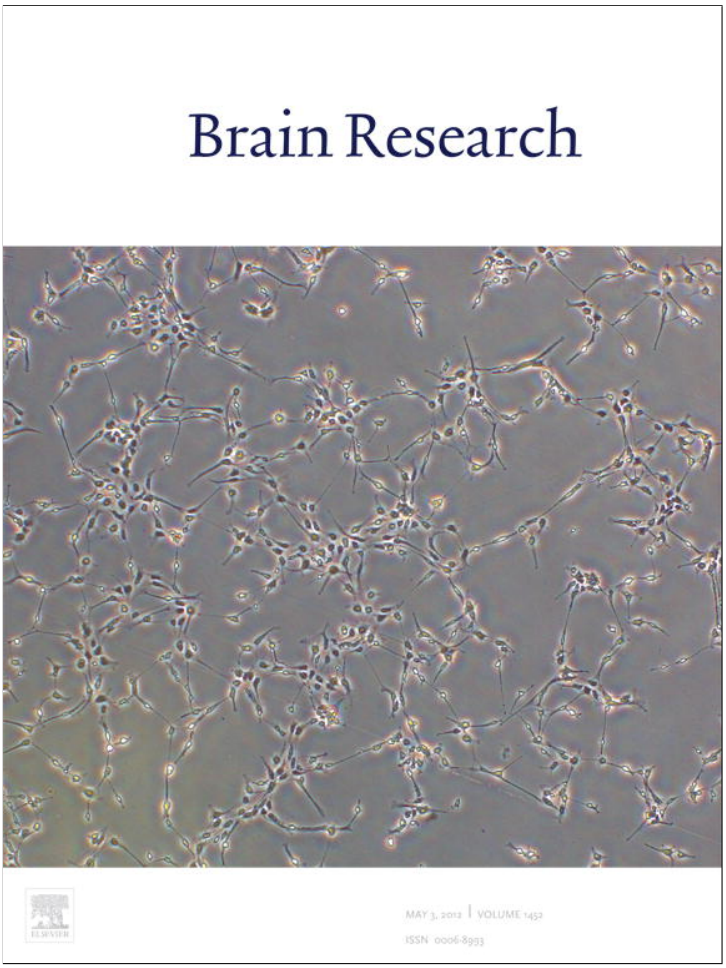

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Research Report

Netrin-1 improves spatial memory and synaptic plasticity impairment following global ischemia in the rat

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ABSTRACT

Cerebral ischemia, which is the second and most common cause of mortality, affects millions of individuals worldwide. The present study was performed to investigate whether intrahippocampal administration of netrin-1 could improve spatial memory impairment in radial arm maze task and restore long-term potentiation (LTP) in 4-vessel occlusion model of global ischemia. The results showed that intrahippocampal infusion of nerin-1 24 h after ischemia (at both doses of 400 and 800 ng) significantly ameliorated spatial memory impairment and at a dose of 800 ng was capable to improve synaptic dysfunction as observed by recovery of population spike component of basal evoked potential and LTP through enhancement of excitability and normalization of paired pulse response. Taken together, the present study shows that netrin-1 dose-dependently ameliorates spatial memory impairment and improves synaptic dysfunction as observed by recovery of population spike component of basal evoked potential and LTP in rats with global ischemia.

1. Introduction

Focal and global cerebral ischemia, which is the second and most common cause of mortality and with long-term debilitating complications, affects millions of individuals worldwide (Lopez et al., 2006; Paul et al., 2007). Stroke remains a significant clinical unmet condition, with only 3% of the ischemic patients benefiting from current treatment strategies, such as the use of thrombolytic agents. The latter drugs are often themselves limited by a narrow therapeutic time window and their possible ensuing risk of hemorrhage (Heuschmann et al., 2004; Kwiatkowski et al., 1999). Therefore, understanding the mechanisms responsible for brain injury due to global ischemia and finding new neuroprotective/neurorestorative strategies is critical and will be useful in clinical settings. Global ischemia (following cardiac arrest) can lead to serious cognitive deficits in humans (Peskine et al., 2010). Experimental models of global ischemia are accompanied with severe loss of neurons in the CA1 area of the hippocampus, while the adjacent dentate gyrus (DG) neurons are spared (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984). Although DG neurons do not show signs of delayed cell death following ischemia, but this does not rule out possible functional impairment of synaptic plasticity in DG (Wang et al., 2005).

Netrins belong to an evolutionary-conserved family of laminin-related proteins (Harris et al., 1996). During development of nervous system, members of netrin family act as

