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Forelimb use after focal cerebral ischemia in rats treated with an α2-adrenoceptor antagonist

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Abstract

Atipamezole, a selective α2-adrenoceptor antagonist, enhances recovery of sensorimotor function after focal cerebral ischemia in rats. The aim of the present study was to further characterize the effects of atipamezole treatment combined with enriched-environment housing in ischemic rats by evaluating spontaneous exploratory activity in the cylinder test. The right middle cerebral artery (MCA) of rats was occluded for 120 min using the intraluminal filament method. Atipamezole (1.0 mg/kg) or 0.9% NaCl was administered on postoperative days 2 through 11 and 15, 19, and 23. Spontaneous behavior of rats in a transparent cylinder was videotaped before, and 6 and 23 days after surgery 20 min after drug administration. Constant asymmetry in forelimb use was observed in the cylinder test on postoperative days 6 and 23. Ischemic rats used the impaired forelimbs (contralateral to lesion) during lateral exploration less than did sham-operated rats ($P < .001$). Ischemic rats also preferred to turn contralateral to the lesion ($P < .05$). Atipamezole increased the simultaneous, but not independent, use of the forelimbs during lateral exploration ($P < .05$). The data suggest that noradrenergic manipulation does not significantly enhance recovery in a test that does not depend on practice following focal cerebral ischemia.
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1. Introduction

Most stroke patients exhibit some degree of motor recovery following the initial severe disabling state (Nakayama et al., 1994). Similarly, rats subjected to cerebral ischemia or other brain trauma show spontaneous recovery of function. The recovery rate seems to depend on the task and occurs over days or weeks as tested by simple sensorimotor tests and is more long-lasting or even permanent as tested by more complex motor tests (Butovas et al., 2001; Grabowski et al., 1993; Hudzik et al., 2000; Jolkkonen et al., 2000; Markgraf et al., 1992).

Enhancement of spontaneous recovery following cerebral ischemia using pharmacologic interventions has been accepted as a potential approach for treatment of stroke patients. For example, experimental studies with amphetamine suggest that the noradrenergic system has an important role in the recovery process (Feeney, 1998). These studies, which

have been undertaken mainly in the sensorimotor cortex ablation model, indicate the importance of noradrenaline both in the promotion and maintenance of the recovered state (Sutton and Feeney, 1992). Even low doses of amphetamine, however, induce severe circling in rats subjected to transient occlusion of the middle cerebral artery (MCA), possibly due to the almost complete striatal damage typical of this model (Wakayama et al., 1993). An alternative way to stimulate the noradrenergic system is to selectively block presynaptic α2-adrenoceptors (e.g., yohimbine, atipamezole), which increases the release and turnover of noradrenaline (Haapalinna et al., 2000; Laitinen et al., 1995).

We previously demonstrated facilitation of sensorimotor recovery by atipamezole in rats following transient MCA occlusion (Jolkkonen et al., 2000; Puurunen et al., 2001). The aim of the present study was to further characterize behavioral effects of atipamezole combined with enriched-environment housing in ischemic rats using the cylinder test. The cylinder test measures asymmetry in forelimb use during spontaneous exploration. The advantage of the cylinder test is that repeated testing does not influence asymmetry, rats do

not develop compensatory strategies, and it is also possible to assess turning behavior in response to drug treatment (Schallert and Tillerson, 2000).

2. Methods

2.1. Animals

Studies were performed using male Wistar rats (HsdBrl: WH, Hannover origin; National Laboratory Animal Centre, Kuopio, Finland) weighing 275–325 g. The rats had free access to food and water throughout the experiment and were housed under 12:12 h light/dark conditions in a temperature-controlled environment (20 ± 1 °C). Experimental procedures were conducted in accordance with the European Community Council directives 86/609/EEC and were approved by the Committee for the Welfare of Laboratory Animals of the University of Kuopio and by the Provincial Government of Kuopio.

Two days after the operation, rats were placed in an enriched environment, which facilitates recovery of function after focal cerebral ischemia (Held et al., 1985; Puurunen et al., 2001). The enriched environment consisted of two large cages ($61 \times 46 \times 46$ cm) connected together by a short tunnel and ladders, containing tunnels, shelves, a running wheel, and different kinds of manipulable objects (e.g., glass balls, bottles, and wooden pieces), which were replaced every other day (Puurunen et al., 1997).

