

Combined enriched environment/atipamezole treatment transiently improves sensory functions in stroke rats independent from neurogenesis and angiogenesis

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Abstract

Functional recovery after cerebral ischemia may be enhanced by activation of the noradrenergic system and by environmental enrichment. The underlying mechanisms have remained elusive, but endogenous neurogenesis and perilesional angiogenesis have been speculated to contribute to the behavioral improvement. To address this question, neurogenesis in the subventricular zone (SVZ) and perilesional angiogenesis (RECA-1) were correlated with behavioral performance in forty Wistar rats subjected to transient middle cerebral artery occlusion (MCAO) or sham-operation. Atipamezole, an α_2 -adrenoreceptor antagonist (1 mg/kg, i.p.), was administered for 10 days together with housing of rats in an enriched environment. MCAO rats and sham-operated rats housed in single non-enriched cages were used as controls. Histological analysis after 28-day behavioral follow-up showed a massive increase in doublecortin (DCX)-positive cells in the SVZ both in MCAO rats housed in single cages and in the enriched environment together with atipamezole treatment whereas perilesional RECA-1 staining for new blood vessels was not altered. Time to the first contact and time to remove sticky stimuli from the forelimbs indicated improved sensory processing, which disappeared after cessation of atipamezole administration. Skilled forelimb use as measured by performance in Montoya's staircase test was not affected by the treatment. There were no correlations between behavioral measures and histology. Thus, sensory facilitation or reversal of hypometabolism by the combined therapy may be the mechanism accounting for the improved behavior after stroke independent from neurogenesis and angiogenesis.

Keywords: angiogenesis, behavioral recovery, cerebral ischemia, enriched environment, neurogenesis, noradrenaline.

Introduction

The current treatment options in stroke are extremely limited and many patients are left with permanent disabilities with upper extremity motor impairment being the most prominent [1]. Thus, restorative therapies are urgently needed, especially those based on activation of intrinsic brain repair mechanisms. The major advantage of these kinds of restorative treatments is their extended therapeutic time window, which may remain open for several weeks after the stroke.

One of these putative restorative mechanisms involves augmenting endogenous neurogenesis. Knowledge is emerging explaining how new neurons can be produced and cell migration encouraged towards the site of brain injury; these seem to be attractive new ways to enhance functional recovery after stroke [2, 3]. The subgranular zone of the hippocampal dentate gyrus and the subventricular zone (SVZ) are the two brain regions with the capability to undergo neurogenesis throughout life. Cerebral ischemia is known to increase the number of doublecortin (DCX)-positive neuroblasts in the SVZ [4]. It has been reported that newborn cells migrate from SVZ to the damaged striatum and once there generate new neurons for an extended period of time after the initial ischemic insult [5]. Unfortunately, the long-term survival of these cells seems to be low [5, 6]. Angiogenesis is another promising strategy for promoting post-stroke recovery [7, 8]. Several genes related to angiogenesis are

immediately activated after the ischemic insult, promoting survival of endothelial, glial and neuronal cells [9]. Subsequently, those newly formed vessels may take part in the removal of damaged tissue [10] and support migration of neural stem cells [11].

Recently, Beltran *et al.* [12] demonstrated that a short-term increase in the noradrenergic activity by administration of the α_2 -adrenoceptor antagonist, atipamezole (ATI), could lead to a sustained motor improvement after stroke when paired with housing of rats in enriched environment (EE). However, the underlying mechanisms have remained elusive. Differential types of intensive rehabilitative training have been shown to induce neurogenesis [13] as well as angiogenesis [14, 15] possibly capable of achieving some level of functional recovery after a stroke. Thus, in the present work we (1) studied the effect of combined EE/ATI treatment on forelimb function after focal cerebral ischemia and (2) correlated behavioral data with neurogenesis in the SVZ and angiogenesis in the perilesional cortex.

Materials and Methods

Animals

Forty male Wistar Han rats (Harlan, The Netherlands), weighing 290–340 g were used in the study. The animals were housed individually in a controlled environment (temperature $21 \pm 1^\circ\text{C}$, humidity 50–60%, light period

07:00–19:00 h) with access to food (2016S, Teklad) and fresh water available *ad libitum*. Animal care procedures were carried out according to European Community Council Directives 86/609/EEC guidelines and all procedures were approved by the Animal Ethics Committee (Hämeenlinna, Finland).

