysfunction seen after ischaemic injury. In addition to neuronal loss in the hippocampus at the time of injury, delayed neurodegeneration is observed that is preceded by apoptosis [2,4]. Given that neurodegeneration in ischaemic injury is linked to delayed neuronal loss, it is critical to implement an early intervention so as to mitigate this process. One therapeutic paradigm used in experimental studies to facilitate

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recovery from ischaemic injury is enriched environment (EE) housing (reviewed in [5]).

Enriched environment refers to housing conditions that facilitate enhanced sensory, cognitive and motor stimulation, as well as social interaction relative to standard housing conditions [6]. In this housing condition, animals are housed together and have increased opportunities for exercise or physical activity. Numerous reports show that EE facilitates the recovery from cerebral ischaemia using different assessment paradigms ranging from morphological to behavioural. For example, at the morphological level post-ischaemic EE housing decreases neurodegeneration [7,8], enhances cell proliferation in the subventricular zone [9,10] and increases dendritic spine density [11,12]. At the behavioural level, evidence suggests that the recovery of motor [13,14] and cognitive [12,15–18] functions following cerebral ischaemic injury is enhanced by environmental enrichment.

Although the benefits of EE housing on recovery following cerebral ischaemic injury are well documented, the mechanisms underlying their effects remains unclear. Several studies report that the beneficial effects of EE housing after cerebral ischaemia may be associated with elevations in nerve growth factor and brain-derived neurotrophic factor [17,19,20]. However, some reports show that the neuroprotective effects of nerve growth after cerebral ischaemia only last a few hours after injury while expression of brain-derived neurotrophic factor after ischaemic injury did not consistently result in decreased neuronal loss even though it contributed to post-ischaemic improvement in cognitive function [21,22]. Here we hypothesize that the beneficial effects of EE housing on delayed neurodegeneration are associated with increased insulin-like growth factor-1 (IGF-1) signalling.

The importance of IGF-1 during recovery from ischaemic injury is illustrated in studies demonstrating that in patients with ischaemic stroke, serum levels of IGF-I during the recovery period positively correlate with functional measures after ischaemic stroke [23,24]. Furthermore, in animal models of cerebral ischaemia, IGF-I treatment reduced infarct size and neurological deficits when administered after induction of stroke [25,26]. Thus, in the present study we examined whether the beneficial effects of EE housing on ischaemia-induced delayed neurodegeneration and cognitive impairment is associated with increased IGF-1 signalling in the hippocampus.

Materials and methods

Animal model

Male Wistar rats approximately 3 months of age (body weight of 350–375 g at the time of surgery) were obtained from Harlan Laboratories (Madison, WI, USA). Animals were housed in pairs in a pathogen-free vivarium under controlled condition (temperature 22 ± 1°C and humidity 70 ± 5%) and a 14:10 h light : dark cycle was maintained. All animals were housed in the same room so that temperature, humidity and lighting conditions were similar for all groups. Animals had free access to food and water delivered through an automated and filtered system. Animals were also handled daily throughout the study so that they could get acclimated to the research personnel thereby decreasing stress. Experiments started 1 week after arrival of the animals from the breeder and all protocols in this study were approved by the Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health guidelines.

Cerebral ischaemia

The four-vessel occlusion method was used to induce transient global cerebral ischaemia as described previously [15]. Briefly, rats were anaesthetized with isofluorane/oxygen (2.5% isofluorane and 30% oxygen) mixture and both common carotid arteries were isolated. Immediately following isolation of both carotid arteries, the vertebral arteries were electrocauterized. Body temperature was kept at 37–37.5°C using a heating pad during the surgical procedure and until the animals were fully recovered. The next day, both common carotid arteries were occluded for 12 min while the animals were awake. Animals that developed postoperative complications such as excessive weight loss (>20% of preoperative body weight, $n = 0$) were excluded from the study. In addition, observing for sluggishness, extreme aversion to being touched, and weight loss assessed pain level. Animals were not given post-operative analgesia but were euthanized immediately when persistent pain was observed ($n = 0$). Sham-operated animals were subjected to the same anaesthesia and surgery that consisted of a neck incision without carotid manipulation and an incision behind the occipital bone without cauterization of the vertebral arteries. All efforts were made to minimize animal distress and reduce the number of rats used.

Animal housing

Immediately upon recovery from anaesthesia, rats were randomly placed in either EE housing or paired housing (control). The rats remained in their assigned housing condition throughout the duration of the study. Animals in the EE group ($n = 11$ ischaemia and $n = 11$ shams) were housed together in a sensory-rich living condition (wire cage measuring 2 m × 1 m × 1.65 m) consisting of a variety of objects as described previously [15,27]. In addition, these rats were placed each day in an open field (1.2 × 1.2 × 1.2 m) during the evening hours with a novel arrangement of toys and objects and allowed to explore for 30 min while the objects in the home cage were being changed. Objects in both EE housing and open field were changed daily to maintain novelty.

Animals assigned to the control (CON) group ($n = 10$ ischaemia and $n = 10$ shams) were housed in pairs in standard laboratory cages (16.5 × 22.5 × 13.5 cm). Although rats in this group were able to observe ongoing activity in the room, they did not receive any stimulation and contact was limited to daily handling and routine cage changing. Paired housing was used to control for the social interaction effect of the EE.

Behavioural testing

Two weeks after injury (ischaemia or sham), rats were tested in the water maze to evaluate cognitive impairment. All testing were performed approximately 2 h prior to the onset of the dark cycle to ensure that it is close to the rats' active period. Behavioural testing was performed in the water maze for both spatial (cued-learning and memory) and nonspatial (discrimination learning) cognitive tasks.

