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Research report

Methanesulfonyl fluoride, an acetylcholinesterase inhibitor, attenuates simple learning and memory deficits in ischemic rats

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Abstract

Methanesulfonyl fluoride (MSF), a highly selective CNS inhibitor of acetylcholinesterase, has been recently demonstrated to promote improvement in cognitive performance in patients with senile dementia of Alzheimer type. Because a similar cognitive impairment may accompany stroke, we investigated in the present study whether treatment with MSF could produce beneficial effects in adult rats subjected to an experimental stroke model. Sprague–Dawley rats received transient 60 min intraluminal occlusion of the right middle cerebral artery (MCAo) and were given i.p. injections of either MSF (1 mg/kg at 24 and 48 h post-MCAo and 0.3 mg/kg thereafter every other day) or the vehicle, peanut oil, for 4 weeks. Behavioral tests and biochemical assays were performed at 28 days post-surgery. MSF treatment produced about 90% inhibition of acetylcholinesterase in the brain. Ischemic animals that received the vehicle displayed significant elevated body swing biased activity (84.8 ± 10%) and significantly prolonged acquisition (398 ± 62 s) and shortened retention (79 ± 26 s) of the passive avoidance task. Interestingly, while the ischemic animals that received the MSF exhibited elevated body swing biased activity (87.7 ± 8%), they performed significantly better in the passive avoidance task (255 ± 36 s and 145 ± 18 s in acquisition and retention) than the vehicle-treated animals. Moreover, whereas brains from both groups of animals revealed similar extent and degree of cerebral infarction, the MSF-treated ischemic animals showed more intense immunoreactivity, as well as a significantly higher number (10–15% increase) of septal choline acetyltransferase-positive cells than the vehicle-treated ischemic animals. These results show that MSF, possibly by preserving a functional cholinergic system, attenuated stroke-induced deficits in a simple learning and memory task.
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Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Acute stroke; Animal models; Cerebral infarct; Learning and memory; Vascular cognitive impairment; Cholinergic system; Neuroprotection

1. Introduction

The central nervous system (CNS) is highly vulnerable to brain injury. Because CNS neurons are solely dependent on the glucose and oxygen delivered by the blood [14,23,30,42,48], inadequate blood supply to the brain, such as that produced by cerebral ischemia, can easily trigger neuronal death. Stroke is one of the leading causes of death in the world. In the US, an estimated 700,000 suffer from stroke; 30% of these stroke-afflicted patients die, while 20–30% become severely and permanently disabled. Although tissue plasminogen activator (tPA) is an FDA-approved drug for stroke, it has very limited therapeutic window and only benefits 1–3% of ischemic stroke patients [35,41].

The unpredictable nature of the majority of stroke cases entails that treatment cannot be initiated until the injury becomes apparent. Patients that survived acute stroke are left with motor, verbal, cognitive or affective dysfunctions. In recent years, a concerted research effort in the treatment

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of stroke is provided for rehabilitation of the survivors. Indeed, the need for exercise training or a similar physical therapy program for stroke survivors has been shown to aid in recovery of lost motor skills [12,24]. Restoration of cognitive functions in stroke survivors has been less examined, thus we became interested in testing a pharmacological treatment that could protect against cognitive deficits in an animal model of stroke. Moss and colleagues [29] recently reported that methanesulfonyl fluoride (MSF), a very selective CNS acetylcholinesterase (AChE) inhibitor, improves cognitive performance in patients with senile dementia of the Alzheimer type (SDAT). Stroke patients display similar "cognitive performance deficits" termed "vascular cognitive impairments" or VCI [37], which are milder (i.e., no dementia) than those seen in SDAT patients. However, VCI can progress and eventually present as severe cognitive deficits. At this severe stage, the symptoms are now categorized as vascular dementia and mimic those seen in SDAT patients. The present study examined whether treatment with MSF could protect against deficits in a simple learning and memory task produced by an experimental stroke model in adult rats.

2. Materials and methods

2.1. Experimental protocol

A total of 40 eight-week old, male Sprague–Dawley rats weighing about 250 g served as subjects in the present study. All animals were virus antibody free, and a 3-day acclimation period was allowed prior to using the animals. Animals were kept under a 12–12 h light/dark cycle and allowed free access to food and water before and after surgical procedures. All experimental procedures followed UTEP IACUC guidelines for use of animals in research to minimize discomfort of the animals during surgery and during the recovery period. All tests were run blind, and the animal codes were revealed only at the end of the behavioral and histological analyses. In the present experiment, the 0.3 mg/kg dose of MSF, which was previously reported as efficacious in ameliorating cognitive deficits in animals [25,26,29], was used to examine the potential protective effects of MSF in stroke animals. Sixteen animals underwent MCAo surgery, while another sixteen animals underwent sham surgery; these two groups were further subdivided into animals that received either MSF or vehicle (see Fig. 1). An additional eight age-matched normal animals were used for analysis of MSF inhibition of AChE activity in the rat brain.

2.2. Stroke surgery

Rats underwent the MCAo surgery as described elsewhere [6,7,31]. The MCA suture technique involved insertion of a filament through the carotid artery to reach the junction of the MCA, thus blocking the blood flow from the common carotid artery, as well as from the circle of Willis. Under deep anesthesia using chloral hydrate (400 mg/kg, i.p.), the right common carotid artery was identified and isolated through a ventral midline cervical incision. The filament size was 4-0, made of sterile, non-absorbable suture (Ethicon, Inc.), with the diameter of the tip tapered to 24 to 26-gauge size using a rubber cement. About 15 to 17 mm of the filament was inserted from the junction of the external and internal carotid arteries to block the MCA. The right MCA was occluded for 1 h. Based on our studies and several others [4–6], a 1-h MCAo results in maximal infarction. In addition, the length and size of the tip of the filament have been found to produce complete MCAo in 2-month old rats weighing between 250 to 350 g [6–8]. For sham surgery, all of the surgical procedures described above were followed, except that the filament was not inserted into the artery. Monitoring of physiological parameters including arterial blood gases, blood pressure and plasma glucose of animals undergoing such surgical procedure remained