Transient middle cerebral artery occlusion

Anesthesia was induced in a gas chamber (30% O₂/70% N₂O) with 5% halothane for 2–3 minutes and then maintained at 0.5–1.5% using a nose mask. Body temperature was maintained at 37°C throughout the surgery by means of a heating pad connected to a rectal probe (Harvard Homeothermic Blanket Control Unit). Cerebral ischemia was induced by the filament technique [16]. The right common carotid artery (CCA) was exposed and a heparinized nylon filament of Ø 0.23 mm diameter was inserted *via* the ECA stump into the internal carotid artery (1.8–2.1 cm) to occlude the middle cerebral artery (MCA). After MCAO (90 minutes occlusion), the filament was gently pulled out and ECA was closed by electro-coagulation leaving a long ECA stump to permit the subsequent cell infusion. Sham-operated control animals underwent the same procedure but without filament insertion. Buprenorphine (0.03 mg/kg) was administered to relieve post-operative pain.

Experimental groups

Twenty-four hours after MCAO, the forelimb-placing test was conducted to assess the success of the MCAO [17]. In this test, the rat was held facing towards the table, resting its forelimbs on the table edge. The forelimb was gently pulled down and subsequent retrieval and limb placement was checked. The test was repeated by holding the head upward at a 45° angle so that the rat was not able to see the table or make vibrissal contact. The lateral placing of the forelimb was achieved by pulling down the forelimb while the rat was placed along the table edge. In addition, forelimb flexion was evaluated when the rat was lifted in the air by the base of its tail.

The tests were scored in following manner: two points for normal performance, one point for delayed/incomplete response and zero points for no response.

After the limb-placing test, magnetic resonance imaging (MRI) was performed with a Bruker 7 T horizontal scanner. The rats were anesthetized with 5% isoflurane in 30% O₂/70% N₂O. After induction, the anesthesia was maintained throughout the imaging session with 2.5% isoflurane inhaled through a nose mask. T2-weighted multi-slice images were acquired using a RARE sequence with the following parameters: time-to-repetition TR = 2.5 s, effective time-to-echo effTE = 40 ms, RARE factor 8, matrix size of 256×256, field-of-view of 30×30 mm, 15 slices with a slice thickness of 1 mm. Infarct volumes were analyzed using ImageJ.

MCAO rats with no limb-placing impairment or incomplete MCAO based on MRI were excluded from the study. The exclusion criteria were defined prior to the start of the experiment. In addition, based on the limb placing scores and MRI, the rats were assigned to equal experimental groups: MCAO+single (*n*=11), MCAO+EE/ATI (*n*=7), and SHAM+single (*n*=11).

EE consisted of two large metal cages (61×46×46 cm) that were connected by a tunnel. The cages (*n*=8–9 animals per cage) contained objects to encourage sensorimotor activity (*i.e.*, toys, ladders, wooden tubes, tunnels, shelves) that were changed every three days. Atipamezole hydrochloride (Orion) was dissolved in 0.9% NaCl and administered at a dose of 1 mg/kg (*s.c.*) for 10 days. The drug dose was selected based on previous studies [17]. The MCAO rats in single cages and sham-operated rats were housed individually in standard cages (53×32.5×20 cm).

Behavioral testing

All behavioral tests were carried out in a blinded manner one day before the operation and on post-operative days 11 and 28 (Figure 1). Behavioral outcome was assessed using the sticky label and Montoya's staircase tests. These are sensitive at detecting treatment effects, but are minimally affected by repeated testing.