Water maze cued learning During testing, the water maze tub was filled with tepid water (22 ± 2°C) and made opaque by the addition of powdered milk. The pool was divided into four quadrants of equal surface area and the starting locations for testing were assigned north, south, east and west (not actual compass positions but rather relative to the behavioural testing room). The behavioural testing walls were already painted white so just the other distal (to the water maze) visual cues in the rooms were removed during the habituation training and actual testing. The cued spatial learning and memory (acquisition and recall) task is sensitive to hippocampal dysfunction [28,29]. The day before actual testing started, rats were given habituation training for the purpose of teaching the rats to swim and locate the platform using the visible cue. Habituation training consisted of allowing the rats to swim to locate a visible goal, which is a 10-cm-diameter flower pot positioned in one quadrant halfway between the centre and the side of the pool and submerged 2 cm below the surface with a small brass rod mounted on it vertically protruding 10 cm above the water and a red plastic ball mounted at the top as a visual cue. The day of habituation training, rats received four trials with the goal/platform in a fixed location and fixed starting location (start position is east quadrant and the platform located in the west quadrant). Actual testing was conducted the day after completion of the habituation training, wherein the rats received three trials per day for 4 consecutive days and the starting location and visual goal positions were changed randomly for every trial. During the trials, swim latency (time to reach the platform) to reach the platform was recorded by a video camera connected to an image analyser (Water Maze System Version 4.20, Columbus, OH, USA). In addition, swimming speed (path length/swim latency) was used to assess the motoric activity in performing the task.

Water maze discrimination-learning task The rats were tested in the discrimination-learning test after 2 days of rest following the cued learning and memory task. The discrimination-learning task is sensitive to both hippocampal and nonhippocampal (prefrontal cortex) dysfunctions [30]. Rats were given two trials per day × 4 days and had to discriminate between black and white visible goals to find the hidden platform and all extra-maze cues in the room were again covered. The visible goal painted white was placed on top of the hidden platform to provide escape (P^+) from the water (located in the south-east quadrant); whereas the other one painted black was floating (P^-) and not able to offer sufficient buoyancy to support the rat (located in the south-west quadrant). Both visible goals were placed 10 cm above the water level. For this task, path taken to reach the correct goal was recorded as well as search errors based on the choices of P^+ compared with P^- as the aim was to train the rats to avoid P^-. Search errors represent the number of P^- choices made during the daily trials.

Tissue preparation

The day after the completion of behavioural testing, all rats were euthanized using CO_2 inhalation, the brains removed and cut in half sagitally. Half of the brain was immediately placed in liquid nitrogen until processed and used for Western blot (Akt, and IGF-1 receptor activation) and ELISA (IGF-1 protein expression). The other half was used for caspase 3 immunohistochemistry, and Fluoro-Jade staining. For immunohistochemistry and Fluoro-Jade, brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3 overnight then cryoprotected before sectioning.

Immunohistochemistry

The fixed brains were sectioned at 30 μm thickness using a cryostat. Tissue sections were obtained covering the entire hippocampal region in its rostro-caudal extension and the free-floating sections were first treated with 0.3% hydrogen peroxide in PBS to inactive endogenous peroxidase activity. After inactivation, the tissues were rinsed and placed in the blocking solution of 3% serum, 0.1% Triton-X and 1% bovine serum albumin for 1 h then washed in 0.1M phosphate-buffered saline (pH 7.3) followed by incubation for 48 h at 4°C with antibody recognizing caspase 3 (rabbit monoclonal, 1:500, Signet Laboratories, Dedham, MA, USA). The primary antibody was detected using biotinylated IgG secondary antibodies (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature followed by incubation in avidin–biotin complex (ABC kit, Vector Laboratories, Burlingame, CA, USA). Immunoreactions were visualized with hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride (DAB) in Tris buffer (pH 7.3) enhanced with nickel. After thorough rinsing, the tissue sections were mounted on gelatin-coated slides, dried and coverslipped. Tissues from all experimental groups were run simultaneously and under identical conditions to ensure reproducibility of results. A predilution test was performed to ensure specificity of the antibody and negative controls, involving deletion of the primary antibody, were used to rule out any nonspecific interactions.

Caspase 3 immunoreactivity was quantified using the area-fractionator grid defined by the StereoInvestigator (MicroBrightfield, Colchester, VT, USA) computerized analysis system as described previously [7]. The counting frame was set to 80 × 120 μm, the scan grid 340 × 340 μm and the Cavalieri grid spacing 30 μm at 40× magnification. The percentage of coverage in four sections was averaged to obtain a final estimate of caspase immunoreactivity for each animal.

Fluoro-Jade staining

To assess the degree of neuronal loss due to ischaemia, tissue sections adjacent to the ones used for immunohistochemistry were stained with Fluoro-Jade as described previously [8,27]. Briefly, sections were treated in 0.06% potassium permanganate after rehydration in descending grades of alcohol. After pretreatment, the slides were rinsed in distilled water then transferred to the Fluoro-Jade staining solution (0.001% Fluoro-Jade in acetic acid) for 30 min, rinsed in distilled water again then air-dried. Finally, sections were immersed in Histoclear and coverslipped using DPX mounting medium. The stained sections were examined under an epifluorescence microscope (Nikon E800) with a FITC fluorescence filter cube and counting of degenerated neurones was done in the hippocampus proper (CA1–CA3 areas) but was restricted to the stratum pyramidale layer. The optical fractionator technique was used for quantitative analysis as previously described [7,8] using the StereoInvestigator (MicroBrightfield, Colchester, VT, USA) computerized analysis system.