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cues that guide migrating cells and axons to their targets (Cook et al., 1998; Tessier-Lavigne and Goodman, 1996). These diffusible proteins are required for proper development of the nervous system due to their ability to stimulate outgrowth and to reorient growth of commissural axons. These proteins are expressed with a diffusible gradient in some regions of the nervous system and in this way could attract commissural axons (Kennedy et al., 1994). In mammals, the netrin family proteins are composed of four members, i.e. netrin-1, netrin-2, netrin-3 and β netrin (Kennedy et al., 1994; Koch et al., 2000). Several receptors for netrin-1 have been discovered and characterized including DCC (deleted in colorectal cancer), Neogenin (Cho and Fearon, 1995; Keino-Masu et al., 1996), a receptor family related to UNC-5 (uncoordinated gene 5) (Ackerman et al., 1997; Leonardo et al., 1997) and the adenosine A2b receptor family (Corset et al., 2000). Netrin-1 is a bifunctional guidance cue, attracting some axons while repelling others during neuronal development. The response to netrin-1 is regulated by the expression of netrin receptors and their regulated presentation on the cell surface. While repulsive effect of netrin-1 has been attributed to its UNC-5 receptors, it also functions as an axonal attractant through binding to DCC family of receptors which comprise DCC and Neogenin (Chisholm and Tessier-Lavigne, 1999), (Ackerman et al., 1997). In adult mammals, netrin-1 also exhibits anti-inflammatory effect by inhibition of leukocyte migration (Ly et al., 2005) and it is considered as an important factor for angiogenesis (Li et al., 2011; Nguyen and Cai, 2006; Tian et al., 2011). In addition, netrin-1 has been introduced as a survival factor (Furne et al., 2006; Llambi et al., 2005; Wang et al., 2008a), acting through DCC and UNC5H receptors which themselves belong to the dependence receptor family. These types of receptors could stimulate apoptosis in the absence of ligand. Thus, DCC or UNC5H expression leads to apoptosis unless netrin-1 is present (Llambi et al., 2001; Mehlen et al., 1998). Protective effect of netrin-1 against ischemia–reperfusion injury of the kidney (Brian Reeves et al., 2008; Wang et al., 2008b) and myocardial infarction (Zhang and Cai, 2010) and its anti-apoptotic property (Wu et al., 2008) have already been established. Of interest, this protein is strategically situated to positively influence axon regeneration in adult peripheral nervous system (Lee et al., 2007; Madison et al., 2000). Since widespread expression of netrin-1 occurs by neurons and oligodendrocytes in the adult mammalian spinal cord (Manitt et al., 2001) and its expression increases following cerebellar and spinal cord injury (Low et al., 2008; Wehrle et al., 2005) and brain ischemia (Tsuchiya et al., 2007), these facts strongly indicate that netrin-1 may have beneficial effect under normal and pathological conditions. Therefore, this study was undertaken to investigate, for the first time, whether netrin-1 could improve spatial memory and synaptic plasticity impairment in an experimental model of global ischemia in the rat.

2. Results

2.1. General consideration

Five, four and three rats were excluded from vehicle-treated ischemic and netrin400- and netrin-800-treated ischemic groups due to respiratory distress and severe convulsions, respectively. Also, one rat from vehicle-treated ischemic group did not show any sign of ischemia and one rat died within 24 h.

2.2. The effect of netrin-1 on ischemia-induced impairment of spatial memory

As shown in Fig. 1, rats subjected to global ischemia (vehicle-treated group) exhibited a significantly higher number of working errors ($p<0.001$), reference errors ($p<0.0001$) and also an increased latency in RAM task ($p<0.0001$). Post-ischemia treatment with netrin-1 at a dose of 800 ng significantly decreased the spatial memory impairment induced by ischemia in 8-arm radial maze test. In contrast, although post-ischemia treatment

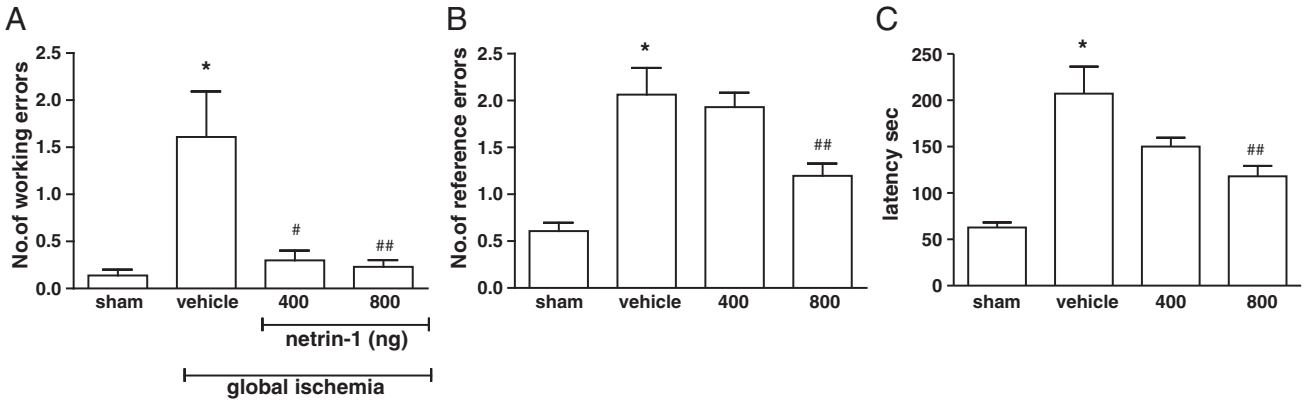

Fig. 1 – The effect of post-ischemic treatment with netrin-1 on cerebral ischemia-induced impairment of spatial memory in RAM task. Animals were administered two doses of netrin-1 (400 and 800 ng) 24 h after ischemia. A) Number of working errors in experimental groups (n=10–12/group). $^{*}p<0.001$ versus the sham group, $^{\#}p<0.01$, $^{\#\#}p<0.001$ versus the vehicle group, B) number of reference errors in experimental groups (n=10–12/group). $^{*}p<0.0001$ versus the sham group, $^{\#\#}p<0.001$ vs. the vehicle group, C) Mean value of the latency (n=10–12/group). $^{*}p<0.0001$ versus the sham group, $^{\#\#}p<0.001$ versus the vehicle group (two-way repeated measure ANOVA with Bonferroni multiple comparisons).

with netrin-1 (400 ng) did not significantly decrease the number of reference errors and latency, but it significantly decreased number of working errors ($p<0.01$).