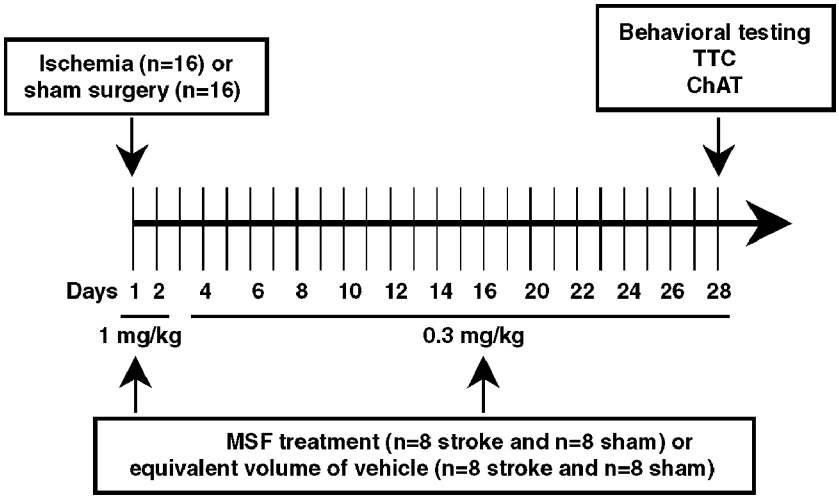

Fig. 1. Experimental protocol. Timeline of surgical procedures, drug treatment and behavioral and biochemical assays.

within normal limits. In addition, body temperature was maintained at 37 °C using a rectal probe-controlled heating pad. Finally, a laser Doppler (Perimed) was used to confirm successful MCAo in all animals.

2.3. Behavioral testing

At 4 weeks after ischemia/sham surgery, the elevated body swing test (EBST) and the passive avoidance test were performed to measure motor asymmetry and simple learning/memory performance, respectively [3,8]. Briefly, the EBST involves handling the animal by its tail and recording the direction of the swings made by the animal. The test apparatus consisted of a clear Plexiglas box (40 × 40 × 35.5 cm). The animal was gently picked up at the base of the tail and elevated by the tail until the animal's nose was at a height of 2 in. (5 cm) above the surface. The direction of the swing, either left or right, was counted once the animal's head moved sideways approximately 10° from the midline position of the body. After a single swing, the animal was placed back in the Plexiglas box and allowed to move freely for 30 s prior to retesting. These steps were repeated 20 times for each animal. We have previously utilized the EBST and noted that ischemic animals displayed >75% biased swing activity as early as 24 h post-ischemia surgery [6–8]. The passive avoidance test followed the procedures described in detail elsewhere [6,7]. Briefly, training and testing were carried out using a step-down passive avoidance box (27 × 27 × 30 cm; Lafayette Inst.) made of Plexiglas. A Plexiglas platform shelf (7.5 × 26.7 × 9.4 cm) was located in one corner of the box. Upon stepping off the platform, the rat received scrambled foot shock (approximately 2 mA; generated by a DC shock scrambler BRS Foringer No. SCS-003). Acquisition of the task was measured in terms of the amount of time it took the rat to remain on the platform continuously for up to 3 min. Twenty-four hours later, a retention test was conducted by placing the rat on the platform exactly as before and recording the latency to step-down measured to a maximum of 3 min. Ischemic animals display significant impairments in acquisition and retention of the task as early as 24 h post-ischemia, which persists for at least 6 months post-ischemia [6–8].

The EBST and passive avoidance test have been routinely utilized in stroke models to justify their use in the present study [18,19,38,43,45,47,50,51]. Although the passive avoidance test was used here and in other studies [43,45,47,50,51] as a simple learning and memory task, other studies have used it as an index of attentional and emotional factors [9,20,36,40].

After the behavioral testing, we randomly assigned animals for TTC staining and ChAT immunohistochemistry to eliminate the bias of assigning animals that were good or bad performers in the behavioral tasks to either histopathological assay. Because of the small sample size, counterbalancing was not possible. In addition, because the same brain region (striatum with the septum) was used for TTC and ChAT immunohistochemistry, and since TTC staining does not allow subsequent ChAT immunohistochemistry, animals had to be randomly assigned to only one assay.

2.4. Cerebral infarction assay

The triphenyltetrazolium chloride (TTC) staining procedures followed those described elsewhere [48]. Randomly selected animals (n = 4 per group) were sacrificed at 4 weeks after ischemia/sham surgery. Under deep anesthesia (chloral hydrate, 500 mg/kg, i.p.), animals were perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0 mm sections. The brain slices were incubated in 2% TTC dissolved in PBS for 30 min at 37 °C and then transferred to 5% formaldehyde solution for fixation. The volume of infarction, as revealed by negative TTC stains indicating dehydrogenase-deficient tissue, was measured in each slice (6 brain slices per animal) and summed using computerized planimetry (PC-based Image Tools software). The volume of infarction = 2 mm (thickness of the slice) × [sum of the infarction area in all brain slices (mm^2)] [48]. To minimize artifacts produced by post-ischemic edema in the infarcted area, the infarct volume was calculated with an alternate technique. In brief, the infarcted area in the ipsilateral hemisphere was indirectly measured by subtracting the non-infarcted area in the ipsilateral hemisphere from the total intact area of the contralateral hemisphere.

2.5. Choline acetyltransferase (ChAT) immunohistochemistry

Following the behavioral test at 4 weeks post-ischemia, randomly selected animals (n = 4 per group) were deeply anesthetized and perfused intracardiacally with 150 ml saline followed by 150 ml of 4% paraformaldehyde. The brains were post-fixed overnight in 4% paraformaldehyde and subsequently immersed in 20% sucrose in 4% paraformaldehyde and stored at 8 °C until sectioning. A vibrotome (Vibroslice, FL) was used to cut the brain (40 um sections). Ten serial brain sections that included the septal region (+1.4 to +0.2 mm from the bregma) were processed for ChAT immunohistochemistry. Sections were pre-incubated in 5% blocking serum (normal goat, Jackson ImmunoResearch Laboratories, PA) in 0.1% Triton-X in 0.1 M NaPBS for 60 min, followed by incubation in the primary ChAT antibody (Boehringer Mannheim Biochemicals, IN; 1:10 dilution in 0.1 M phosphate-buffered saline, NaPBS) at room temperature overnight. The sections were reacted with the appropriate biotinylated secondary antibody (1:2000) for 45 min followed by an avidin–biotin–peroxidase complex system (ABC Elite Vectastain Kit, Vector, Burlingame, CA) for 45 min. Finally, the Vector VIP Kit (Vector, Burlingame, CA) was used to visualize the reaction product. The sections were mounted on gelatin-