Cresyl violet staining

Tissue sections adjacent to those used for Fluoro-Jade staining were used for nissl staining to validate the Fluoro-Jade results. Sections were mounted on gelatin-coated slides and air-dried, rehydrated in descending grades of alcohol, and then dipped in Cresyl violet for 7–10 min. Tissue sections were then differentiated in 70% acetic alcohol, dehydrated in ascending grades of alcohol, and cleared in Histoclear, and coverslips were applied using Permount.

Western blot

To assess activation of IGF-1 receptors (IGF-1R) and Akt, its downstream signalling pathway, 0.5 g of frozen hippocampal tissue was used for Western blot. Briefly, tissues were homogenized in extraction buffer (50 mM Tris, pH 8.0), 150 mM NaCl, 5 mM EDTA, protease inhibitor mixture (Sigma, St. Louis, MO, USA), and 100 mM

phenylmethylsulphonyl fluoride (Sigma Aldrich) using ground glass microhomogenizers for Western blot. Following addition of 1% SDS, the homogenates were centrifuged at 10 000 **g** for 10 min, the supernatants collected, and total protein concentration was determined using the BCA-Protein assay (Pierce, Rockford, IL, USA). After determination of protein concentration, the supernatant was mixed with solubilizer containing SDS, glycerine, EDTA, Tris, bromphenol blue and dithiothreitol, boiled for 5 min at 95°C, and stored at −20°C until use.

For Western blot, equal amounts of protein (40 μg) from each rat were loaded and separated by SDS-PAGE gel electrophoresis as previously described [7]. Briefly, the protein bands were electrophoretically separated then transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). The membranes were stained with 0.5% Ponceau Red to visualize total proteins, then destained and nonspecific binding sites were blocked by incubation of the membranes in 5% powdered milk in Tris-buffered saline containing 0.5 ml/l Tween-20. After blocking, membranes were incubated overnight at 4°C with either: (1) anti-Akt (1:1000, monoclonal mouse); (2) anti-phosphorylated Akt (1:1000, monoclonal rabbit); (3) anti-IGF-1R (1:1000, monoclonal rabbit); or (4) anti-phosphoIGF1R (1:1000, monoclonal rabbit) with gentle agitation. All antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Immunocomplexes were visualized using horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich) and chemiluminescence (Pierce, Rockford, IL, USA). Samples were analysed in triplicates and measurements were averaged and used as one individual data point for statistical analysis. Quantification was done by densitometric analysis (Scion Image Beta 4.0.2; Frederick, MD, USA) using actin as an internal control.

Enzyme-linked immunosorbent assay (ELISA)

The Quantikine Rat/Mouse IGF-1 Immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) was used to determine IGF-1 protein expression. ELISA was performed according to the manufacturer's instructions. Briefly, 96-well plates were precoated with monoclonal antibody specific for IGF-1. Assay diluents were added to each well (50 ml/well). Standards were diluted serially (1:2) from 6 to 0 ng/ml or with the respective calibrator diluents and plated. Both standards and samples were incubated together for 2 h at room temperature. The wells were then rinsed in wash buffer five times then incubated in polyclonal anti-mouse IGF-1 antibody overnight at 4°C. The plates were washed then incubated with anti-mouse IgG horseradish peroxidase conjugate for 1 h in the dark at room temperature. Tetramethyl benzidine/peroxidase substrate solution was added to the wells to produce the colour reaction. Colour reaction was stopped with 1N HCl (100 μl) and reaction was read in a microplate reader (Bio-Tek, Winooski, VT, USA) at a wavelength of 450 nm (650-nm reference wavelength). Protein concentration was determined from the regression line for the IGF-1 standard corrected for the total amount of protein in the sample and assay sensitivity ranged from 1.6 to 8.4 pg/ml. Assay was performed in triplicates and measurements were averaged and used as one individual data point for statistical analysis.

Statistical analysis

The SAS general linear model (SAS Institute, North Carolina) procedures for two-way analysis of variance (ANOVA) were used to examine effects of injury and EE, and injury and EE interaction. When appropriate, the SAS CONTRAST statement was used for planned comparisons of the effects of injury (ischaemia groups *vs.* sham groups), differential housing (standard laboratory cage *vs.* EE), and the combination of injury and differential housing on Fluoro-Jade staining, IGF-1 expression, Akt levels and caspase 3 immunoreactivity. Independent t-test was used to examine EE effects on ischaemia-induced neurodegeneration in the hippocampus. Linear regression was also performed to assess the relationship between IGF-1 levels and caspase immunoreactivity as well as the relationship between Fluoro-Jade staining and mean number of errors on the discrimination learning task. For the behavioural data, the SAS general linear model procedures for two-way ANOVA with repeated measures were used to determine differences in swim latency, path length, swimming speed and search errors. All experimental protocols were coded to preclude bias.

Results

Protective effects of EE against ischaemia-induced neuronal loss

Quantification of ischaemia-induced neuronal loss in the Fluoro-Jade-stained tissues showed selective neurodegeneration in the pyramidal neurones at the CA1 sector of

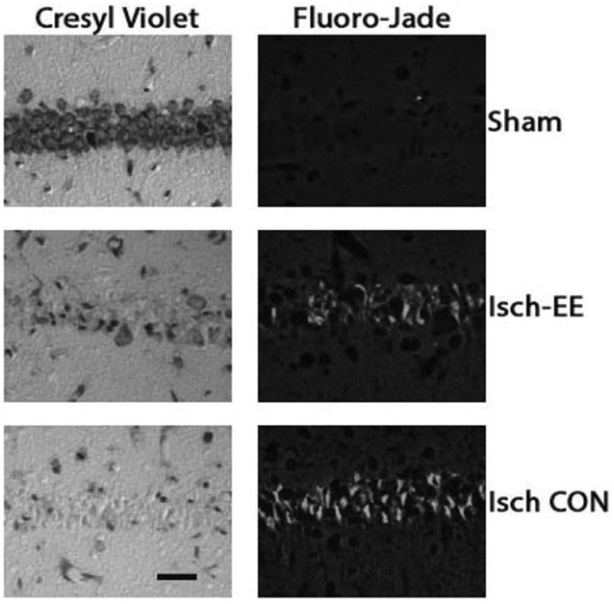

Figure 1. Representative photomicrographs of Fluoro-Jade and Cresyl Violet staining. Images on the left panel are cresyl violet-stained tissues. Fluoro-Jade images are depicted on the right panel. Scale bar: 100 μm. Legends: Isch, ischaemia; CON, control; EE, enriched environment.