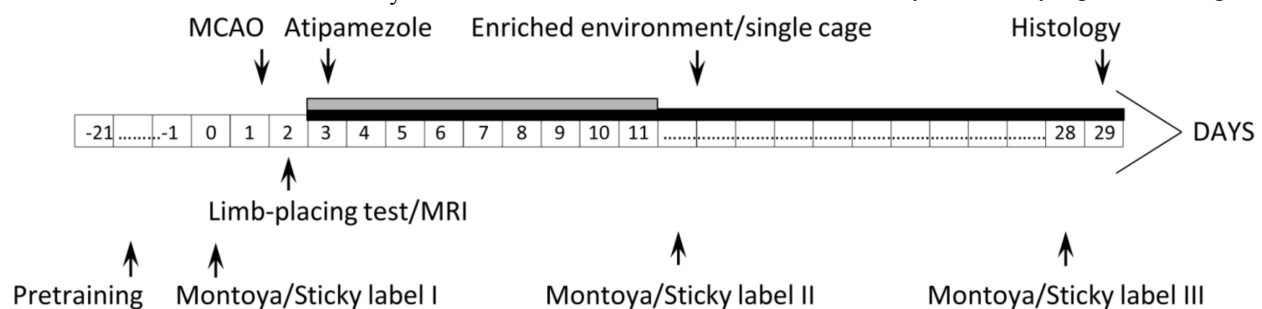


Figure 1 – Study design. Animals were pre-trained to perform Montoya's staircase and sticky label test before MCAO. Animals were placed either in single cages or in an enriched environment on post-operative day 2. Housing in enriched environment was combined with atipamezole treatment for 10 days. Behavioral tests were carried out at baseline and on post-operative days 11 and 28. After behavioral follow-up, the rats were perfused for histological analysis.

The sticky label test was used to evaluate sensory function and motor learning; it was performed as previously described [18]. Before testing, the animals were familiarized with handling and the testing cage. In the test, a white colored circular label (Ø 9 mm, Tough-Spots, Diversified Biotech) was placed on the distal-radial region of both wrists and rat was moved to a test

cage. The time for the first contact with the label and the time to remove the label were measured. A maximum time of 120 seconds was set if the rat was not able to remove the label.

Montoya's staircase provides a quantitative measure of a rat's independent skilled forelimb use [19]. Rats were trained for a period of three weeks (five consecutive

days/week). During training, the animals were on partial food deprivation (15 g/day given after training sessions) to enhance motivation. They maintained about 85% of their normal body weight. After MCAO, the food was taken away on the evening before the test. In this test, the rat was placed in a transparent staircase box made of plexiglass (285 mm long \times 90 mm high \times 60 mm wide). Each well of the staircase was filled with three rodent precision pellets (BioServ 45 mg). After the 10 minutes session, the total numbers of eaten and dropped pellets were counted. Rats that failed to eat at least 10 pellets at baseline were excluded from the study.

Immunohistochemistry

On post-operative day 29, rats were perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were carefully removed from the skull and postfixed. Brain sections (35 μ m) were cut using a sliding microtome and sections were stored in antifreeze solution at -20°C .

Immunostaining for doublecortin was applied as a marker for neurogenesis in the SVZ. The sections were washed in 0.1 M PBS, pH 7.4, pretreated in citrate buffer (85°C) for 30 minutes and incubated with the primary antibody (1:1000, Santa Cruz, SC.8066) in Tris-buffered saline with 0.5% Triton X-100 (TBS-T) overnight. After washing in TBS-T, the sections were incubated with the secondary antibody (rabbit anti-goat, 1:400; Vector BA-5000) for two hours. After washing, the sections were incubated with mouse Streptavidin (GE Healthcare), and finally for 2–3 minutes with Ni-intensified diaminobenzidine. Stained sections were evaluated in a blind manner with the Adobe Photoshop CC software. Images were captured under $5\times$ magnification using Axio Imager 2 upright microscope (Carl Zeiss GmbH, Germany) equipped with AxioCam ERe5s camera. In the analysis, images were merged together, converted to gray scale and areas with staining intensity above threshold (background) were extracted and measured.

Immunostaining with rat endothelial cell antibody (RECA-1) was applied for blood vessel visualization. The sections were washed in 0.1 M PBS, pH 7.4, and incubated with the primary antibody (1:1000, AbD Serotec, Oxford, UK) in Tris-buffered saline with 0.5% Triton X-100 for 18 hours at room temperature. After washing in Tris-buffered saline with 0.5% Triton X-100, the sections were incubated with the secondary antibody (goat anti-mouse*biotin, Vector) for two hours. After washing, the sections were incubated with mouse Streptavidin (GE Healthcare), and finally for 2–3 minutes with diaminobenzidine. Images were captured with a Zeiss Axio Imager M2 microscope. Perilesional cortical areas were sampled (six samples of 250×250 pixels), the area occupied by RECA-1 immunoreactivity was extracted and the immunoreactive area measured.