coated glass slides, dried overnight, dehydrated in increasing alcohol concentrations, and coverslipped using Permount mounting solution. Serial sections were digitized using a PC-based Image Tools computer program. To determine the ChAT alterations in the septum, comparisons of mean total number (average counts from ten brain sections per animal) of septal ChAT-immunoreactive (ir) neurons from MSF- and vehicle-treated ischemic animals were conducted. Individual counts of ChAT-ir neurons were made using the following criteria: heavily stained nuclei (HN), pale stained nuclei (PN) and presence of elaborate processes (P). Each criterion or combined counts of nuclei and processes were used as raw data for statistical analyses. In addition, a 5-point semi-quantitative scale was used to assess the intensity of ChAT immunoreactivity. These evaluations helped to characterize the morphology of ChAT-ir neurons. Two observers blind to treatment conditions carried out the examination of ChAT immunoreactivity. Examination of soma size and neurite length using ChAT immunohistochemistry has been performed in other studies [10,15,27,32,49].

2.6. Acetylcholinesterase assays

Two additional groups of normal control (non-ischemic) rats were treated with MSF ($n = 4$) or peanut oil vehicle ($n = 4$) in accordance with the exact procedures used with the other groups to estimate the level of AChE inhibition produced. At the end of the experiment, the rats were sacrificed and AChE assays were conducted according to the spectrophotometric method [13] with minor modifications (pH set at 7.4 and using I mM acetyl-B-methylthiocholine iodide as substrate). We initially considered using ischemic brain tissues, but pilot studies revealed that AChE levels in the stroke brains from either vehicle or MSF-treated animals were negligible, likely due to the necrotic tissue within the ipsilateral striatum and cortex. This prompted us to use non-ischemic animals. Moreover, since MSF was initiated immediately after stroke, MSF is likely exerting its neuroprotective effect primarily on normal tissues. Accordingly, the use of non-ischemic animals is justified to reveal MSF inhibition of AChE activity.

2.7. Drugs

For animals that underwent ischemia ($n = 16$) or sham ($n = 16$) surgery, they received i.p. injections of either MSF (1 mg/kg at 24 and 48 h post-MCAo and 0.3 mg/kg thereafter every other day; $n = 8$ ischemia and $n = 8$ sham) or the vehicle peanut oil (the same volume; $n = 8$ ischemia and $n = 8$ sham) for 4 weeks. Each animal received 0.5 ml of the solution. For the AChE inhibition assay, normal, non-ischemia rats received MSF ($n = 4$) or vehicle ($n = 4$) using the same treatment regimen as above.

The purpose of the study was to determine as clearly as possible whether or not significant AChE inhibition would affect the pathological events to cerebral ischemia. Significant inhibition of AChE begins at a minimum of 50% inhibition. The drug treatment strategy was to induce AChE inhibition as strongly as possible within the first 48 h. Accordingly, 1.0 mg/kg MSF was administered, the highest dose that is tolerated without signs of toxicity, according to the dose–response curve [29], this dose produces 51.7% inhibition of the remaining active enzyme with each administration. Therefore, applying the pharmacodynamic calculations for repeated injections with enzyme replacement [44] with a half-time of 12 days in rat brain [28], it was expected that AChE inhibition at 24 h was 51.7% (minimum for a pharmacological effect) and at 48 h it was 75% inhibited. This is a good pharmacological effect induced as rapidly as possible (well tolerated by the animals without toxicity). The continued treatment with 0.3 mg/kg, according to the above dose–response function, produced an additional 30.8% inhibition of remaining active enzyme with each administration. Continuing the pharmacodynamic calculations for repeated injections (every other day) with enzyme replacement for the remaining portion of the experiment (three plus weeks) shows that the predicted level of AChE inhibition would be between 91 and 93% inhibition. This is about the maximum tolerated level of inhibition. The actual AChE assays showed about 90% inhibition, validating the general computational and conceptual strategies used in this experiment to induce the maximum level of AChE inhibition and determine its effects of stroke. An additional point is that traditional dose–response studies are designed for short-acting drugs. Because of the very long half-life of the effect of MSF (an irreversible AChE inhibitor, half-life determined by the rate at which new enzyme is synthesized, 12 days), it would have required enormous numbers of animals. The present experiment was designed to test the maximum AChE inhibition effect that is tolerated without toxic effects.

We considered examining the beneficial effects of MSF long after the treatment has been terminated or incorporating a longer course of MSF treatment. However, because the stroke pathology in the clinic, as well as the one produced by the MCAo model, evolves over a short period of time, with the infarct becoming fixed within a few days (3 days), we believe that the same beneficial effects would be detected at time points after MSF treatment has been terminated or when a longer time course of MSF treatment is conducted. Thus, for the present study, only the 4-week MSF treatment paradigm was used.

2.8. Statistical analyses

Animals were tested twice (24-h interval between test sessions) in each behavioral test and the individual averages were used as raw data. Student t test was used to evaluate statistical differences between MSF- and vehicle-treated groups. Differences were considered significant at $P < 0.025$. Values are expressed as means ± SEM.

3. Results

3.1. MSF inhibits acetylcholinesterase activity

As expected from earlier reports, MSF treatment produced profound inhibition of AChE. Whole brain AChE activity, estimated from assays of half of whole brain, was 90.2% inhibited compared to the peanut oil controls. Specific brain parts, dissected from the other half of the brains, showed similar inhibition: 88.5% in hippocampus; 85.9% in cortex; and 95.0% in striatum/nucleus accumbens. Vehicle-treated animals did not show any detectable inhibition of AChE activity either in the whole brain or in the specific brain parts examined.

3.2. MSF does not reduce stroke-induced motor asymmetry deficit

MSF does not protect against ischemia-induced motor asymmetry. Ischemic animals that received MSF exhibited 87.7 ± 8% biased swing activity, while those that were treated with vehicle displayed 84.8 ± 10% biased swing activity (Fig. 2A). There were no significant differences in the motor asymmetries between the two groups ($P > 0.1$), indicating that MSF did not correct the biased motor behaviors induced by unilateral ischemia. In contrast, sham operated animals that received either MSF or vehicle displayed no observable motor asymmetry. All animals exhibited similar levels of pain sensitivity to the 2 mA shock intensity as revealed by similar thresholds (latency or frequency) for jumping and flinching.