the dorsal hippocampus with relative sparing of CA3 neurones following 12 min of bilateral carotid occlusion (Figure 1). Meanwhile, no staining was evident in the ventral hippocampus. Also, no Fluoro-Jade-stained cells were seen in the sham-operated animals. The susceptibility of the CA1 region and relative antero-ventral gradient vulnerability of hippocampal neurones to cerebral ischaemia as reported by others [2,7,8,31] was confirmed and extended in this study. Examination of tissue sections revealed quantifiable Fluoro-Jade-positive cells mainly in the stratum pyramidale of the CA1 region. Significantly decreased number of Fluoro-Jade-positive cells was seen in the ischaemic rats housed in EE compared with ischaemic rats housed in standard laboratory cages [813 ± 34 and 1306 ± 51 respectively; $t(20) = 10.31$, $P < 0.05$] suggesting that EE housing immediately after injury was effective in 'rescuing' neurones that are highly vulnerable to ischaemia.

EE attenuation of ischaemia-induced apoptosis correlates with IGF-1R upregulation

Quantification of caspase 3 immunoreactivity as an index of apoptosis showed significant increase staining in the hippocampal regions in ischaemic rats compared with the sham groups [$F(3,38) = 11.21$, $P < 0.05$]. *Post hoc* comparisons showed that caspase 3 immunoreactivity was 50% greater in the ischaemia CON animals in comparison with the ischaemia rats housed in EE (Figure 2**A**). Negligible caspase 3 immunoreactivity was seen in the hippocampal region of the sham animals and the groups were not significantly different. Also, a significant negative correlation ($r^2 = -0.88$, $P < 0.05$) was seen between IGF-1R phosphorylation and caspase 3 immunoreactivity in the hippocampus (Figure 2**B**). Together, these results suggest that IGF-1R signalling is involved in attenuating apoptosis following transient global cerebral ischaemia.

EE mitigated ischaemia-induced cognitive deficits

After ensuring that visual skills were intact, the rats were tested in a series of water maze tasks designed to assess contextual and discrimination learning. A significant within-subjects effect [$F(3,133) = 12.19$, $P < 0.05$] was seen for swim latency in the cued-learning test given that all rats learned to perform the task efficiently over the four testing days. Significant group differences were also seen in that the ischaemia CON group did not perform the task as well as the ischaemia EE group and the sham groups [$F(3,40) = 9.42$, $P < 0.05$]. *Post hoc* comparisons showed no significant differences in mean swim latency among the ischaemia EE group and the sham CON group (Figure 3**A**). Overall, performance of the sham EE rats was significantly better when compared with all groups. However, a trend towards an interaction effect [$F(3, 38) = 5.87$, $P = 0.07$] was seen between transient global cerebral ischaemia and EE housing in mean swim latency. Comparison of swimming speed did not show significant group differences.

Measurement of path length in the discrimination learning task also showed significant within subjects effect wherein a continuous decrease in distance covered to reach the goal was seen in all groups throughout the testing days [$F(3,133) = 11.87$, $P < 0.05$]. Again, *post hoc* comparisons showed that the ischaemia CON group mostly chose the path to the incorrect goal (Figure 3**B**) leading to a longer indirect route, when compared with the ischaemia EE group and the sham groups [$F(3,38) = 9.49$, $P < 0.05$]. Path length to reach the goal was not significantly different between the ischaemia EE and sham CON groups. Comparison of path length in the