2.3. Electrophysiological experiments

In the recordings from DG area of the hippocampus, slope of excitatory post-synaptic potentials (EPSP) and amplitude of population spike (PS) obtained from input/output curve, 30 minute basic recordings, paired-pulse10–20–30–50 ms and 60 min long-term potentiation (LTP) were analyzed in sham-operated, vehicle (ischemic), and netrin-1 (400–800 ng)-treated ischemic animals. Stimulant pulses were delivered at 0.1 Hz, and a total of 10 responses at each current level were averaged. As illustrated in Fig. 2 for I/O curves, there was a significant difference in PS amplitude at stimulus intensities from 100 to 1000 μA between the sham group (from 11.19±5 to 432.2±40) and the vehicle group (from 1.5±0.9 to 181.5±39) in all currents ($t=2.9–4.5$, $p=0.0019$) and between the vehicle and ischemia+netrin800 groups (from 23.3±6.4 to 425±78.5) ($t=2.5–4.9$, $p=0.0016$). There was also a significant difference in EPSP slope at stimulus intensities from 100 to 1000 μA between the sham group (from 46.67±16.90 to 268.2±37.58) and the vehicle group (from 8.5±1.1 to 114.9±21) in all currents ($t=2.7–4.7$) and there was no significant differences between the vehicle and ischemia+netrin800 groups (from 23.80±8 to 203.8±85) ($t=0.02–1.6$). In addition, there was also no significant difference between the vehicle and ischemia+netrin400 groups regarding PS amplitude and EPSP slope at all currents. In netrin800-treated ischemic animals, there was a significant increase in PS amplitude with no significant change of EPSP slope that indicates an increase in neuronal excitability. The PS/EPSP to intensity ratio is a marker of neuronal excitability. According to Fig. 3, there was no significant difference in neuronal excitability in baseline recording (40% max PS) between sham, vehicle and netrin400 groups. After tetanic stimulation, although this ratio remained constant in these groups, but this ratio significantly increased in ischemic animals that received netrin-1 at a dose of 800 ng in comparison with other groups ($p<0.0001$). There were also no significant differences between the groups regarding normalized EPSP slope and normalized PS amplitude in 30 minute basic recordings.

Although high frequency stimulation (HFS; 200 HZ) of medial perforant path induced a long-lasting synaptic potentiation of sham-operated animals up to 60 min after tetanus (Fig. 4), but in ischemic animals, HFS did not induce a synaptic potentiation of EPSP and PS ($p<0.0001$).

In netrin800-treated ischemic animals, although tetanic stimulation induced a significant increase of PS amplitude ($p<0.001$), but it did not induce synaptic potentiation of EPSP slope. Meanwhile, in netrin400-treated ischemic animals, HFS did not induce a synaptic potentiation of EPSP and PS.

2.4. The effect of netrin-1 on paired-pulse plasticity in the dentate gyrus

When the perforant path is stimulated by two closely paired stimuli, a series of temporal changes occur in granule cell excitation to the second pulse. Usually, granule cell response to the second stimulus is more than that to the first evoked response at interpulse intervals (IPI) 40–200 ms because of facilitation of presynaptic Ca^{2+} influx that leads to paired-pulse facilitation (PPF) (Dobrunz and Stevens, 1997). When the second stimulus is less than that to the first evoked response at interpulse intervals 10–20 ms, paired-pulse depression (PPD) occurs due to recurrent inhibition (Brucato et al., 1992; Tuff et al., 1983). In our study, induction of global ischemia (10 min) significantly increased PSA evoked by the second pulse at IPIs 10–20 ms and induced PPF (sham versus vehicle in pp10, $p=0.0007$, sham versus vehicle in pp20, $p=0.0008$). Under netrin-1 (at doses of 400 and 800 ng) post-ischemic treatment, the rate of the paired-pulse response at IPIs 10–20 significantly reduced. There was also no significant difference in PSA evoked by the second pulse at IPIs 30–50 ms (sham versus vehicle pp30, $p=0.6$, pp50, $p=0.3$) (Fig. 5).