3.3. MSF attenuates stroke-induced passive avoidance impairment

MSF ameliorates ischemia-induced passive avoidance deficits. Ischemic animals that received MSF acquired the task (255 ± 36 s) in a significantly shorter time than ischemic animals that received the vehicle (398 ± 62 s) ($P < 0.025$) (Fig. 2B). In addition, MSF-treated ischemic animals retained the task in a significantly longer time (145 ± 18 s) than vehicle-treated ischemic animals (79 ± 26 s) ($P < 0.025$). Thus, MSF preserved near normal acquisition and retention of the passive avoidance task in ischemic animals. Inc contrast, sham operated animals exhibited normal acquisition and retention of the passive avoidance task, and their performance in this task was not altered by MSF or vehicle treatment.

3.4. MSF does not protect against cerebral infarction

Ischemic animals treated with vehicle had a mean volume of 87.4 ± 9.4 mm^3 of infarcted tissue, while ischemic animals treated with MSF had 78.1 ± 14.2 mm^3 infarction (Fig. 3). These volumes of infarction between the two groups were not significantly different ($P > 0.025$). The

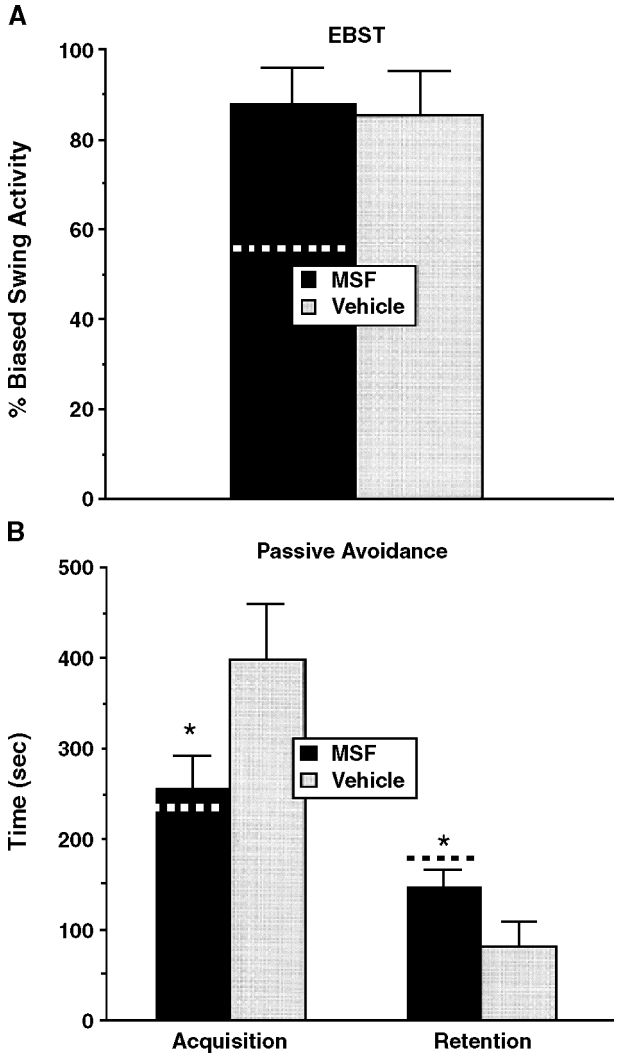

Fig. 2. MSF functional effects. The first set of bars represents the mean total of time to acquire the task, while the second set of bars indicates mean step down latency. Both groups of ischemic animals that received MSF or vehicle displayed significant motor asymmetry as revealed by EBST (A). However, MSF-treated ischemic animals exhibited near normal acquisition and retention of the passive avoidance task compared to vehicle-treated ischemic animals (B). Data are expressed in means ± SEM. Asterisk * indicates significance at $P < 0.025$. Dashed lines correspond to sham control animals; since sham animals treated with vehicle or MSF did not differ between each other, their data were pooled and presented as means.

core of infarction was located around the lateral aspect of the striatum, with portions of the medial striatum and the lateral frontal cortex immediately adjacent to the ischemic core identified as the ischemic penumbra. The core of infarction displayed a small area of tissue loss surrounded by some necrosis. The rest of the TTC-deficient brain sections, mainly consisted of the lateral striatum, revealed widespread cell loss but the tissues remained intact. The TTC data showed that MSF was not effective against necrotic cell death associated with ischemia. Sham operated animals that received MSF or vehicle did not exhibit any detectable brain damage.

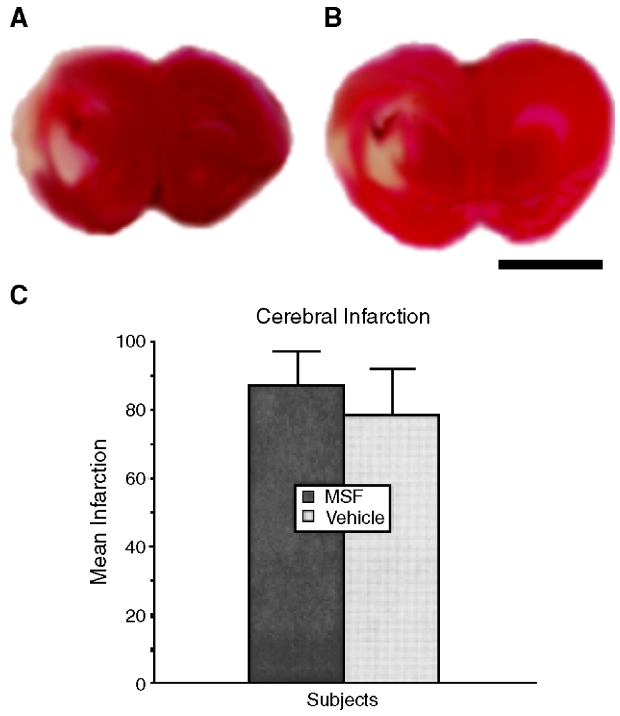

Fig. 3. MSF and cerebral infarction. There was no significant difference in the total volume of cerebral infarction between MSF- and vehicle-treated ischemic animals (shown in panels A and B, respectively) as revealed by triphenyltetrazolium chloride staining. Mean volumes of infarction (C) are expressed in mm^3 ± SEM. Scale bar = 1 cm.