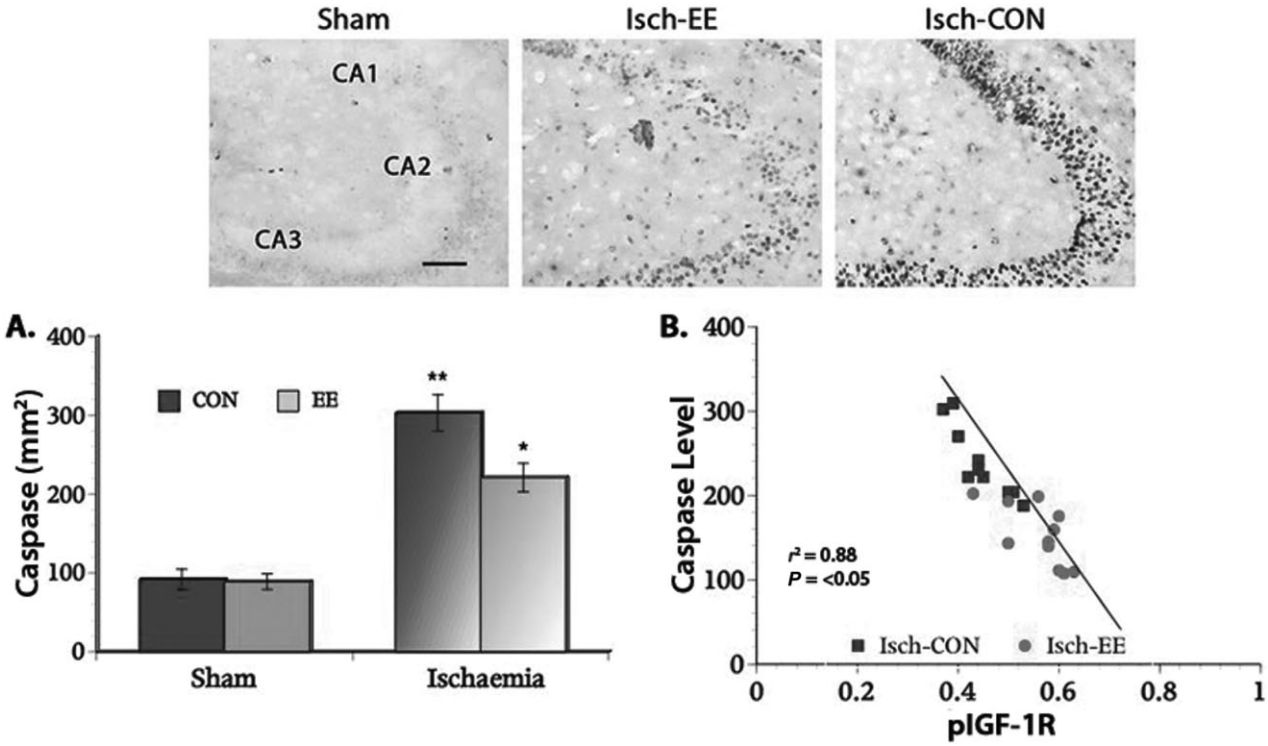

Figure 2. Caspase expression. Representative photomicrographs of caspase 3 immunoreactivity in the hippocampal region (upper panel). Significantly increased caspase 3 expression was seen in the ischaemic rats compared with the sham groups (**A**). However, *post hoc* comparisons showed that the ischaemia EE group has significantly decreased caspase 3 immunoreactivity in relation to the ischaemia CON animals. Regression analysis showed a significant negative correlation between IGF-1R activation and caspase immunoreactivity where decreased levels of IGF-1R phosphorylation is related to increased caspase expression in the ischaemic groups (**B**).*$P < 0.05$, **$P < 0.01$. Legends: Isch, ischaemia; CON, controls; EE, enriched environment; pIGF-1R, phosphorylated IGF-1 receptor. Scale bar: 100 μm.

sham groups showed that rats housed in EE took the most direct route to the correct goal.

Correspondingly, the ischaemia CON group made more errors in the discrimination-learning task overall while the sham EE group made the least amount of errors (Figure 3**C**). *Post hoc* comparison showed no significant difference between the ischaemia EE and sham CON groups in the number of errors committed in the discrimination-learning task. No significant interaction effect was seen between transient global cerebral ischaemia and EE housing in the mean number of errors across the test days. Moreover, a positive correlation ($r^2 = 0.91$, $P < 0.05$) was seen in the mean number of errors made on the last day of the discrimination-learning test and number of Fluoro-Jade-positive cells (Figure 3**D**). Taken together, these results suggest that regardless of injury or housing condition, rats were able to perform the cued learning and discrimination-learning tasks suggesting some degree of spontaneous recovery after ischaemic injury but housing rats in EE can enhance the behavioural recovery.

EE-increased IGF-1 expression and signalling

We examined hippocampal tissues for IGF-1 protein expression using ELISA and the activation (phosphorylation) of IGF-1 receptor (IGF-1R) using Western blot. Our results showed that transient global cerebral ischaemia [$F(3,38) = 8.01$, $P < 0.05$] and EE housing [$F(3,38) = 8.79$, $P < 0.05$] induced a significant upregulation of IGF-1 protein levels (Figure 4**A**). *Post hoc* comparison showed no significant difference in IGF-1 levels between the ischaemia group and the sham EE animals. As well, total IGF-1R levels also increased after transient global cerebral ischaemia [$F(3,38) = 7.88$, $P < 0.05$] and EE housing [$F(3,38) = 8.09$, $P < 0.05$]. *Post hoc* compari-