Statistical analysis

The statistical analyses were done with SPSS software. Statistical differences in infarct volumes and doublecortin and RECA-1 staining between groups were analyzed using one-way analysis of variance (ANOVA) with the LSD *post-hoc* test, if needed. Behavioral data for the

overall group effect or group \times time interactions were analyzed using repeated measures ANOVA followed by LSD *post-hoc* test. One-way ANOVA followed by LSD was used to detect differences between groups during testing days. Correlations between behavioral data and DCX-positive neuroblasts or perilesional RECA-1 staining were determined using Pearson's correlation coefficients.

Results

Six of the 40 (15%) operated rats died. As verified by MRI, the typical infarct area extended over large parts of the parietal sensorimotor cortex and there was severe damage detected in the striatum. There were no differences in the total infarct sizes between the experimental groups. Five animals were excluded, since they failed to meet the predetermined MRI criteria (*e.g.*, no cortical damage). One further MCAO rat was excluded due to a minor behavioral impairment in the limb-placing test.

Sticky label test

Deficit in sensory (time to contact) and motor (time to removal) performance of rats were analyzed using the sticky label test. Rats did not differ significantly in behavioral performance before the operation (Figure 2).

A significant overall group effect ($p<0.001$) and group \times time interaction ($p<0.001$) were observed for impaired forelimb in the time to the first contact to label. These were due to the difference between sham-operated and MCAO rats ($p<0.001$). On post-operative day 11, the removal time was increased in MCAO+single ($p<0.01$) and MCAO+EE ($p<0.001$) rats in comparison to sham-operated rats. In addition, MCAO+single rats displayed more severe impairment compared to MCAO+EE rats ($p<0.05$). On post-operative day 28, both MCAO groups differed from sham-operated animals ($p<0.01$). For the time to removal the label, there was a significant overall group effect ($p<0.01$) and group \times time interaction ($p<0.05$). MCAO+single group was different from sham-operated animals ($p<0.01$). On post-operative day 11, MCAO+single ($p<0.001$) and MCAO+EE ($p<0.01$) groups were different from sham-operated rats. In addition, MCAO+single needed more time to remove the label compared to MCAO+EE rats ($p<0.05$). On post-operative day 28, both MCAO groups were different from the sham-operated rats ($p=0.01$).

There was a significant group effect ($p=0.01$) in the time to the first contact with non-impaired forelimb, but no significant group \times time interaction ($p=0.286$). There was a significant group effect ($p=0.01$) and also a group \times time interaction ($p=0.01$) in the time to remove the label. Both MCAO groups were different from sham-operated controls ($p<0.01$). On post-operative day 11, MCAO+single ($p<0.001$) and MCAO+EE ($p<0.01$) groups needed more time to remove the label compared to sham-operated rats.

Montoya's staircase test

Montoya's staircase was used to assess the skilled forelimb use. The number of pellets eaten or dropped by ipsilateral and contralateral forelimbs in all experimental groups was similar before surgery (Figure 3).