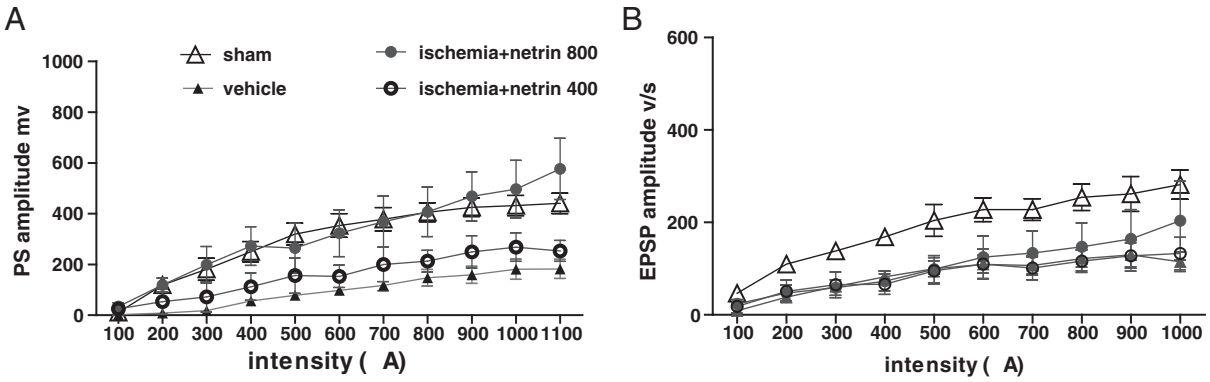

Fig. 2 – The effect of netrin-1 on I/O curves in the DG region of the hippocampus on post-ischemic day 3. A) In I/O curves, there was a significant difference in PS amplitude between the sham and vehicle groups and between the netrin800 and vehicle groups at each stimulus intensity from 100 to 1000 μA, B) In the I/O curves, there was significant difference in EPSP slope between the sham and vehicle groups and there was no significant difference between the netrin800 and vehicle groups. Each point represents data obtained from 6 rats.

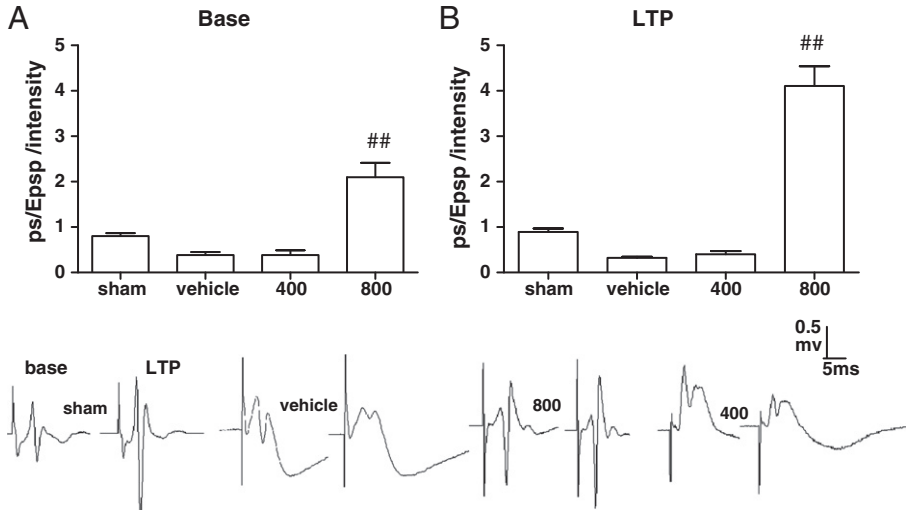

Fig. 3 – The effect of netrin-1 on neuronal excitability in the DG region of the hippocampus on post-ischemic day 3. A) PS/EPSP to intensity ratio in baseline recording. Although there was no significant difference in neuronal excitability between the sham and vehicle groups, however, there was a significant difference between the netrin800 and vehicle groups ($p<0.001$), B) PS/EPSP to intensity ratio in LTP recording. There was no significant difference in neuronal excitability between the sham and vehicle groups and there was a significant difference between the netrin800 and vehicle groups ($p<0.001$). In netrin800-ischemic group, after tetanic stimulation, neuronal excitability increased as compared to baseline recording, but in the other groups, there was no significant difference in neuronal excitability level between baseline and LTP recordings. Each point represents data obtained from 6 rats.

3. Discussion

The major findings of the present study showed that the intrahippocampal injection of netrin-1 dose-dependently and significantly ameliorated memory impairment and improved synaptic dysfunction as observed by recovery of population spike component of basal evoked potential and LTP in rats with global ischemia.

In this study, we induced a mild transient ischemia using a 4-vessel occlusion method (4VO) for 10 min to avoid severe injury to the hippocampus. This method has been reported to cause relatively mild cell death in the CA1 pyramidal neurons but not in the DG neurons, which serves as a gateway to the hippocampus and plays a key role in learning and memory and is also quite resistant (Aoyagi et al., 1998; Katsuta et al., 2003; Kirino, 1982; Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982; Smith et al., 1984). In other words, CA1 pyramidal cells are particularly vulnerable against transient forebrain ischemia (Kirino, 1982; Pulsinelli et al., 1982).

The hippocampus plays an important role in learning and memory. Short- and long-term synaptic plasticity in the hippocampus is postulated to be a neural substrate of memory trace. In this regard, LTP is considered as a major synaptic mechanism for evaluation of long-term synaptic plasticity and paired-pulse stimulation is a standard technique for evaluating short-term synaptic plasticity in rodents. Post-tetanic LTP has been considered to be a physiological form of synaptic plasticity and its occurrence either in cortical or in subcortical areas has been regarded as a cellular substrate for learning and memory (Bliss and Collingridge, 1993; Nicoll and Malenka, 1995). After the 10-min ischemia, granule cells in the dentate gyrus are relatively resistant to cell death but suppression of LTP in surviving DG neurons has been reported and this can be suitable for studying functional changes of surviving neurons following transient forebrain ischemia. According to previous studies, after the 10 min transient forebrain ischemia, marked memory impairment finally develops (Hartman et al., 2005; Langdon et al., 2008; Sandstrom and Rowan, 2007; Schwartz et al., 1998; Squire and Zola, 1996). Our behavioral data are consistent with previous reports indicating that after brief transient forebrain ischemia, two parameters of spatial memory (i.e. reference memory and working memory) are impaired (Langdon et al., 2008) and this has been associated with changes in neuronal plasticity and LTP in survived DG neurons (Aoyagi et al., 1998).