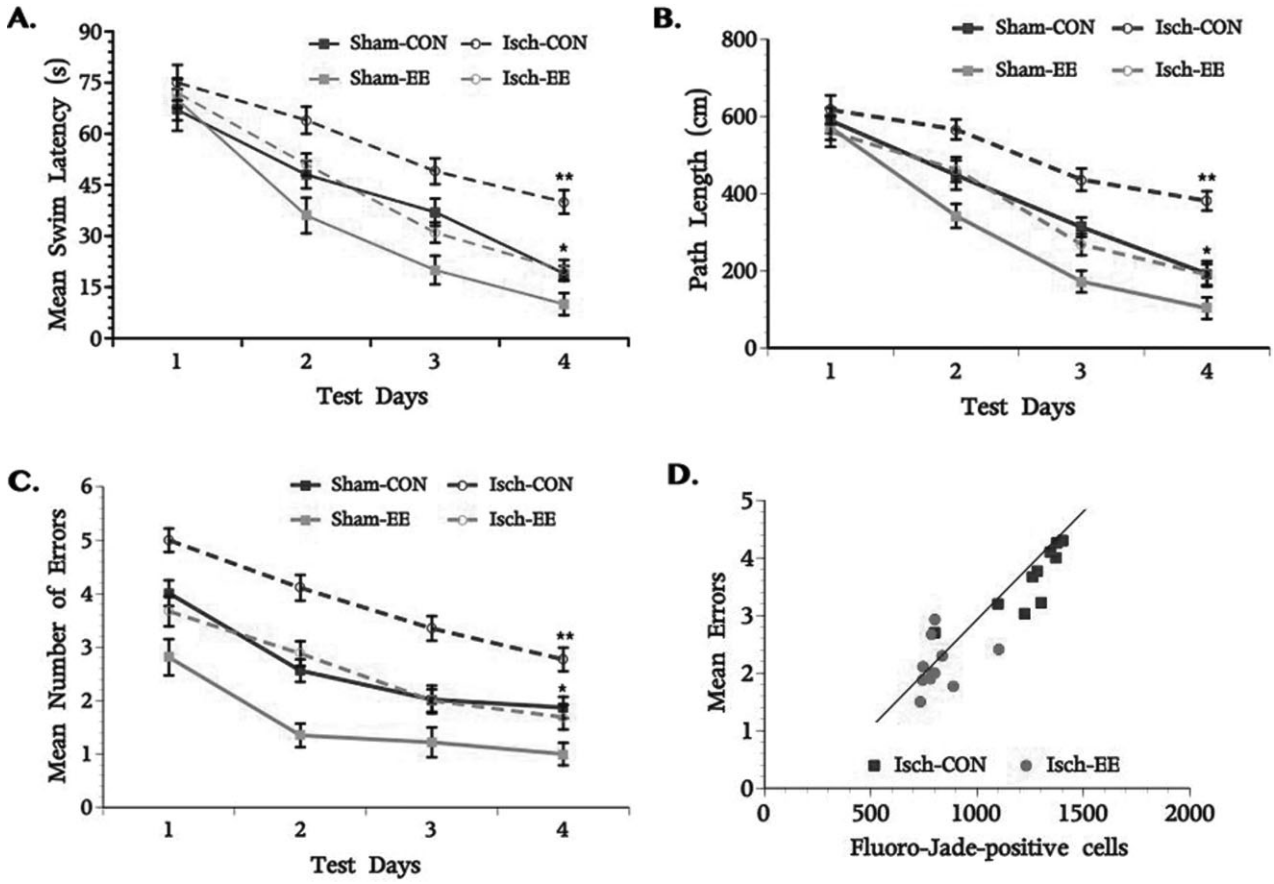

Figure 3. Behavioural testing. Performance in the cued learning and memory task (**A**) shows significant impairment in the ischaemia CON rats in swim latency compared with all groups. Meanwhile, performance of the ischaemia EE rats was not significantly different from the sham CON group. Sham rats housed in EE showed the best performance overall. Analysis of performance in the discrimination learning task also show that overall, the ischaemia CON rats took the most indirect path to reach the correct goal (**B**) and made significantly more errors in discriminating between the correct and incorrect goals (**C**). No significant difference was seen in the performance of the ischaemia EE and sham CON groups in the discrimination learning task. Sham rats housed in EE showed the best performance overall in the discrimination learning task. Regression analysis showed significant positive correlation between the number of Fluoro-Jade-positive cells and performance during the last day of discrimination learning test in that increased number of ischaemia-induced neuronal degeneration as labelled by Fluoro-Jade is related to greater number of errors made in the behavioural task (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$. Legend: Isch, ischaemia; CON, control; EE, enriched environment.

son showed no significant difference in total IGF-1R levels between the ischaemia groups and the sham EE animals. However, when IGF-1 receptor (IGF-1R) activation was analysed, increased phosphorylation [$F(3,38) = 9.05$, $P < 0.05$] was seen only in the ischaemia and sham rats housed in EE compared with their corresponding control groups with the highest levels seen in the sham EE group (Figure 4**B**). Meanwhile, comparison of phosphorylated IGF-1R levels between the sham and ischaemia CON rats did not show any significant difference (Figure 4**C**).

As IGF-1R phosphorylation involves activation of downstream signalling, we also examined the Akt pathway using Western blot. No significant group differences were seen in total Akt levels (Figure 4**C**). However, our results on phosphorylated Akt showed significant increase in the ischaemia and sham groups housed in EE [$F(3,38) = 10.01$, $P < 0.05$] compared with their corresponding control groups (Figure 4**D**) with the highest level seen in the sham EE animals. Conversely, no significant group difference in phosphorylated Akt levels was seen in the ischaemia CON and sham CON groups. These results suggest that EE housing and ischaemic injury can increase IGF-1 protein expression in the hippocampus but only enrichment was able to upregulate IGF-1R signalling.