3.5. MSF enhances septal ChAT immunoreactivity

Ischemic animals that were treated with MSF showed more intense staining of ChAT-ir neurons in the lateral and medial septum compared to vehicle-treated ischemic animals. The septal ChAT immunoreactivity of MSF-treated ischemic animals was characterized by heavily stained nuclei that were noted in clusters along the intermedialis area of the lateral septum and the medial septum (Fig. 4). From the heavily stained nuclei, elaborate ChAT-ir fibers of MSF-treated ischemic animals formed denser networks than those seen in vehicle-treated ischemic animals. Furthermore, MSF-treated ischemic animals demonstrated larger somas of ChAT-ir neurons with long processes and extensive dendritic arborizations compared to those of vehicle-treated ischemic animals (Table 1). There were no obvious differences in the ChAT immunoreactivity between the ipsilateral (to the ischemic side) and the contralateral septum when comparing within treatment groups. However, the general septal ChAT immunoreactivity appears to be increased in the MSF-treated ischemic animals as compared to the vehicle-treated ischemic animals. In contrast, MSF- or vehicle-treated sham operated animals did not differ in their septal ChAT immunoreactivity. Moreover, the septal ChAT immunoreactivity in these MSF- or vehicle-treated sham operated animals appears only slightly more intense and dense than MSF-treated ischemic animals, but markedly more intense, elaborate and dense than vehicle-treated ischemic animals.

4. Discussion

The present study demonstrated the efficacy of MSF in ameliorating the passive avoidance deficits in ischemic adult rats. This positive effect was noted despite the lack of protective effects of MSF on the ischemia-induced cerebral infarction. The observed increase in septal ChAT immunoreactivity in MSF-treated ischemic animals suggests that performance in simple learning and memory tasks, such as the passive avoidance, can be preserved by specifically enhancing the activity of this group of cholinergic neurons outside the infarcted brain area.

Previously, chronic MSF treatment has been demonstrated to enhance the acquisition of a one-trial discriminative

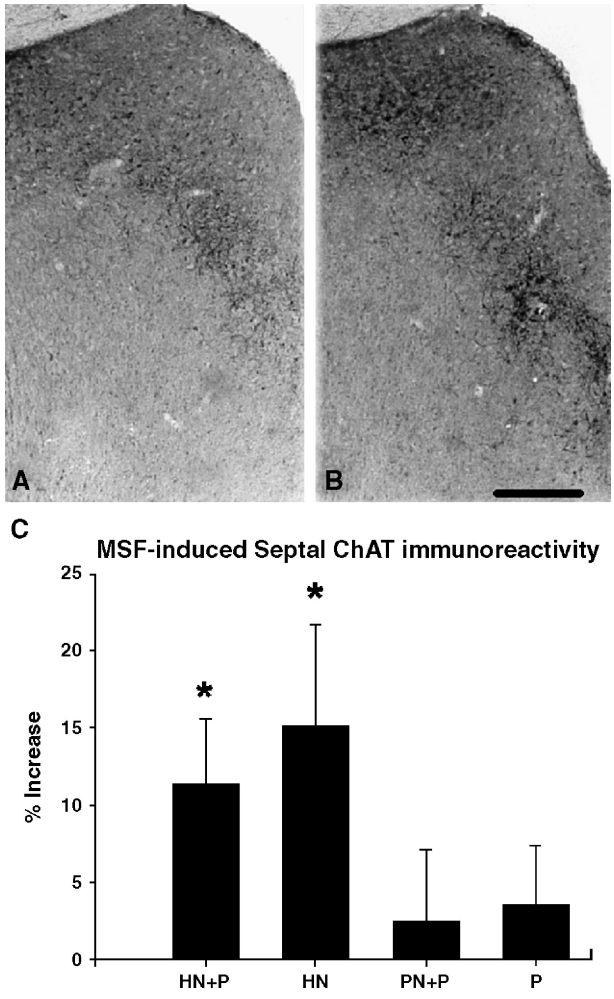

Fig. 4. MSF and septal ChAT immunoreactivity. Compared to vehicle-treated ischemic animals (A), MSF-treated ischemic animals (B) displayed enhanced septal ChAT immunoreactivity characterized by heavily stained nuclei (HN) and elaborate processes (P) forming clusters of dense networks of fibers, which can be found along the medial septum and the lateral septum. Further examination of the morphology of the ChAT-ir cells revealed significant increments (10–15%) in mean total number of HN + P and HN in MSF-treated ischemic animals compared to vehicle-treated ischemic animals (C). Asterisk * indicates significance at $P < 0.025$. PN, pale nuclei. Scale bar = 250 μm.

Table 1
Septal ChAT immunoreactivity

Treatment	Fibers	Nuclei
Stroke + MSF 001	+++	++++
Stroke + MSF 002	+++++	+++++
Stroke + MSF 003	+++++	+++++
Stroke + MSF 004	+++	+++++
Stroke + Vehicle 009	++	+++
Stroke + Vehicle 010	+	++
Stroke + Vehicle 011	+	++
Stroke + Vehicle 012	++	++
Sham + MSF 021	++++	+++++
Sham + MSF 022	+++++	+++++
Sham + MSF 023	+++++	+++++
Sham + MSF 024	+++++	+++++
Sham + Vehicle 029	++++	+++++
Sham + Vehicle 030	++++	+++++
Sham + Vehicle 031	+++++	+++++
Sham + Vehicle 032	+++++	+++++

Septal ChAT immunoreactivity was rated as follows: +, very lightly stained; ++, lightly stained; +++, moderately stained; ++++, heavily stained; +++++, extremely heavily stained. Randomly selected animals (n = 4) from each treatment group were euthanized at 4 weeks post-ischemia for ChAT immunohistochemistry. A 5-point semi-quantitative scale was used to assess the intensity of ChAT immunoreactivity.