Our study also showed that 3 days after ischemia, basal fEPSP and PS amplitude significantly reduces and the I/O curve shifted to the right, indicating that basal synaptic transmission decreases and after tetanic stimulation, LTP was significantly impaired. This reduction of basal synaptic transmission and synaptic plasticity may be caused by impairment of synaptic functions, as reported by other researchers (Aoyagi et al., 1998). Paired-pulse tests with high intensity stimuli eliciting clear PSs have been used to evaluate the recurrent (and feed forward) inhibition in the dentate gyrus of rodents. When local inhibitory interneurons in the dentate gyrus receive excitatory inputs from the granule cells, they elicit impulses and these cells then give rise to a recurrent inhibition of the granule cells. For this reason, the response induced by the second pulse to the perforant path is depressed due to this recurrent inhibition in a manner that is dependent on interpulse intervals (IPIs). Complete blockage of 2nd PS usually appears at short IPIs (10–20 ms).

In our obtained results, a shift from paired pulse depression toward paired pulse facilitation (increased paired pulse

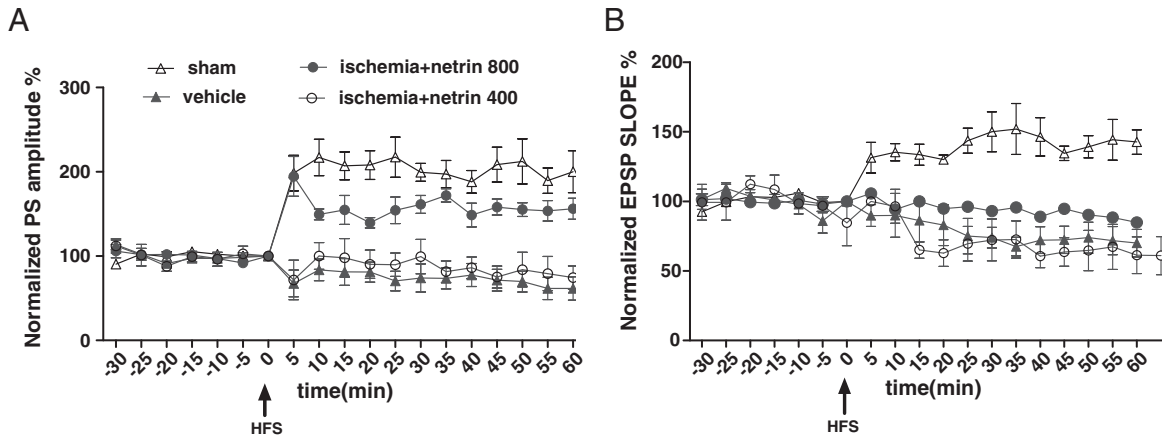

Fig. 4 – The effect of netrin-1 on LTP. The comparison of normalized PS amplitude baseline recording and LTP in sham, vehicle and treatment groups showed that LTP was blocked in PS amplitude (A) and EPSP slope (B) in ischemic animals and PS amplitude recovered in netrin800-treated ischemic group, but did not recover in EPSP slope. Each point represents data obtained from 6 rats. Specimen recordings showed changes in baseline recording and LTP recording in 60 min after HFS. Each recording is the average of 10 consecutive recordings at 100 s with an interval of 10 s.

ratio) occurred in ischemic animals. These findings suggest that recurrent inhibition in the dentate gyrus was possibly blocked due to functional alterations in GABAergic presynaptic terminals on DG neurons. The effect of ischemia on GABA metabolism is variable, depending on the region of the brain being studied. There is a rapid loss of GABAergic neurons in the cerebral cortex and striatum after transient global ischemia (Francis and Pulsinelli, 1982; Sloper et al., 1980). However, GABAergic neurons in the hippocampus appear to be more resistant to the ischemia (Francis and Pulsinelli, 1982). Nuclear magnetic resonance studies have shown that hippocampal GABA levels increase during and immediately after transient forebrain ischemia but decline significantly at 1 h after reperfusion (Peeling et al., 1989). An increase in GABAergic activity during and after ischemia results in a reduction of hippocampal neuronal loss (Johansen and Diemer, 1991).

In this study, we used two doses of netrin-1 (a total of 400 and 800 ng/rat) and after 48 h, retention test of 8-arm radial maze and field potential recording was done. Intrahippocampal infusion of 400 ng netrin-1 had no obvious effect on the field potential recordings (LTP recovery and basal synaptic transmission) and performance of animals in 8-arm radial maze in ischemic animals. In contrast, working and reference errors were significantly reduced and in field potential recording, the population spike recovered in LTP and basal synaptic transmission in ischemic animals that received 800 ng of netrin-1. For the first time, we showed that intrahippocampal infusion of netrin1 (800 ng) 24 h after ischemia can rescue the surviving neurons to a large extent from synaptic dysfunction and impaired LTP and can restore the memory capacity and this could be a good result in functional recovery after stroke.