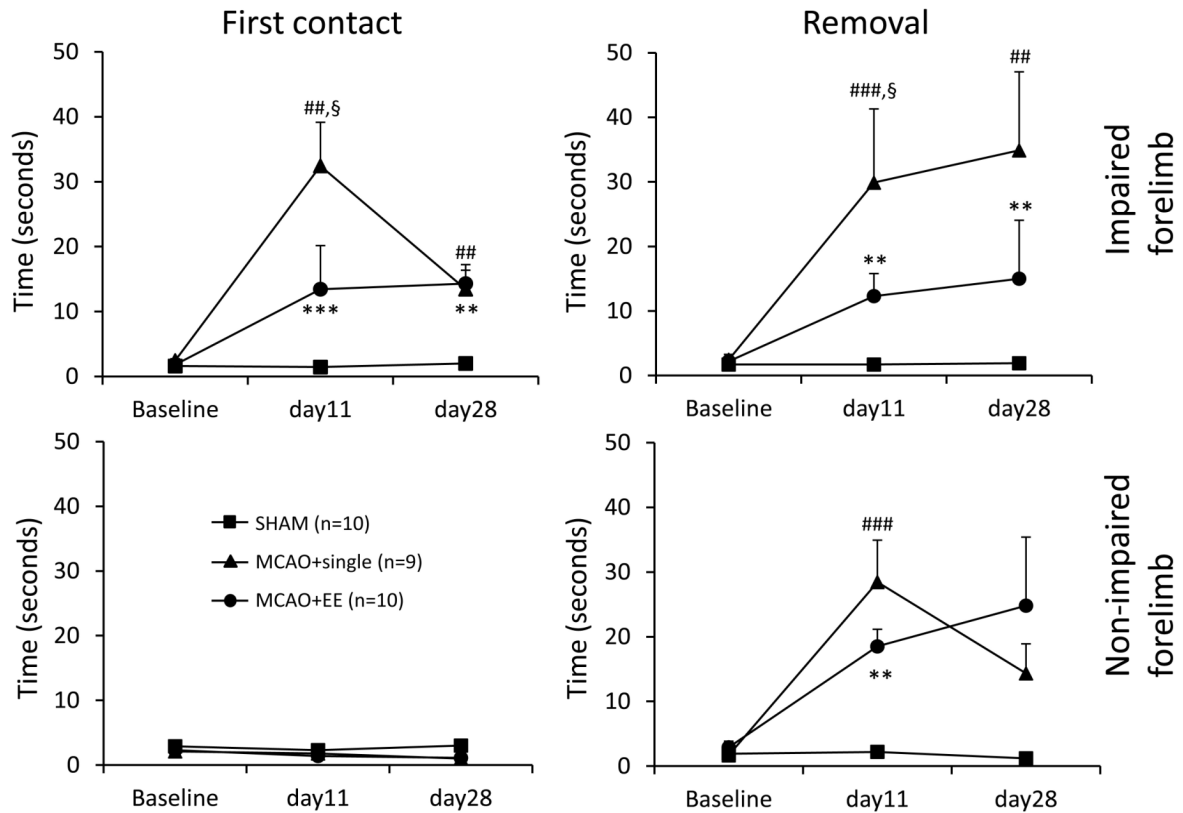


Figure 2 – Sticky label test. The mean time to first contact and the time required to remove the label from the impaired (contralateral) forelimb were increased in MCAO+single compared to MCAO+EE rats on post-operative day 11. Removal of the label but not time to contact with the non-impaired (ipsilateral) forelimb was increased in MCAO rats. Data are mean \pm SEM. Statistical significance: $**p<0.01$; $***p<0.001$ (SHAM vs. MCAO+EE); $^{\#}p<0.01$; $^{\#\#}p<0.001$ (SHAM vs. MCAO+single); $^{\S}p<0.05$ (MCAO+EE vs. MCAO+single).