reward learning task in mid-adult and aged rats [25,26]. Because MSF is a selective inhibitor of AChE [33], this minimizes toxic side effects seen with non-selective AChE inhibitors. Indeed, chronic MSF treatment even at the low dose of 0.22 mg/kg given three times per week produces a significant decrement (about 50%) in brain AChE activity, but without discernable locomotor side effects and no liver damage [26]. Similarly, brain AChE activity is reduced by 70% after a single systemic injection of 1.5 mg/kg MSF with no observable behavioral alterations [28]. Interestingly, memory dysfunctions have been consistently correlated with abnormal synthesis of acetylcholine in the brains of Alzheimer's disease (AD) patients [1]. Indeed, the cholinergic hypothesis, which states that a serious loss of cholinergic function in the CNS contributes significantly to the cognitive symptoms of AD, has been advanced over the last 20 years [2]. Thus, the robust and selective inhibition of brain AChE activity by MSF implicates the drug's potential use for treating memory deficits associated with abnormal cholinergic system. Recently, a double-blind, placebo-controlled study concluded that MSF produces significant clinical improvements in the cognitive performance of SDAT patients [29]. Such cognition enhancing effects of MSF persisted up to 8 weeks after withdrawal of the drug. Here, we presented data extending the possible utility of MSF for preservation of performance in passive avoidance, a simple learning and memory task, in animals subjected to ischemic stroke.

The dichotomy of CNS control over motor behavior and learning/memory function is exemplified in the present results. Since administration of MSF did not protect against striatal and cortical infarction, both MSF- and vehicle-treated ischemic animals displayed asymmetrical behaviors. Conversely, because MSF enhanced septal ChAT immunoreactivity, MSF-treated ischemic animals exhibited better performance in the passive avoidance task than vehicle-treated ischemic animals. A 27% reduction in ChAT-ir neurons has been shown to coincide with significant performance deficits on water maze and other motor tasks in mice with null mutations in the neurotrophin receptor p75 [34], while treatment with nerve growth factor can protect against decrements in ChAT-ir neurons, as well as memory impairments induced by brain insults [11,52]. The beneficial effects of MSF on passive avoidance imply that near normal performance in simple learning/memory tasks can be preserved even with concomitant cerebral infarction. The use of a battery of behavioral tests would further characterize the functional effects of MSF. Additional studies are warranted to reveal clinical applications of MSF treatment for stroke patients with existing cerebral infarction to recover their learning and memory skills.

The absence of observable protective effects of MSF on cerebral infarction may be due to the limited window of treating stroke with pharmacologic agents. Because CNS neurons begin to degenerate rapidly after the onset of ischemia, the brain tissue (the necrotic ischemic core) deprived of oxygen and glucose cannot be rescued from neuronal degeneration by current methods. Pharmacological treatment, therefore, when delayed (i.e., more than the 3-h therapeutic window for tPA) has been a challenged for achieving good clinical outcome in stroke therapy. Notwithstanding, the ischemic penumbra (the periphery of the injured vascular territory) can be normalized with timely restoration of the blood flow [35]. Accordingly, the ischemic penumbra is a target area for prevention of neuronal degeneration, as well as restoration of function following a stroke episode [17]. The present 24-h post-stroke initial treatment with MSF may be too late to rescue the necrotic ischemic core but may have some effects on the ischemic penumbral neurons. Indeed, pretreatment with another AChE inhibitor, ENA-173, has been shown to preserve the levels of hippocampal acetylcholine and to protect against ischemia-induced loss of pyramidal cells in the hippocampus [39,46]. Alternatively, MSF may act solely on cholinergic neurons, which represent only a subset of many neuronal populations altered following ischemia. Thus, it is interesting to examine the effects of pretreatment with MSF, as well as combining MSF with other drugs that may protect neuronal populations other than the cholinergic neurons to achieve optimal protection against ischemic cell death. Finally, the use of more sensitive assays, such as apoptotic markers for degenerating penumbral neurons, in addition to the routine TTC staining of the necrotic core, may reveal more direct effects of MSF on the ischemic brain region.

The protective effects of MSF on the cholinergic system and the resulting preservation of near normal passive avoidance performance appear to be limited to the presence of a pathologic condition. Compared to ischemic animals,

MSF treatment in sham operated animals produced no obvious increase in septal ChAT immunoreactivity or enhancement of passive avoidance performance. Although we found here and in our previous studies [25,26,29] that MSF resulted in brain AChE activity inhibition in normal animals, MSF neither altered septal ChAT immunoreactivity nor impaired passive avoidance performance in non-ischemic animals. Some studies have reported memory-enhancing effects of AChE inhibitors in normal rodent subjects [21,22], as well as in healthy humans [16]. The absence of memory enhancement in the present intact animals treated with MSF is likely due to MSF being a highly selective AChE inhibitor, compared to other drugs with dual AChE and BChE inhibitory effects, thereby limiting its neuroprotective effects to diseased states, such as stroke. Thus, while passive avoidance is subject to emotional and attentional activity, in addition to its use as a learning and memory task, the neuroprotective effects of MSF seen here support MSF use for stroke therapy rather than a memory or emotional enhancing drug for intact animals. These observations taken together suggest that, while MSF may not overtly enhance learning and memory in normal subjects, the drug is safe even in the absence of a diseased state. In the clinic, preventive medication may benefit at-risk stroke patients.

In summary, we described for the first time that the AChE inhibitor MSF maintained near normal passive avoidance performance in adult rats subjected to experimental stroke. While it did not exert protective effects against cerebral infarction, MSF increased septal ChAT immunoreactivity, which possibly mediated the preservation of memory functions in MSF-treated ischemic animals. We propose further investigations of MSF as an adjunct treatment for facilitating preservation of learning and memory functions in stroke.