It has been reported that netrin-1 is expressed in the peri-ischemic area with a peak at 14 days after focal ischemia. Since the receptor for netrin-1 is upregulated after ischemia in the same brain structures, a neuroprotective role for endogenous netrin-1 has been suggested (Tsuchiya et al., 2007). It has also been demonstrated that administration of netrin-1 could reduce the infarct volume and preserve the cortex in focal ischemia (Wu et al., 2008). The protective effect of netrin-1 is partly mediated by its anti-apoptosis activity, which might result from a reduced p53 level (Wu et al., 2008). It is also possible that netrin1 in some ways could act like growth factors including brain-derived neurotrophic factor (BDNF) (Manitt et al., 2009). It has been shown that LTP is lost in mutant mice lacking BDNF production and could be recovered after administration of exogenous BDNF (Patterson et al., 1996). In addition, BDNF could improve LTP and cognitive functions after transient forebrain ischemia in the CA1 region of the rat (Ferrer et al., 1998). Since expression of some growth factors like BDNF increases 20 min after induction of global ischemia in CA3 and in the dentate gyrus (Ferrer et al., 1998; Uchino et al., 1997), this may indicate its beneficial potential in attenuation of post-ischemic injuries. The latter mechanism may have also occurred for netrin-1 in our study, which requires further investigation. Of interest, there are various similarities between netrin-1 and BDNF including neurotrophic and chemotactic activity and their important roles in branching and innervations at target (Manitt et al., 2009), in calcium homeostasis and cAMP production (Glazner and Mattson, 2000; Ming et al., 1997; Song and Poo, 1999), and in angiogenesis (Kermani and Hempstead, 2007; Li et al., 2011). We designed our study to evaluate the therapeutic potential of intrahippocampal administration of netrin-1 on the recovery of cognitive function and LTP induction after transient forebrain ischemia. Forty-eight h after infusion of 800 ng netrin-1, working and reference errors significantly reduced, LTP recovered in PS amplitude but not in EPSP, and paired pulse indexes shifted to more negative potentials at IPI10 and IPI20 ms. In contrast, infusion of netrin-1 at a dose of 400 ng only significantly reduced working error and shifted paired pulse indexes to more negative potentials at IPI10 and IPI20 ms. For evaluation of synaptic strength and basal synaptic transmission, we studied the changes in EPSP slope and PS amplitude at similar intensities (I/O curve, 0.1–1 mA) between different groups. To investigate possible change in synaptic plasticity, we examined the influence of transient

forebrain ischemia on induction of LTP and its recovery by netrin-1. Recovery of basal population spikes was observed 3 days after ischemia in animals that received 800 ng of netrin-1. At this time point, according to the I/O curve that shifted to the right, the basal fEPSP remained significantly reduced, indicating that basal synaptic mechanisms have not been altered and neuronal excitability has increased. Since the PS/EPSP ratio to intensity significantly increased in treatment groups and this ratio is a good marker for neuronal excitability level, then, these results suggest that the netrin-1 may have increased neuronal excitability and this could put forward as a compensating mechanism for the impaired synaptic transmission in 4-VO model of our study. The recovery of LTP in population spike was observed in ischemic animals that received 800 ng of netrin-1, although basal population spike remained reduced at this time. This observation clearly suggests that the recovery of neural plasticity precedes that of basal neuronal transmission. Since information transmission in the neural network is finally determined by the generation of action potential, netrin-1 by increasing neuronal excitability may have contributed to the recovery of brain function and this compensatory mechanism could really restore the memory capacity in 8 arm radial maze in our study. Since following netrin-1 (400 and 800 ng) treatment, the rate of the paired-pulse response at interpulse intervals 10–20 significantly reduced, and as mentioned before, this may show the neuroprotective effect of netrin-1 on GABAergic interneurons. In our study, an improvement of working memory in RAM task and paired-pulse response as an indicator of short-term plasticity was observed following netrin-1 (400 and 800 ng) treatment of ischemic rats, indicating that both doses of netrin-1 have had an improving effect regarding short-term memory. However, netrin-1 at a dose of 800 ng only improved LTP as an indicator of long-term memory.

Although there are some reports indicating abnormal expression of some kinds of netrins in an experimental model