2.2. Middle cerebral artery occlusion

Focal cerebral ischemia was induced by the intraluminal filament technique (Longa et al., 1989). Anesthesia was induced in a chamber using 3% halothane in 30% O_2/70% N_2O. A surgical depth of anesthesia was maintained throughout the operation with 0.5–1% halothane delivered through a nose mask. The right common carotid artery was exposed through a midline cervical incision under a surgical microscope and gently separated from the nerves. Microvascular clips were placed around the right common carotid artery and the internal carotid artery to prevent bleeding during the insertion of the filament. The external common carotid artery was closed with a suture and cut with microscissors and electrocoagulated. A heparinized nylon filament ($\emptyset$ 0.25 mm, rounded tip) was inserted into the stump of the external

common carotid artery. The filament was advanced 1.9–2.1 cm into the internal common carotid artery, to occlude the blood flow to the MCA territory, until resistance was felt. The filament was held in place by tightening the suture around the internal common carotid artery and placing a microvascular clip around the artery. Body temperature was monitored and maintained at 37 °C using a heating pad connected to a rectal probe (Harvard Homeothermic Blanket Control Unit, 50-7061). After 120 min of MCA occlusion, the filament was removed and the external carotid artery was permanently closed by electrocoagulation. In the present study, blood pressure or blood gases were not measured, because cannulation of a femoral artery would have interfered with the behavioral assessment. In a previous publication, however, we have shown that blood gasses and blood pressure are stable in the described procedures (Jolkkonen et al., 1999). At the end of the operation and on postoperative days when necessary, supplementary 0.9% NaCl solution was administered (ip) to the animal. The sham operation was undertaken in a similar manner, except the carotid arteries were only exposed. Successful MCA occlusion was verified on postoperative day 2 using the limb-placing test, which correlates with infarct volume (Puurunen et al., 2001).

2.3. Study design and drug treatment

Two days after surgery, a modified version of the limb-placing test (De Ryck et al., 1989) was used to assess the behavioral deficit of the animals and to assign them to behaviorally equivalent experimental groups: (1) SHAM + NaCl ($n = 11$), (2) SHAM + ATI ($n = 11$), (3) ISCH + NaCl ($n = 9$), and ISCH + ATI ($n = 11$). Only the rats with total scores less than 10 were included into ischemic groups.

Atipamezole (supplied by Orion Pharma, Finland) was dissolved in 0.9% NaCl and administered at a dose of 1.0 mg/kg (sc). The drug dose was selected on the basis of previous experiments (Jolkkonen et al., 2000; Puurunen et al., 1997, 2001). The ischemic control rats and sham-operated control rats were injected with 0.9% NaCl (sc). The injections were administered on postoperative days 2 through 11 and 15, 19, and 23 (Fig. 1).

Drug treatment (atipamezole) began on the second day after ischemia induction. The aim was to avoid interference of the maturation of acute ischemic damage in the early phase and to focus on behavioral effects of atipamezole on recovery of function.
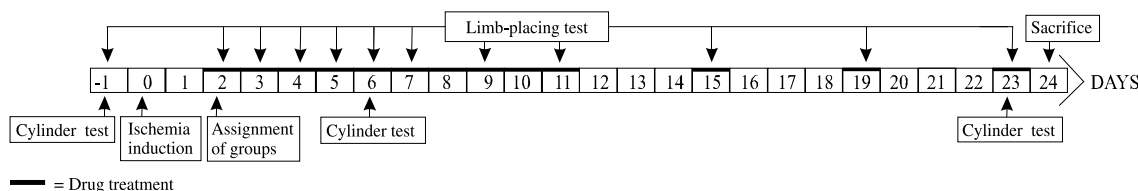

Fig. 1. The study design. The bars and arrows indicate the timing of drug dosing and behavioral testing.