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References

[1] G. Aston-Jones, J. Rogers, R.D. Shaver, T.G. Dinan, D.E. Moss, Age-impaired impulse flow from nucleus basalis to cortex, Nature 318 (1985) 462–464.
[2] R.T. Bartus, On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis, Exp. Neurol. 163 (2000) 495–529.
[3] C.V. Borlongan, P.R. Sanberg, Elevated body swing test: a new behavioral parameter for rats with 6-hydroxydopamine-induced hemiparkinsonism, J. Neurosci. 15 (1995) 5372–5378.
[4] C.V. Borlongan, D.W. Cahill, P.R. Sanberg, Locomotor and passive avoidance deficits following occlusion of the middle cerebral artery, Physiol. Behav. 58 (1995) 909–917.
[5] C.V. Borlongan, R. Martinez, R.D. Shytle, T.B. Freeman, D.W. Cahill, P.R. Sanberg, Striatal dopamine-mediated motor behavior is altered following occlusion of the middle cerebral artery, Pharmacol. Biochem. Behav. 52 (1995) 225–229.
[6] C.V. Borlongan, H. Hida, H. Nishino, Early assessment of motor dysfunctions aids in successful occlusion of the middle cerebral artery, NeuroReport 9 (1998) 3615–3621.
[7] C.V. Borlongan, Y. Tajima, J.Q. Trojanowski, V.M. Lee, P.R. Sanberg, Cerebral ischemia and CNS transplantation: differential effects of grafted fetal rat striatal cells and human neurons derived from a clonal cell line, NeuroReport 9 (1998) 3703–3709.
[8] C.V. Borlongan, Y. Tajima, J.Q. Trojanowski, V.M. Lee, P.R. Sanberg, Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats, Exp. Neurol. 149 (1998) 310–321.
[9] C. Brandner, F. Schenk, Septal lesions impair the acquisition of a cued place navigation task: attentional or memory deficit? Neurobiol. Learn. Mem. 69 (1998) 106–125.
[10] M.L. Derby, R. Giuliano, D.A. Figlewicz, M.C. Bohn, GDNF is trophic for mouse motoneurons that express a mutant superoxide dismutase (SOD-1) gene, Amyotrophic Lateral Scler. Other Mot. Neuron Disord. 1 (2000) 113–122.
[11] C.E. Dixon, P. Flinn, J. Bao, R. Venya, R.L. Hayes, Nerve growth factor attenuates cholinergic deficits following traumatic brain injury in rats, Exp. Neurol. 146 (1997) 479–490.
[12] B.H. Dobkin, Activity-dependent learning contributes to motor recovery, Ann. Neurol. 44 (1998) 158–160.
[13] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[14] M. Endres, S. Namura, M. Shimizu-Sasamata, C. Waeber, L. Zhang, T. Gomez-Isla, B.T. Hyman, M.A. Moskowitz, Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family, J. Cereb. Blood Flow Metab. 18 (1998) 238–247.
[15] M. Ferrini, V. Bisagno, G. Piroli, C. Grillo, M.C. Deniselle, A.F. De Nicola, Effects of estrogens on choline-acetyltransferase immunoreactivity and GAP-43 mRNA in the forebrain of young and aging male rats, Cell. Mol. Neurobiol. 22 (2002) 289–301.
[16] M.L. Furey, P. Pietrini, G.E. Alexander, M.B. Schapiro, B. Horwitz, Cholinergic enhancement improves performance on working memory by modulating the functional activity in distinct brain regions: a positron emission tomography regional cerebral blood flow study in healthy humans, Brain Res. Bull. 51 (2000) 213–218.
[17] C. Iadecola, M.E. Ross, Molecular pathology of cerebral ischemia: delayed gene expression and strategies for neuroprotection, Ann. N. Y. Acad. Sci. 835 (1997) 203–217.
[18] S. Ishibashi, T. Kuroiwa, S. Endo, R. Okeda, H. Mizusawa, A comparison of long-term neurological symptoms after two different focal ischemic models in Mongolian gerbils, Acta Neurochir., Suppl. 86 (2003) 159–162.
[19] S. Ishibashi, T. Kuroiwa, S. Endo, R. Okeda, H. Mizusawa, Neurological dysfunctions versus regional infarction volume after focal ischemia in Mongolian gerbils, Stroke 34 (2003) 1501–1506.
[20] H. Jeltsch, F. Bertrand, R. Galani, C. Lazarus, S. Schimchowitsch, J.C. Cassel, Intraseptal injection of the 5-HT1A/5-HT7 agonist 8-OH-DPAT and working memory in rats, Psychopharmacology (Berlin) 175 (2004) 37–46.
[21] L. Lamirault, C. Guillou, C. Thal, H. Simon, (-)-9-Dehydrogalanthaminium bromide, a new cholinesterase inhibitor, enhances place and object recognition memory in young and old rats, Neurobiol. Learn. Mem. 80 (2003) 113–122.
[22] L. Lamirault, C. Guillou, C. Thal, H. Simon, Combined treatment with galanthaminium bromide, a new cholinesterase inhibitor, and RS 67333, a partial agonist of 5-HT4 receptors, enhances place and object