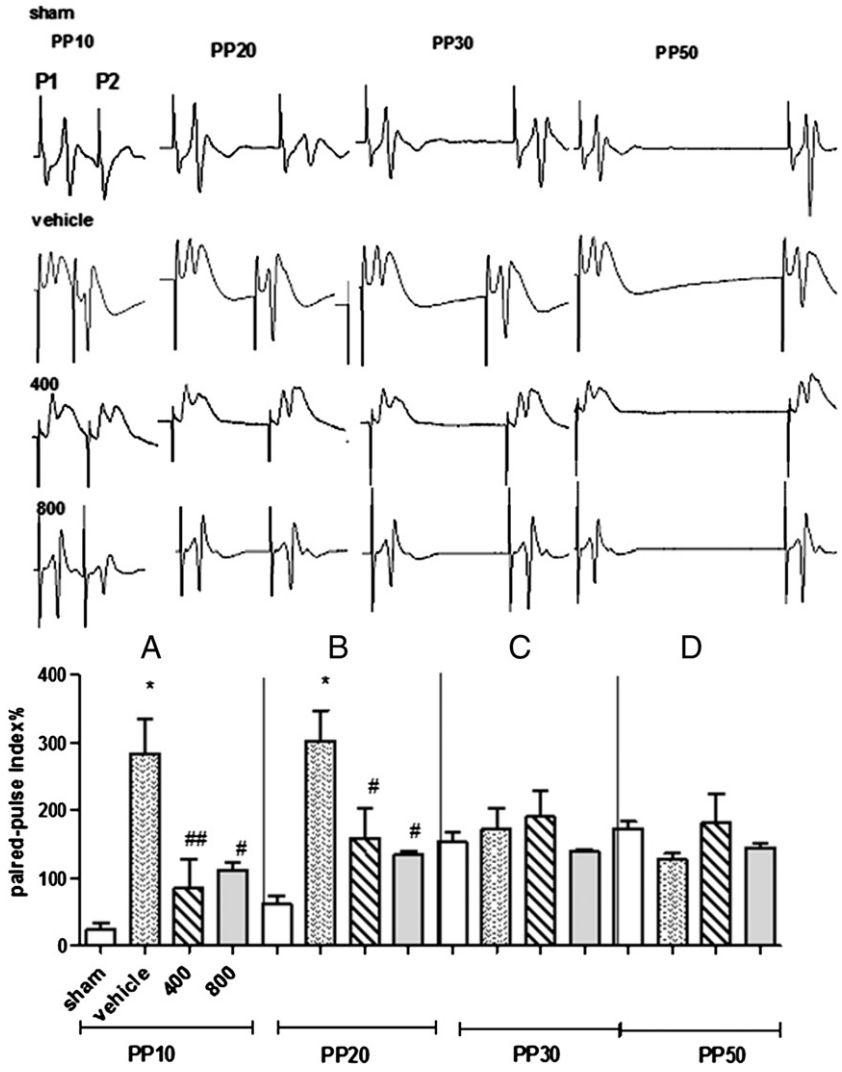

Fig. 5 – The effect of netrin-1 on paired-pulse plasticity at PP10 (A), PP20 (B), PP30 (C), and PP50 (D). The paired-pulse ratio of PSA second (P2)/first (P1) responses × 100 was calculated (n = 6/group). Specimen recordings showed changes in the P1 and P2. Each recording is the average of 10 consecutive population spikes evoked by paired stimuli in 100 s. *Sham vs. vehicle ($p < 0.001$), #vehicle vs. 800 ($p < 0.05$), ##vehicle vs. 400 ($p < 0.01$).

of temporal lobe epilepsy (Pan et al., 2010) and its role in mossy fiber sprouting (towards the molecular layer of the hippocampus) as an important pathogenic mechanism for hyperexcitable recurrent neural networks in epilepsy has already been established (Muramatsu et al., 2010), but with regard to short duration of our study, this may not have occurred in the present study. However, such critical and important issues should be considered in the design of future studies on the possible beneficial effect of netrins for the treatment of central nervous system disorders.

Taken together, the present study showed that netrin-1 dose-dependently ameliorates spatial memory impairment and improves synaptic dysfunction as observed by recovery of population spike component of basal evoked potential and LTP in rats with global ischemia and this may have an implication for new therapeutic regimens for patients with global ischemia.

4. Experimental procedures

Sixty male Wistar rats (Pasteur institute, Tehran, Iran; weighing 300–320 g) were used in this study. They were housed 3 per cage at a room with controlled temperature (23±2 °C) and a relative humidity of 50±10%, and maintained on a 12-h light–dark cycle with ad libitum access to food and water unless otherwise indicated. All procedures were conducted in accordance with NIH guidelines for animal care and treatment and those of Tehran University of Medical University (Tehran, Iran).

4.1. *Induction of transient global ischemia*

Transient global cerebral ischemia (10-min duration) was induced by the four-vessel occlusion method according to the method of Pulsinelli and Brierley (1979) with slight modification. In brief, the test animals that had achieved criterion of spatial memory test were briefly anesthetized with i.p. administration of ketamine (80 mg/kg) and xylazine (10 mg/kg) and were positioned in a stereotaxic apparatus (Stoelting Company, USA), a midline incision (1 cm in length) was made in the dorsal neck (behind the occipital bone). The paraspinal muscles were separated from the midline and the right and left alar foramina of the first cervical vertebrae were exposed. The rat vertebral arteries travel within the vertebral canal and pass beneath the alar foramen before entering into the posterior fossa. A pin 0.5 mm in diameter was inserted through each alar foramen and both vertebral arteries were cauterized and permanently occluded. The muscles and fascia were closed in layers. The rats were then allowed to recover for 24 h. Food was withheld for 24 h before ischemia induction. For induction of global ischemia, rats were anesthetized with chloral hydrate (250 mg/kg, i.p.) and both common carotid arteries were isolated and freed from vagus and surrounding tissues. When the animal was becoming conscious, the common carotid arteries were occluded by carotid clips. Criteria for effective forebrain ischemia were loss of righting reflex, full mydriasis, unresponsiveness to gentle touch, and tonic extension of the paws. Body temperature was maintained at 37 °C throughout the experiment. Only those animals which achieved the above-mentioned criteria for ischemia were used for further experiments. Rats were allowed to survive for 3 days after surgery.