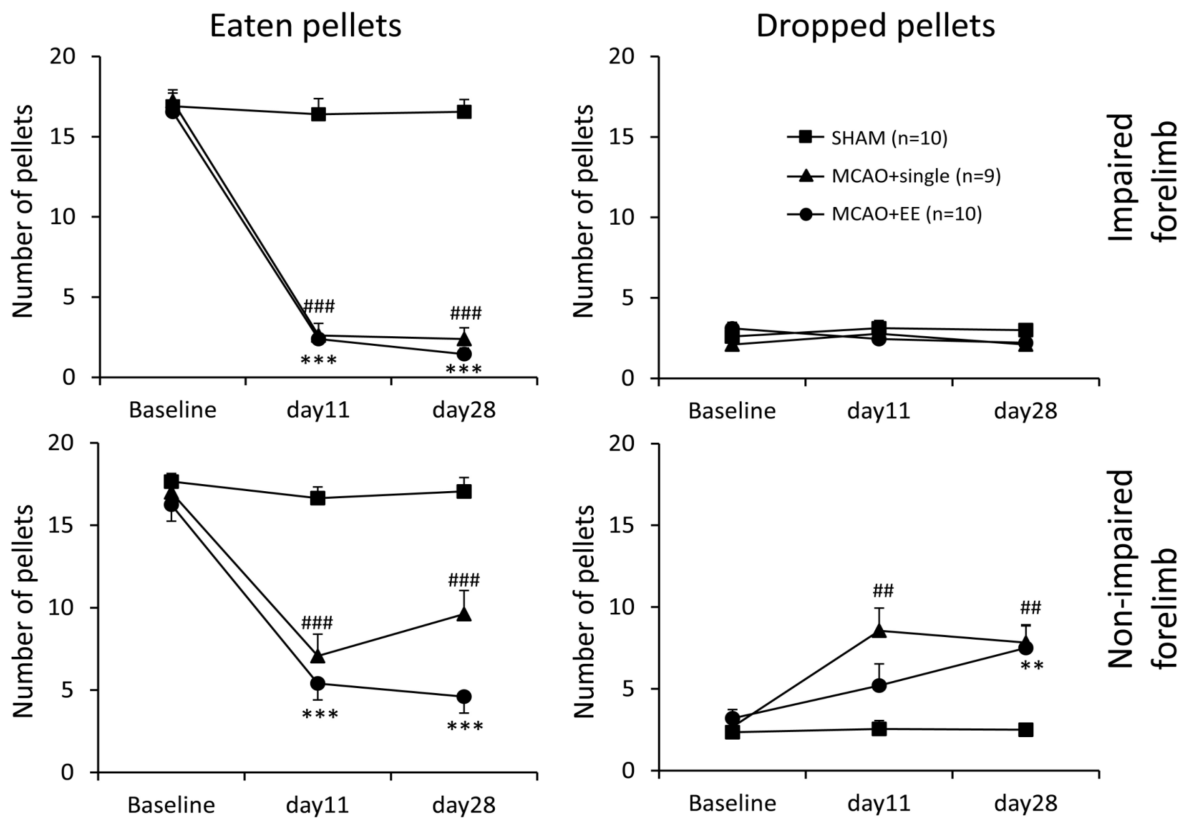


Figure 3 – Skilled forelimb use in Montoya's staircase test. The number of eaten pellets was decreased both by the impaired (contralateral) forelimb and the non-impaired (ipsilateral) forelimb in the MCAO rats. The number of dropped pellets by the non-impaired forelimb was increased in MCAO rats. Data are mean \pm SEM. Statistical significance: $**p<0.01$; $***p<0.001$ (SHAM vs. MCAO+EE); $^{\#}p<0.01$; $^{\#\#}p<0.001$ (SHAM vs. MCAO+single).

There was a significant overall group effect in the number of pellets eaten with the impaired forelimb ($p<0.001$). A significant group \times time interaction ($p<0.001$) was observed due to the differences between sham-operated and MCAO rats. A more detailed analysis showed a significant decrease in the number of eaten pellets between sham-operated and MCAO groups ($p<0.001$). The reaching performance was reduced in both MCAO groups compared to sham-operated rats on post-operative days 11 and 28 ($p<0.001$). The number of dropped pellets did not differ between the experimental groups ($p=0.674$) and there was no group \times time interaction ($p=0.344$).

There was also a significant overall group effect ($p<0.001$) and group \times time interaction ($p<0.001$) in the number of pellets eaten with the non-impaired forelimb. A more detailed analysis of retrieved pellets revealed a significant decrease in number of eaten pellets between sham-operated and MCAO groups ($p<0.001$). The MCAO groups differed from sham-operated rats on post-operative days 11 and 28 ($p<0.001$). There was also a significant overall group effect ($p<0.001$) and group \times time interaction ($p<0.001$) in the number of dropped pellets for the non-impaired forelimb, which was also due to the difference between sham-operated and MCAO rats ($p<0.01$). The MCAO+single rats made more mistakes compared to the sham-operated controls on post-operative days 11 and 28 ($p<0.01$) and MCAO+EE rats made more mistakes compared to sham-operated rats post-operative day 28 ($p<0.01$).

DCX and RECA-1 staining

The antibody for DCX was used to examine the newborn neuroblasts in the SVZ on post-operative day 29 after MCAO (Figure 4, B and D). One-way ANOVA revealed a significant overall group effect in DCX staining in the ipsilateral SVZ ($p<0.001$). Group comparisons revealed that staining was increased both in MCAO rats housed in single cages ($p<0.001$) and enriched

environment ($p<0.001$) compared to sham-operated rats (Figure 4A). There was no significant group effect in RECA-1 staining in the ipsilateral or contralateral perilesional cortex (Figure 5, A–D).

No correlations were found between DCX-positive staining in the SVZ and behavioral performance on post-operative day 28. Furthermore, no correlations were observed between RECA-1 staining in the perilesional cortex and behavioral performance.