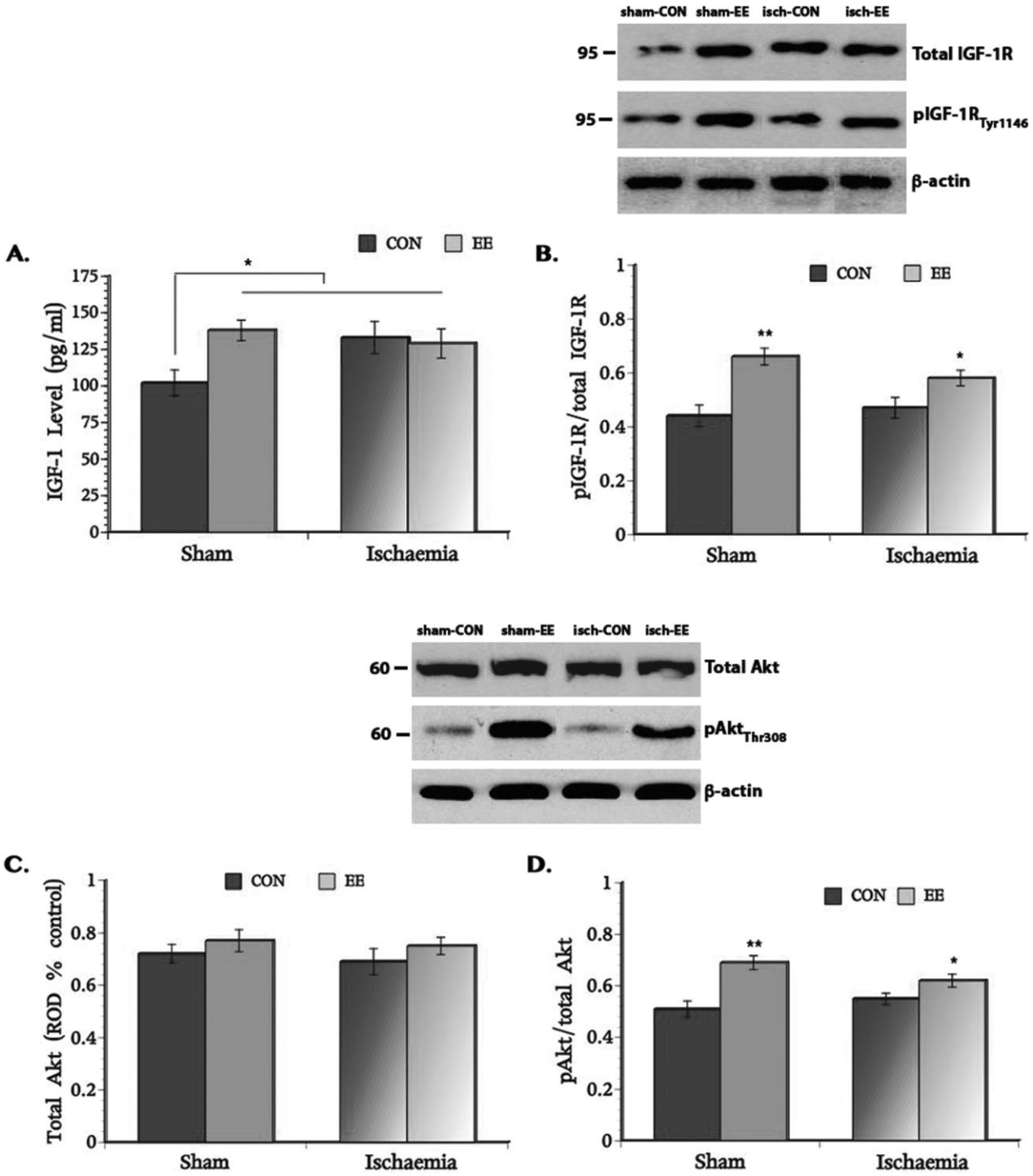

Figure 4. IGF-1 expression and signalling. Levels of IGF-1 protein significantly increased after transient global cerebral ischaemia and EE housing (**A**). Analysis of total IGF-1R levels showed significant increased in the ischaemic groups and in the sham EE animals compared with the sham control group. However, IGF-1R phosphorylation showed significant increased only in the ischaemia EE and sham EE (**B**). No significant difference was seen in IGF-1R phosphorylation between the ischaemia CON and sham CON groups. Analysis of total Akt levels showed no significant group differences (**C**) but significantly increased Akt phosphorylation was seen in the ischaemia EE and sham EE groups (**D**). No significant difference was seen in levels of phosphorylated Akt between the ischaemia CON and sham CON groups. $^{*}P < 0.05$, $^{**}P < 0.01$. Legends: Isch, ischaemia; CON, controls; EE, enriched environment; pAkt, phosphorylated Akt.

Discussion

In the present study we show that transient global cerebral ischaemia-induced neurodegeneration and behavioural impairment may be modifiable by EE housing. These findings are supported by our data that EE housing: (1) mitigated ischaemia-induced neuronal loss; (2) attenuated ischaemia-induced increase in caspase 3 immunoreactivity in the hippocampus; (3) ameliorated ischaemia-induced cognitive impairments; and (4) increased IGF-1 receptor activation and signalling through the Akt pathway after ischaemic injury. Ultimately, these findings suggest that the beneficial effects of EE housing as a therapeutic intervention in optimizing recovery after cerebral ischaemic injury may be mediated by increased IGF-1 signalling.

Our results show significant decreased in neurodegeneration in the hippocampus of ischaemic rats housed in EE immediately following recovery compared with the ischaemia CON group. Our results parallel earlier reports that show EE housing can lead to better preservation of pyramidal neurones in hippocampal CA1 following transient global cerebral ischaemia [7,8]. However, the current data are at odds with our earlier studies that demonstrate no significant difference in hippocampal neurodegeneration between the ischaemia EE housed and ischaemia CON animals [16,27,32]. The discrepancy between the results of our earlier studies and the current data may be attributed to the timing of EE housing; that is, ischaemic rats were housed in EE immediately following recovery in the current study, whereas in our earlier studies ischaemic rats were housed 3 days following recovery when delayed neurodegeneration may have started to occur.

Another related key point in the present study is the decreased caspase 3 immunoreactivity in the hippocampus of ischaemic rats housed in EE compared with the ischaemic CON group suggesting that enriched housing was also able to modulate apoptosis. Apoptosis plays an important role is ischaemia-induced delayed neurodegeneration that can occur days to weeks after reperfusion in ischaemic injury [33]. Induction of apoptosis is a tightly regulated process characterized by a discrete set of biochemical and morphological events mediated by members of the caspase family [34]. Together, our data on caspase 3 immunoreactivity and Fluoro-Jade staining indicate the likelihood that caspase-mediated apoptosis can be mitigated by EE housing following ischaemic injury.