4.2. *Experimental groups*

The rats were divided into sham-operated (n=12), vehicle-treated ischemic (n=16), and netrin-1-treated ischemic groups (in two subgroups, n=10 for each), except for excluded animals. Netrin-1 was purchased from R&D Systems (Minneapolis, MN, USA). In our study, netrin-1 (200 and/or 400 ng for each side) was dissolved in 5 μl of PBS and infused into the left and right hippocampal fissures 24 h after cerebral ischemia. Dose of netrin-1 was chosen according to our pilot studies and existing references (Wu et al., 2008). For intrahippocampal injections, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), placed in a stereotaxic apparatus and the skull was exposed through an incision on the midline and the following coordinates from bregma were used: –4.8 mm posterior, 3.5 mm lateral and 3.5 mm ventral from surface of the skull. Drug was injected at a rate of 0.5 μl/min with a Hamilton microsyringe. After the injection, we waited 5 min before the syringe was withdrawn at a rate of 1 mm/min.

4.3. *Radial arm maze task (RAM)*

The rats for the radial arm maze studies were food-restricted to 90% of their free-feeding body weight. The radial arm maze was constructed of black-painted Plexiglas. The center platform was 26 cm in diameter with 8 arms (10 cm wide×50 cm long) extending from the center. A 2.5 cm diameter hole at the end of each arm held a plastic disposable cup.

Radial arm maze testing was performed in three phases, i.e. shaping, acquisition, and retention according to earlier reports (Davis et al., 2010). Shaping was done with all of the arms baited with the food. It was initially done in groups and then individually. During the social shaping, 3 rats were allowed to explore the baited maze simultaneously for 10 min to become acclimated to the apparatus. There were at least 2 days of social shaping. Food rewards eaten were recorded and used as a criterion for exclusion. For the individual shaping, each rat was allowed 3 min to explore the maze with all eight arms baited, in a way that the rats received experience in gaining a food by completely traversing an arm. Acquisition followed the shaping trials. During the training trials, 4 out of the 8 arms were baited with the food. The pattern of baited arms was chosen to minimize non-spatial search strategies and to ensure that the difficulty level was similar for all rats. The same arms remained baited for all training and retention trials for each rat. There were 20 massed trials for each subject. The trial terminated when the rat entered all 4 baited arms. Between trials, the food was replenished and the maze was cleaned.

The observer was blind to treatments and recorded the sequence of arm entries and trial latency. Retention testing was conducted 7 days after the final acquisition trials (3 days after ischemia). The rats received 3 trials as described under the acquisition procedure. For acquisition and retention testing, the

pattern of arm entries was analyzed for correct choices and types of errors. Reference memory errors were defined to be a visit to an arm that had never been baited. Working memory errors were defined as a revisit to an arm in which the food reward had previously been obtained within that trial.

4.4. *Electrophysiological experiments*

Dentate gyrus LTP was recorded under anesthesia. Three days following transient forebrain ischemia, the rats were anesthetized with urethane (1.5 g/kg) and placed in a stereotaxic apparatus. The rectal temperature was monitored and maintained at 37±0.5 °C with an automatic heating pad. Bipolar stimulating and recording electrodes were made of stainless steel wire (0.125 mm diameter, Advent, UK). It was positioned stereotaxically so as to selectively stimulate the medial perforant path while recording in the dentate gyrus. The electrode stimulating the medial perforant path fibers was implanted 4.2 mm lateral to the true lambda. The recording electrode was ipsilaterally implanted 3.8 mm posterior and 2.2 mm lateral to the bregma.

The electrical signals from the DG were amplified 1000-fold, digitized at 10 kHz, and band-pass filtered at 0.1 Hz–10 kHz. Recording of field potentials was started at least 15 min after placing the stimulation and recording electrodes. All the stimuli were biphasic square wave pulses (200 μs width) and their intensities for baseline recording were set at the current that evoked 40% of the maximum population spike amplitude (PSA). Test stimuli (0.1 Hz) were delivered at 10 s intervals to monitor field excitatory postsynaptic potentials (fEPSP) and population spike (PS).

At the beginning of the experiments, to determine the stimulus intensity that evoked 40 maximal field responses, an input/output (I/O) curve was constructed, that comprised 10 stimulus intensities (at intervals of 10 s), ranging from 100 μA to maximal response, to evaluate synaptic potency. The strength of a field potential was evaluated from the slope of the EPSP and amplitude of the population spike (PS). The maximal EPSP slope was obtained on the first positive deflection of the field potential. The PS amplitude was measured by averaging the distance from the negative peak to the preceding peak and the following positive peak.

After stable baseline recording for at least 30 min, the response to paired-pulse stimulation was subsequently recorded, delivered at 40%-maximal stimulus intensity with inter-stimulus intervals of 10, 20, 30, and 50 ms and then LTP was induced by delivery of high-frequency stimulation (HFS) (10 trains of 15 pulses at 200 Hz separated by 10 s). After the tetanic stimuli, the baseline stimulation was resumed and recording continued for at least 60 min.

4.5. *Data analysis*

Data are presented as the means±SEM. For estimation of LTP, following single pulse recording, the initial slopes of fEPSP and amplitude of PS for 30 min before and 60 min after tetanus were normalized. For data comparison, a two-way analysis of variance (ANOVA) test was applied and further evaluated with Bonferroni post-hoc test. Meanwhile, one-way analysis of variance test with Tukey post-hoc test was applied for comparison of paired-pulse ratio and PS/EPSP/intensity between different groups and unpaired *t*-test for comparison of PS amplitude and EPSP slope in I/O curve. The statistical comparisons for working and reference memory performance were done by two-way repeated measure ANOVA with Bonferroni post-test. Statistically significant level was set at $p<0.05$.

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