2.4. Behavioral assessment

The cylinder test described by Schallert et al. (2000) was used to assess forelimb use and rotation asymmetry. For the test, the rat was placed in a transparent cylinder (∅ 20 cm) and videotaped during the light part of the light/dark cycle before, and 6 and 23 days after surgery. The rat was transported to the videotaping room 20 min after the injection of atipamezole or saline, placed in the cylinder, and videotaped for 10 min in the dark using red light. Mirrors were placed behind the cylinder so that behavior could be clearly viewed when the animal turned away from the camera. Exploratory activity for 1–3 min was analyzed in a manner blind to treatment groups using a video recorder with slow-motion capabilities. Forelimb use was observed during the first contact against the wall after rearing and during lateral exploration of the wall. Independent use of impaired and nonimpaired forelimbs as well as simultaneous use of both forelimbs were calculated. After takeoff, the first forelimb to contact the wall with weight support was scored as an independent wall placement for that limb. A simultaneous (both forelimbs) movement was scored if the first limb maintained its position and the other forelimb was placed on the wall. If the animal placed both forelimbs simultaneously on the wall, that was scored as a simultaneous movement. To score again, the rat had to remove both forelimbs from the surface of the cylinder. After the rat removed both forelimbs from the wall, stood with its hindlimbs, and then contacted the wall with the other forelimb, that was scored as an independent wall placement for that limb. When the rat explored the wall laterally, alternating both limbs, simultaneous movement was scored. If one forelimb was stationary and the other made several movements, it was scored as one simultaneous movement. Forelimb use during takeoff and landing could not be measured reliably from the videotapes and was not analyzed. The numbers of quarter turns in either direction during rearing and ground were also counted from the videotapes to measure turning preference.

The modified version of the limb-placing test described by De Ryck et al. (1989) was used to assess hindlimb and forelimb responses to tactile and proprioceptive stimulation (Jolkkonen et al., 2000; Puurunen et al., 2001). The rats were habituated for handling and testing before ischemia induction. The limb-placing test was used to assign rats to behaviorally equal groups on day 2 and to study sensorimotor recovery on days 2 through 7, and 9, 11, 15, 19, and 23 after surgery (Fig. 1). The limb-placing tasks were performed 30 min after drug and saline injections. The test consisted of seven limb-placing tasks, which were scored by a tester blind to the treatment groups. The following scores were used to detect impairment of the hindlimb and the forelimb: 2 points, the rat performed normally; 1 point, the rat performed with a delay of more than 2 s and/or incompletely; 0 points, the rat did not perform normally. Both sides of the body were tested. The maximum possible score achieved by the sham-operated rats was 14.

2.5. Determination of infarct volumes

The animals were decapitated 24 days after occlusion of the MCA and their brains were rapidly removed from the skull and frozen on dry ice. Coronal sections (40 μm) were cut throughout the brain on a cryostat and sections at 0.5-mm intervals were collected on slides. Sections were stained for 20 min with a solution containing 1.2 mmol/l nitroblue tetrazolium and 0.1 mol/l sodium succinate in 0.1 mol/l sodium phosphate buffer, pH 7.6, at 37 °C (Nachlas et al., 1957). Sections were then rinsed in water, dehydrated in an ascending series of alcohol, cleared in xylenes, and coverslipped with Depex. Estimations of the infarcted areas in the cortex and striatum were performed using an image analysis system (MCID) by an observer blind to the experimental groups. The image of each section was stored as a 1280×1024 matrix of calibrated pixel units. The digitized image was then displayed on a video screen, areas of interest were manually outlined separately for each hemisphere, and surviving gray matter with optical densities above the threshold levels was recognized automatically by the image analysis system. The difference between the size of an intact area in the contralateral hemisphere and its respective residual area in the ipsilateral hemisphere was used as the infarcted area. Total infarct volume was calculated by multiplying infarct area by the distance between the sections and summing together the volumes for each brain.

2.6. Statistics

All data were analyzed using SPSS for Windows. One-way analysis of variance (ANOVA) was used to study overall group effects on behavioral data in the cylinder followed by a post hoc test (LSD) when necessary. Limb-placing test scores were analyzed using the Kruskal–Wallis nonparametric test for several independent samples followed by Mann–Whitney U test. A P-value of less than .05 was considered to be statistically significant.