In this study we also found an attenuation of cognitive deficits in the ischaemia rats housed in EE compared with the ischaemia CON group when tested in the water maze, which is hardly surprising. The two key findings are: (1) decreased swim latency in the cued learning task; and (2) decreased path length and errors committed in the discrimination-learning task. Although all rat groups seemed to exhibit the ability to learn the task of reaching the goal in both tests, the ischaemia CON rats persistently show increased time and longer path taken to reach the goal, as well as made more errors in locating the correct goal in the discrimination-learning tests. The performances of the EE ischaemic animals in the cognitive tasks, however, seem to be similar to the sham CON group. Our findings on improvement in cognitive impairment and concomitant decreased in neurodegeneration in the ischaemic EE group are comparable to previous reports that show post-ischaemic EE housing decreases infarct volume and facilitated cognitive recovery [7,8]. However, our data are also at odds with the report that no reduction in infarct volume is seen in EE housed rats after focal cerebral ischaemia but behavioural recovery is enhanced [14,18,35,36]. These conflicting findings may be attributed to the different ischaemia models used and duration of blood vessel occlusion where the focal ischaemic model used longer occlusion times, and also timing of EE housing after injury (immediately after injury or days after injury). It is noteworthy that as spontaneous behavioural recovery has been reported to occur after cerebral ischaemia regardless of the injury model or timing of EE housing after injury, it is possible that this phenomenon is mediated via plasticity mechanisms rather than degree of infarct volume [35,37].

The mechanisms underlying the protective effects of EE housing against delayed neurodegeneration following transient global cerebral ischaemia are still not fully understood. In this study we examined the possible association between IGF-1 and neuroprotective effects of EE after ischaemic injury as it has pleiotropic functions in the central nervous system including the prevention of neuronal and glial cell death, modulation of neuroinflammation, and stimulation of the proliferation of neural progenitors cells (reviewed in [38]). Although our results show increased IGF-1 protein expression weeks after cerebral ischaemia, these data are consistent with previous reports on the upregulation of both IGF-1 messenger RNA and protein in neurones and glial cells in the hippocampus of rats, gerbils and monkeys within hours to

several days after ischaemic injury [39–41]. The ischaemia-induced upregulation of IGF-1 protein and IGF-1R seen in the present study is possibly a compensatory response triggered after injury but it may not be adequate to provide neuroprotection. This line of reasoning is based on: (1) studies that show exogenous administration of IGF-1 is necessary to mitigate ischaemia-induced neuronal cell loss in the hippocampus [25,26]; (2) IGF-1 levels increased in both ischaemic rats groups compared with the sham CON animals; (3) increased IGF-1R phosphorylation is evident only in ischaemic rats housed in EE compared with the ischaemic CON group; and (4) enrichment-induced upregulation of IGF-1R is associated with the attenuation but not inhibition of apoptosis in the hippocampus of ischaemic rats.

However, a surprising finding in the present study is that evidence of increased phosphorylation of IGF-1R is seen only in the ischaemia EE and sham EE groups despite the presence of elevated IGF-1 and IGF-1R levels in both ischaemic groups. Some explanations for these unanticipated findings include the possibility that the increased glutamate release attenuated IGF-1R phosphorylation in the ischaemia CON group through activation of the *N*-methyl-*D*-aspartate receptors (NMDARs) [42]. Conversely, the increased IGF-1R phosphorylation in the ischaemia EE group suggest that enriched housing was able to modulate the injury-induced glutamate toxicity by decreasing NMDAR1 phosphorylation as we previously reported [7]. Namely, the role of EE housing in preventing ischaemia-induced glutamate release from exceeding the putative excitotoxic threshold may be caused by its ability to activate synaptic NMDARs while moderating extrasynaptic glutamate signalling. Another possible explanation is that EE enhances synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) activation and modulates NMDAR1 phosphorylation resulting in the moderation of glutamate excitotoxicity. That is, because glutamate attenuates IGF-1R phosphorylation via NMDA receptors [43], it is possible that by competitively increasing the activation of the other inotropic glutamate receptor AMPA through EE housing IGF-1 action may be enhanced [42,44]. Although the studies cited to support this line of thinking examined EE effects on glutamate release and activation during the early days post-injury, enrichment-induced effects on NMDAR1 and AMPA activation can persist weeks after ischaemia as shown in an earlier report on EE housing after focal ischaemic injury [44].

IGF-1R signalling is mediated through the downstream Akt pathway, which is implicated in cell growth, proliferation and survival under a wide variety of circumstances [45]. Akt signalling is reported to be sufficient and, in some cases, necessary for the trophic factor-induced cell survival of several neuronal cell types (reviewed in [46]). Here we confirmed that the EE-induced increased signalling by the growth factor IGF-1 through the Akt pathway is associated with decreased neuronal loss and attenuated apoptosis in the hippocampus following transient global cerebral ischaemia. It is also likely that in the intact brain EE-induced IGF-1 upregulation exert its trophic effects through this mechanism and promotes neuronal plasticity and neurogenesis (reviewed in [47]). What is not clear from our data is the cellular phenotype of IGF-1 expression and whether the neuroprotection afforded by the IGF-1R/Akt signalling is triggered during the ischaemia or reperfusion period as we implemented EE housing immediately following injury. In addition, we did not examine pathways downstream of Akt such as the glycogen synthase kinase-3β; and these issues warrant further investigation in future studies.

In sum, the present experiments provide evidence that housing rats in EE after transient global cerebral ischaemia may provide the brain some resiliency to insult. Specifically, apoptosis and neurodegeneration are modulated in the ischaemia EE rats and these protective effects are most likely facilitated by increased IGF-1R signalling through the Akt pathway. Our study also demonstrates that EE housing can enhance functional recovery following ischaemic injury. These findings indicate the importance of using strategies that can counteract some of the effects of cerebral ischaemia by enhancing the endogenous repair mechanisms thereby mitigating the damaging effects of secondary injury.

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Author contributions

M.W. did all the tissue imaging and helped with manuscript preparation; J.W. and M.R. equally contributed in

performing the behavioural testing and tissue processing for Western blots, immunohistochemistry and Fluoro-Jade staining; T.L.B. conceived of the funded project and wrote the manuscript with M.W.

Disclosure statement

The authors do not have any competing interest and no financial conflict of interest to declare.

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