recognition in young adult and old rats, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 27 (2003) 185–195.
[23] J.M. Lee, G.J. Zipfel, D.W. Choi, The changing landscape of ischaemic brain injury mechanisms, Nature 399 (1999) A7–A14.
[24] R.F. Macko, C.A. DeSouza, L.D. Tretter, K.H. Silver, G.V. Smith, P.A. Anderson, N. Tomoyasu, P. Gorman, D.R. Dengel, Treadmill aerobic exercise training reduces the energy expenditure and cardiovascular demands of hemiparetic gait in chronic stroke patients. A preliminary report, Stroke 28 (1997) 326–330.
[25] D.H. Malin, P.J. Toups, L.D. Osgood, D.E. Fowler, C.L. Hunter, K.R. Arcangeli, D.E. Moss, Methanesulfonyl fluoride enhances one-trial reward learning in mid-aged rats, Neurobiol. Aging 12 (1991) 181–183.
[26] D.H. Malin, R.E. Plotner, S.J. Radulescu, R.N. Ferebee, J.R. Lake, P.G. Negrete, P.J. Schaefer, M.K. Crothers, D.E. Moss, Chronic methanesulfonyl fluoride enhances one-trial per day reward learning in aged rats, Neurobiol. Aging 14 (1993) 393–395.
[27] M. Molnar, E. Tongiorgi, E. Avignone, S. Gonfloni, F. Ruberti, L. Domenici, A. Cattaneo, The effects of anti-nerve growth factor monoclonal antibodies on developing basal forebrain neurons are transient and reversible, Eur. J. Neurosci. 10 (1998) 3127–3140.
[28] D.E. Moss, L.A. Rodriguez, S.B. McMaster, Comparative behavioral effects of CNS cholinesterase inhibitors, Pharmacol. Biochem. Behav. 22 (1985) 479–482.
[29] D.E. Moss, P. Berlanga, M.M. Hagan, H. Sandoval, C. Ishida, Methanesulfonyl fluoride (MSF): a double-blind, placebo-controlled study of safety and efficacy in the treatment of senile dementia of the Alzheimer type, Alzheimer Dis. Assoc. Disord. 13 (1999) 20–25.
[30] H. Nishino, K. Koide, N. Aihara, M. Kumazaki, T. Sakurai, H. Nagai, Striatal grafts in the ischemic striatum improve pallidal GABA release and passive avoidance, Brain Res. Bull. 32 (1993) 517–520.
[31] H. Nishino, M. Kumazaki, T. Sakurai, P.R. Sanberg, C.V. Borlongan, Melatonin protects against deficits associated with occlusion of the middle cerebral artery in adult rats, Soc. Neurosci. 24 (1998) 213.
[32] J.S. O'Brien, G.S. Carson, H.C. Seo, M. Hiraiwa, Y. Kishimoto, Identification of prosaposin as a neurotrophic factor, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 9593–9596.
[33] G. Pacheco, R. Palacios-Esquivel, D.E. Moss, Cholinesterase inhibitors proposed for treating dementia in Alzheimer's disease: selectivity toward human brain acetylcholinesterase compared with butyrylcholinesterase, J. Pharmacol. Exp. Ther. 274 (1995) 767–770.
[34] D.A. Peterson, H.A. Dickinson-Anson, J.T. Leppert, K.F. Lee, F.H. Gage, Central neuronal loss and behavioral impairment in mice lacking neurotrophin receptor p75, J. Comp. Neurol. 404 (1999) 1–20.
[35] W.A. Pulsinelli, M. Jacewicz, D.E. Levy, C.K. Petito, F. Plum, Ischemic brain injury and the therapeutic window, Ann. N. Y. Acad. Sci. 835 (1997) 187–192.
[36] L. Ricceri, G. Calamandrei, J. Berger-Sweeney, Different effects of postnatal day 1 versus 7 192 immunoglobulin G-saporin lesions on learning, exploratory behaviors, and neurochemistry in juvenile rats, Behav. Neurosci. 111 (1997) 1292–1302.
[37] G.C. Roman, P. Sachdev, D.R. Royall, R.A. Bullock, J.M. Orgogozo, S. Lopez-Pousa, R. Arizaga, A. Wallin, Vascular cognitive disorder: a new diagnostic category updating vascular cognitive impairment and vascular dementia, J. Neurol. Sci. 226 (2004) 81–87.
[38] R.L. Roof, G.P. Schielke, X. Ren, E.D. Hall, A comparison of long-term functional outcome after 2 middle cerebral artery occlusion models in rats, Stroke 32 (2001) 2648–2657.
[39] S. Sadoshima, S. Ibayashi, K. Fujii, T. Nagao, H. Sugimori, M. Fujishima, Inhibition of acetylcholinesterase modulates the autoregulation of cerebral blood flow and attenuates ischemic brain metabolism in hypertensive rats, J. Cereb. Blood Flow Metab. 15 (1995) 845–851.
[40] M. Sarter, J.P. Bruno, B. Givens, Attentional functions of cortical cholinergic inputs: what does it mean for learning and memory? Neurobiol. Learn. Mem. 80 (2003) 245–256.
[41] P.D. Schellinger, E. Orberk, W. Hacke, Antithrombotic therapy after cerebral ischemia, Fortschr. Neurol.Psychiatr. 65 (1997) 425–434.
[42] B.K. Siesjo, C. Carlsson, M. Hagerdal, C.H. Nordstrom, Brain metabolism in the critically ill, Crit. Care Med. 4 (1976) 283–294.
[43] N. Sugo, P.D. Hurn, M.B. Morahan, K. Hattori, R.J. Traystman, A.C. DeVries, Social stress exacerbates focal cerebral ischemia in mice, Stroke 33 (2002) 1660–1664.
[44] R.J. Tallarida, R.B. Murray, Manual of pharmacologic calculations with computer programs, Springer-Verlag, New York, 1986.
[45] M. Tamura, Y. Aoki, T. Seto, Y. Itoh, Y. Ukai, Cerebroprotective action of a Na+/Ca2+ channel blocker NS-7. II. Effect on the cerebral infarction, behavioral and cognitive impairments at the chronic stage of permanent middle cerebral artery occlusion in rats, Brain Res. 890 (2001) 170–176.
[46] K. Tanaka, N. Ogawa, K. Mizukawa, M. Asanuma, Y. Kondo, S. Nishibayashi, A. Mori, Acetylcholinesterase inhibitor ENA-713 protects against ischemia-induced decrease in pre- and postsynaptic cholinergic indices in the gerbil brain following transient ischemia, Neurochem. Res. 19 (1994) 117–122.
[47] M. Vendrame, J. Cassady, J. Newcomb, T. Butler, K.R. Pennypacker, T. Zigova, C.D. Sanberg, P.R. Sanberg, A.E. Willing, Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume, Stroke 35 (2004) 2390–2395.
[48] Y. Wang, S.Z. Lin, A.L. Chiou, L.R. Williams, B.J. Hoffer, Glial cell line-derived neurotrophic factor protects against ischemia-induced injury in the cerebral cortex, J. Neurosci. 17 (1997) 4341–4348.
[49] N.L. Ward, T. Hagg, BDNF is needed for postnatal maturation of basal forebrain and neostriatum cholinergic neurons in vivo, Exp. Neurol. 162 (2000) 297–310.
[50] A.E. Willing, L. Jiang, P. Nowicki, S. Poulos, M. Milliken, D.W. Cahill, P.R. Sanberg, Effects of middle cerebral artery occlusion on spontaneous activity and cognitive function in rats, Int. J. Neurosci. 112 (2002) 503–516.
[51] A.E. Willing, J. Lixian, M. Milliken, S. Poulos, T. Zigova, S. Song, C. Hart, J. Sanchez-Ramos, P.R. Sanberg, Intravenous versus intrastriatal cord blood administration in a rodent model of stroke, J. Neurosci. Res. 73 (2003) 296–307.
[52] G. Wortwein, J. Yu, T. Toliver-Kinsky, J.R. Perez-Polo, Responses of young and aged rat CNS to partial cholinergic immunolesions and NGF treatment, J. Neurosci. Res. 52 (1998) 322–333.