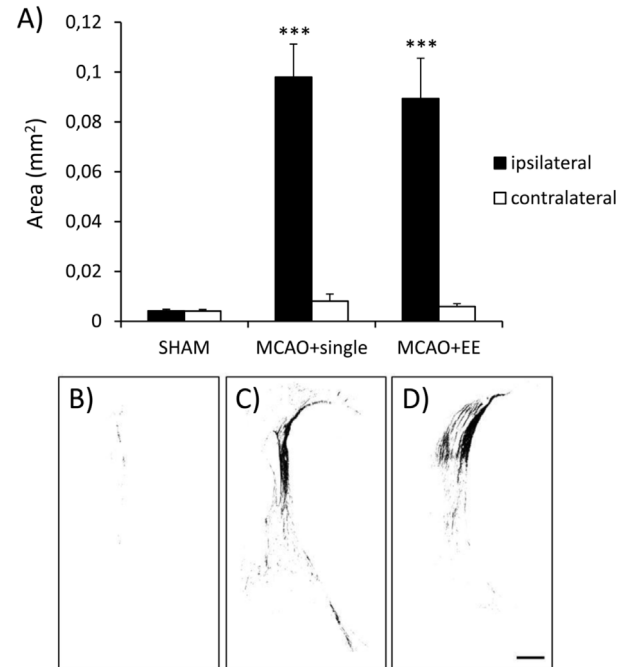


Figure 4 – Doublecortin staining. Doublecortin (DCX) staining in the subventricular zone (SVZ) was increased in MCAO rats (A). Representative images demonstrating the distribution and density of DCX-positive neuroblasts in the ipsilateral SVZ in sham-operated and MCAO rats (B–D). Statistical significance: *** $p<0.01$ (compared to sham-operated rats). Data are mean \pm SEM. Scale bar = 440 μ m.

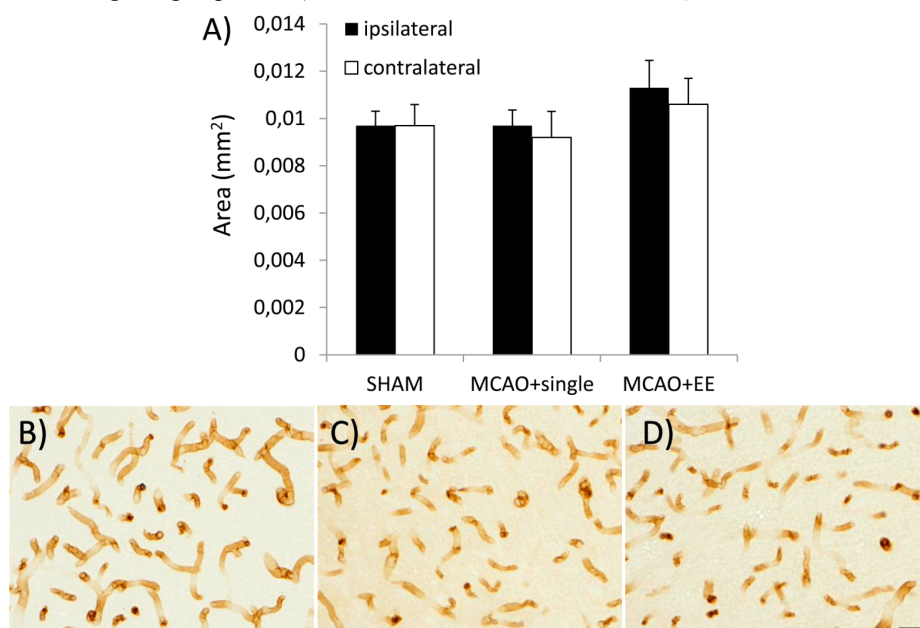


Figure 5 – RECA-1 staining. RECA-1 staining in the perilesional cortex did not differ between sham-operated and MCAO rats (A). Representative images showing RECA-1 positive microcapillaries in the perilesional cortex in sham-operated and MCAO rats (B–D). Data are mean \pm SEM. Scale bar = 20 μ m.

Discussion

The present study examined whether behavioral recovery after focal cerebral ischemia could be facilitated by atipamezole treatment and housing in enriched environment and whether this would be associated with altered neurogenesis in the SVZ or perilesional angiogenesis. A transient sensorimotor improvement was achieved with the combined therapy in the performance of rats in the sticky label test. However, the long-term behavioral outcome was not correlated with DCX-immunoreactivity in the SVZ or with the number of new blood vessels in the perilesional cortex.