3. Results

3.1. Inclusion of the rats to the experiment

From operated rats, 18% died and 9% was excluded from the study because they did not show sufficient behavioral deficit in the limb-placing test (more than 10 points).

Table 1
Infarct volumes (mm³) in rats subjected to transient occlusion of the middle cerebral artery

Group	Hemisphere	Cortex	Striatum
ISCH + NaCl ($n = 9$)	123 ± 42	59 ± 36	26 ± 2
ISCH + ATI ($n = 11$)	127 ± 52	61 ± 35	25 ± 2

Values are mean ± S.D. Number of rats is in parentheses. There were no significant differences in infarct volumes between groups.
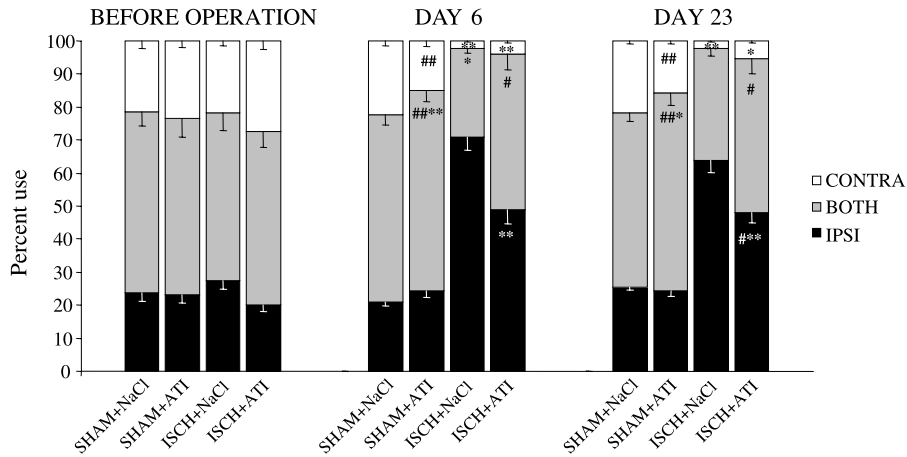
Fig. 2. Forelimb use during lateral exploration in the cylinder. Results are represented separately for different treatment groups before surgery, and 6 and 23 days after ischemia induction. Bars indicate percentages ($\pm$ S.E.M.) of steps made with the contralateral or ipsilateral forelimb and both forelimbs simultaneously. Statistical differences (one-way ANOVA followed by LSD): $*P<.05$, $**P<.01$ (compared to SHAM + NaCl); $\#P<.05$, $\#\#P<.01$ (compared to ISCH + NaCl).

3.2. Infarct volumes

Transient occlusion of the MCA in rats produced consistent and extensive cortical and striatal infarction. There was no statistically significant difference in infarct volumes determined from NBT-stained sections between ischemic control rats and atipamezole-treated ischemic rats (Table 1).

3.3. The cylinder test

Before ischemia induction, the independent use of each forelimb was 23% of all steps made on the wall (Fig. 2). On postoperative day 6, ischemic control rats used the impaired (contralateral to lesion) forelimb significantly less compared to sham-operated controls ($P<.001$). This deficit was still obvious on postoperative day 23 ($P<.001$).

Atipamezole treatment increased simultaneous forelimb use in sham-operated rats on postoperative day 6 ($P<.01$)

(Fig. 2). A similar effect was observed between ischemic rats treated with atipamezole and ischemic controls ($P<.05$). Atipamezole did not affect the independent use of the impaired forelimb significantly.

On postoperative day 23, there was a statistically significant difference between sham-operated rats treated with atipamezole and sham-operated controls in the simultaneous use of both forelimbs ($P<.05$). Ischemic rats treated with atipamezole used both forelimbs simultaneously ($P<.05$) more than ischemic controls on postoperative day 23.

3.4. Turning behavior

There was no preference in turning behavior before surgery. On day 6 after ischemia induction, ischemic controls turned more to the side contralateral to the lesion compared to sham-operated controls ($P<.05$), whereas in the atipamezole-treated ischemic group, turning behavior was similar
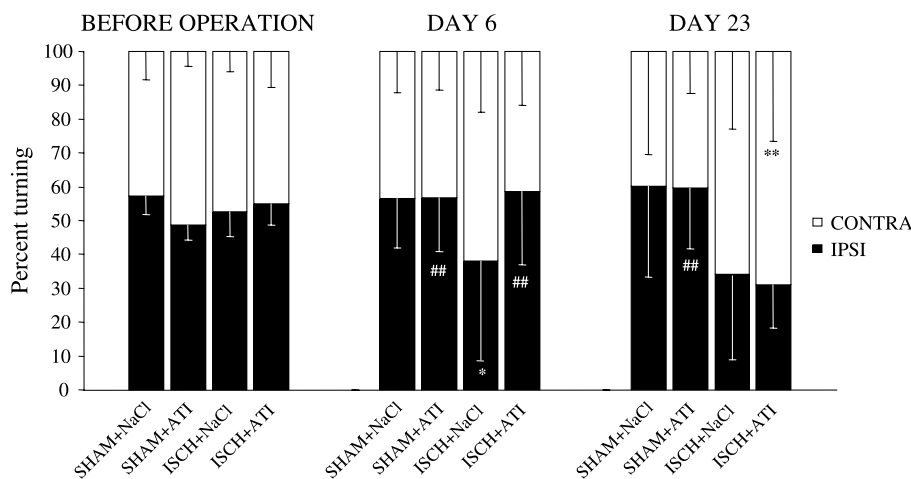

Fig. 3. Quarter turns in the cylinder. Results are represented separately for different treatment groups before surgery, and 6 and 23 days after ischemia induction. Bars indicate percentages ($\pm$ S.E.M.) of turns towards the ipsilateral and contralateral sides. Statistical differences (one-way ANOVA followed by LSD): $*P<.05$, $**P<.01$ (compared to SHAM + NaCl); $\#P<.05$, $\#\#P<.01$ (compared to ISCH + NaCl).
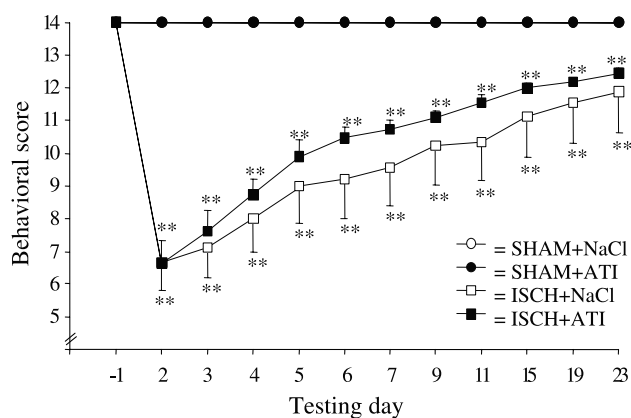
Fig. 4. Mean scores (± S.E.M.) for the groups of rats in the limb-placing test following transient occlusion of the middle cerebral artery. Scores from sham-operated controls (SHAM + NaCl) and drug-treated sham-operated rats (SHAM + ATI) overlap. Statistical differences (Mann–Whitney U test): ** $P < .001$ (compared to SHAM + NaCl).

to that of sham-operated rats (Fig. 3). Ischemic rats treated with atipamezole turned more to the contralateral side on day 23 compared to sham-operated controls ($P < .01$).

3.5. The limb-placing test

Immediately after MCA occlusion, the rats were severely impaired in the limb-placing test followed by a spontaneous recovery almost to the preoperative level by the end of the experiment (Fig. 4). Behavioral performance was different between sham-operated controls and ischemic rats until the end of experiment ($P < .001$). Atipamezole treatment tended to facilitate recovery of impaired limb-placing responses of ischemic rats, but not significantly.