Transient improvement in sensorimotor functions by combined therapy in MCAO rats

The sticky label test measures deficits in sensory (time to the first contact) and motor (time for removal) performance after cerebral ischemia. The MCAO rats were severely impaired in both measures and the impairment seemed to be permanent. The time needed to the first contact and to remove the label was reduced only when atipamezole was administered together with EE on post-operative day 11 indicating that there was enhanced sensory processing following α_2 -adrenoceptor blockade. This could also explain why atipamezole is effective in simple behavioral tests assessing tactile/proprioceptive functions, but not in more demanding motor tests [17, 20]. Another mechanism could be reversal of remote hypometabolism by atipamezole as previously shown in MCAO rats [21]. Interestingly, the time to remove the label by ipsilateral (non-impaired) forelimb was also increased in MCAO rats possibly because the animals were using the contralateral forelimb to support the ipsilateral forelimb in removing the sticky label.

No behavioral improvement by combined therapy in Montoya's staircase test

The performance of ischemic rats in Montoya's staircase test revealed a significant impairment of skilled forelimb functions as reflected in the decrease in the number of picked and eaten pellets with impaired forelimb during the 28-day follow-up. In addition, behavioral testing revealed permanent impairment with virtually no recovery in the affected forelimb. It is likely that the severity of corticostriatal damage after MCAO causes a persistent impairment, but there may also be some kind of bilateral nature of forelimb impairment. This can also be related to the design of the Montoya's test box: while reaching and grasping the pellets with the non-impaired forelimb, the animal has to rely on the impaired forelimb for postural support and adjustment [19]. In addition, plasticity in the intact hemisphere may lead to impairments in skilled movements in the forelimb ipsilateral to injury [22].

Of particular note, there were no differences in skilled forelimb use between the various MCAO groups. The provision of an enriched environment alone or together with atipamezole did not improve skilled use of impaired forelimb. This is in contrast to previous studies demonstrating that atipamezole treatment when combined with housing in an enriched environment could improve sensorimotor outcome even long after the cessation of

drug treatment [12, 17]. Again, this may be attributed to the experimental stroke model used and the severity of the ischemic damage. In the study of Beltran *et al.* [12], a permanent MCAO model was used and this is typically characterized by the development of a more restricted cortical lesion.

Cerebral ischemia enhances neurogenesis in the SVZ, but not perilesional angiogenesis

An enriched environment is known to enhance neurogenesis in both the hippocampus and SVZ in naïve animals [23, 24]. However, after cerebral ischemia, the results have been controversial, ranging from decreases [25] to increases [4]. In the present study, the generation of neuroblast in the SVZ was evaluated with DCX staining. We detected a massive increase in the number of DCX-positive cells in the ipsilateral SVZ after MCAO in agreement with previous studies [6, 26]. Here, one has to note that reliable determination of heavily packed neuroblasts in the SVZ is challenging and saturation of the signal may have led to masking of some minor changes. On the other hand, the present finding is in agreement with Nygren *et al.* [25] showing EE induced enhancement of neurogenesis after MCAO in the dentate gyrus but not in the SVZ.

No behavioral correlations with neurogenesis in the SVZ or perilesional angiogenesis

A recent meta-analysis clearly showed that exposure of animals to EE after cerebral ischemia could enhance sensorimotor functions [27] but the underlying mechanisms have remained unclear. Here we correlated behavioral data in MCAO rats with the levels of DCX immunoreactivity in the SVZ and perilesional RECA-1 staining but could not detect a significant correlation after the 28-day follow-up in MCAO rats indicating that neurogenesis or angiogenesis do not seem to be playing any major role in behavioral recovery. In the case of angiogenesis, it is known that expressions of VEGF and flk-1 and many other angiogenesis related genes become decreased with time, indicating that the major role of the newly produced blood vessels is removal of cell debris rather than promoting long-term plasticity [9]. However, it is possible that the behavioral data may have been biased by the compensation induced by the enriched environment. Indeed, recently it has been speculated that compensatory movements represent the major mechanism behind the behavioral improvement achieved by EE [28].

Conclusions

The present study detected only a transient sensory improvement in MCAO rats after atipamezole and enriched environment, which disappeared after cessation of atipamezole treatment. Thus, enhanced sensory processing or reversal of remote hypometabolism (diaschisis) might be the underlying mechanism activated by blockade of α_2 -adrenoceptors and subsequent increase in noradrenergic activity rather than neurogenesis or angiogenesis.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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