4. Discussion

A long-lasting forelimb use asymmetry was observed in the cylinder test following transient MCA occlusion. Ischemic rats preferred the nonimpaired forelimb (ipsilateral to lesion) during lateral exploration both 6 and 23 days after operation. Forelimb asymmetry in the cylinder test is related to the loss of dopamine in the striatum in 6-hydroxydopamine-lesioned rats, indicating that striatal damage in the transient MCA occlusion model might contribute to this behavioral abnormality (Tillerson et al., 2001). The cylinder test has been used to detect asymmetry in forelimb use in rat models of stroke, parkinsonism, brain trauma, and spinal cord injury (Bland et al., 1999; Johnston et al., 1999; Roof et al., 2001; Schallert et al., 2000; Tillerson et al., 2001). Asymmetry is evident during takeoff, lateral exploration, and landing. Whether the reliance is on the impaired forelimb during takeoff and landing was difficult to differentiate, and thus, only forelimb use was counted during lateral exploration on the wall. This long-lasting asymmetry in

forelimb use contrasts with the rapid recovery of function that occurs in simple sensorimotor tests after MCA occlusion (Jolkkonen et al., 2000; Puurunen et al., 2001). Improvement in simple tests might, at least partially, be a result of compensatory strategies that rats develop to overcome loss of function. Apparently, forelimb asymmetry in rats subjected to MCA occlusion need not be compensated. Thus, the cylinder test might be a reliable tool for preclinical testing of restorative drugs.

Atipamezole enhanced simultaneous use of the forelimbs in sham-operated and ischemic rats on postoperative days 6 and 23. Independent use of the impaired forelimbs, however, was not improved. During lateral exploration on the wall, independent use of the impaired forelimb and initiation of movement might be more demanding for the ischemic rats than continuing the series of movements that began with the nonimpaired forelimb. Facilitation of simultaneous use might be related to plastic alterations in the contralateral cortex, as shown in rats (Jones and Schallert, 1994; Stroemer et al., 1998) and stroke patients (Hallett, 2001). For example, behavioral improvement after amphetamine dosing is related to axonal sprouting in the contralateral sensorimotor cortex, shown by synaptophysin and GAP-43 staining (Stroemer et al., 1998). Another possibility is activation of direct corticospinal pathways (Tracey, 1995) or plasticity of cerebellar pathways (Barbelivien et al., 2002).

Ischemia induced spontaneous turning to the side contralateral to the lesion. This coincides with hypermetabolism of the ipsilateral substantia nigra that occurs several days after striatal lesion (Hosokawa et al., 1984; Kelly et al., 1982). Circling behavior has been used in a behavioral rating scale to evaluate neurologic status immediately after MCA occlusion (Bederson et al., 1986; Campbell et al., 1997). Successful induction of ischemia is associated with contralateral circling. In the present study, ischemic rats were not able to rely completely on the impaired forelimb but this should have led to ipsilateral turning. The preferential use of ipsilateral whiskers during exploration along the walls of the enclosure might not contribute to turning to the contralateral side (Schallert and Whishaw, 1984). Atipamezole increased turning to the side ipsilateral to the lesion in ischemic rats on day 6, but this was no longer apparent on day 23. As with damage to intrinsic neurons of the striatum (Schallert and Lindner, 1990), MCA occlusion induces significant shrinkage to the ipsilateral substantia nigra pars reticulata in 2 weeks (Tamura et al., 1990). Thus, atipamezole could affect turning via nonspecific stimulation of dopamine release 6 days after MCA occlusion when nigral shrinkage is not completely established.

The performance of ischemic control rats in the limb-placing test recovered over time and almost reached the level of sham-operated rats by the end of the experiment. Atipamezole tended to improve performance of ischemic rats in the limb-placing test, which is consistent with previous results (Jolkkonen et al., 2000; Puurunen et al., 2001). The lack of a significant effect in this study might be explained by

increased variability, which might have masked the atipamezole effects.

In summary, the present study demonstrated that atipamezole treatment combined with enriched-environment housing does not enhance spontaneous use of the impaired forelimb independent of the nonimpaired forelimb following transient MCA occlusion in rats. It is well established that noradrenergic drugs enhance motor learning following cerebral insults, but noradrenergic drugs might work only when recovery requires learning of new motor strategies. Thus, further studies are needed to explore whether atipamezole treatment combined with extensive motor training would be more beneficial in promoting functional recovery.

Acknowledgements

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