Zinc chelation reduces traumatic brain injury-induced neurogenesis in the subgranular zone of the hippocampal dentate gyrus

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A B S T R A C T

Numerous studies have demonstrated that traumatic brain injury (TBI) increases hippocampal neurogenesis in the rodent brain. However, the mechanisms underlying increased neurogenesis after TBI remain unknown. Continuous neurogenesis occurs in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) in the adult brain. The mechanism that maintains active neurogenesis in the hippocampal area is not known. A high level of vesicular zinc is localized in the presynaptic terminals of the SGZ (mosaic fiber). The mossy fiber of dentate granular cells contains high levels of chelatable zinc in their terminal vesicles, which can be released into the extracellular space during neuronal activity. Previously, our lab presented findings indicating that a possible correlation may exist between synaptic zinc localization and high rates of neurogenesis in this area after hypoglycemia or epilepsy. Using a weight drop animal model to mimic human TBI, we tested our hypothesis that zinc plays a key role in modulating hippocampal neurogenesis after TBI. Thus, we injected a zinc chelator, cloquinal (CQ, 30 mg/kg), into the intraperitoneal space to reduce brain zinc availability twice per day for 1 week. Neuronal death was evaluated with Fluoro-Jade-B and Neuh staining to determine whether CQ has neuroprotective effects after TBI. The number of degenerating neurons (FJB (+)) and live neurons (Neuh (+)) was similar in vehicle and in CQ-treated rats at 1 week after TBI. Neurogenesis was evaluated using BrdU, Ki67 and doublecortin (DCX) immunostaining 1 week after TBI. The number of BrdU, Ki67 and DCX positive cell was increased after TBI. However, the number of BrdU, Ki67 and DCX positive cells was significantly decreased by CQ treatment. The present study shows that zinc chelation did not prevent neurodegeneration but did reduce TBI-induced progenitor cell proliferation and neurogenesis. Therefore, this study suggests that zinc has an essential role for modulating hippocampal neurogenesis after TBI.

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Introduction

Despite the longstanding notion that the adult brain does not produce new neurons, it has recently been demonstrated that throughout the lifetime of mammals, newly generated neurons are born within the subgranular zone (SGZ) of the dentate gyrus and migrate over a short distance into the granular cell layer [1,2]. Once there, these newly born cells become functionally integrated into existing hippocampal circuitry by sending dendrites into the molecular layer and axons into the CA3 region [3–5]. The full functional implications of this continuous morphological and structural rearrangement is not known, however it may provide a basis for network plasticity for hippocampus-dependent learning and memory. Traumatic brain injury (TBI) increases adult neurogenesis in the subgranular zones (SGZ) of dentate gyrus of hippocampus in rodents and in the human brain. TBI-related injury to this structure has been associated with learning and memory deficits. Recent observations that hippocampal neurogenesis is implicated in learning and memory functions [6] has raised hopes that injury-induced neurogenesis may represent an endogenous repair mechanism or otherwise serve as a basis of therapeutic intervention. Indeed, promising studies have demonstrated that neural progenitors migrate to injured brain regions and differentiate into the neuronal phenotype specific to the area [7–12]. Kleindienst et al. [13] showed the presence of a proliferative response that peaks during the first week after TBI, and a return to baseline levels of proliferation in the dentate gyrus by 35 days after injury. However, the exact mechanisms that regulate progenitor cell proliferation and neurogenesis response to TBI are not well understood.
It has previously been demonstrated that hypoglycemia increases the number of proliferating progenitor cells and immature neurons in the SGZ of rats. However, 4 weeks post-injury, this initial increase is predictably followed by a sustained decline of progenitor cell proliferation and immature neurons [14]. The mechanism underlying the rise and decline of hippocampal progenitor cell proliferation after hypoglycemia is unclear. However, we have proposed that synaptic zinc release from mossy fiber is a key factor in this process. According to this hypothesis, a pathologically large release of synaptic zinc occurs after hypoglycemia, which stimulates neurogenesis at short latency post-injury, but is subsequently depleted, leading to a decline in neurogenesis due to reduced zinc release or a reduced concentration of vesicular zinc [15].

Zinc is an essential component of more than 1000 enzymes and is critical for regulation of a multitude of cellular processes, including cell division and DNA synthesis [16]. Specifically, zinc is involved in hormonal regulation of cell division in cells regulated by insulin-like growth factor-I (IGF-I) [16] or nerve growth factor (NGF) [17]. Divalent zinc is the second most abundant transition metal in the brain following iron. Chelatable zinc is highly localized in the synaptic vesicle of mossy fiber of the dentate granule cell [18,19], sites where neurogenesis and neural migration are most active in the adult brain [20]. Zinc has long been recognized as a biologically essential element for brain function [21–23]. Division and migration of cerebellar granular cells are reduced following a period of severe zinc deficiency [24,25]. Golub et al. [26] showed that zinc deficiency impaired performance in short-term-memory tasks. Recent studies demonstrated that age-related decline of spatial memory, impaired spatial memory and impaired behavior were found in ZnT3 knock-out mice [27–29]. Thus, zinc appears to be an essential element required for cell division, proliferation, migration and development, and further suggests that this element may play a critical role in neurogenesis and cognitive function.

Clioquinol (5-chloro-7-iodo-8-hydroxy-quinoline, CQ) is a metal chelator. Studies on the effects of endogenous zinc under physiological and pathological conditions have exploited chelating agents to elucidate the specific role played by this ion [30–32]. Recent animal studies have demonstrated that CQ decreased basal neurogenesis as well as on seizure-induced transient hippocampal neurogenesis [33].

In the present study, we aimed to elucidate the potential role of brain zinc in modulating hippocampal neurogenesis after TBI by using a membrane-permeable zinc chelator, CQ.

Materials and method

Experimental animals

The care and handling of animals were in accordance with institutional guidelines and were approved by the Institutional Animal Studies Committee of Hallym University in Chuncheon, Korea (protocol # Hallym 2011-67-1). In this study, we used 8 weeks old male Sprague–Dawley rats (250–300 g; DBL Co., Korea). The animals were housed in a temperature- and humidity-controlled environment (22 ± 2 °C, 55 ± 5% and a 12 h light:12 h dark cycle), and supplied with Purina diet (Purina, Gyeonggi, Korea) and water ad libitum.

Weight drop model

Rats were deeply anesthetized with 2% isoflurane and a 7:5:25 mixture of nitrous oxide/oxygen. Rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The scalp and temporalis muscles were reflected, and a 5.0 mm diameter hole was drilled through the skull (3.0 mm lateral to the midline and 4.0 mm lambda to the bregma) [34–37]. TBI was performed using an EM controlled impact device (Impact OneTM Stereotaxic Impactor, Richmond, IL). For the mechanical trauma, a 5 mm blunt steel impactor tip was retracted and positioned above intact dura. The injury was triggered using the myNeuroLab controller at a strike velocity of 4.5 m/s, strike depth of 2.5 mm, and dwell time of 500 ms [38]. All rats were maintained at a core temperature of 36–37.5 °C during and after surgery, until ambulatory. Rats with seizures were excluded from data analysis (n = 1).

Clioquinol administration

To deplete levels of vesicular zinc or to chelate extracellular zinc, a zinc chelator, clioquinol was used. Rats were injected with clioquinol (CQ; 30 mg/kg, i.p.) twice per day (9–10 AM and 5–6 PM) for 1 week after TBI or without TBI. Clioquinol was dissolved with dimethyl sulfoxide (1% DMSO, Sigma). In the TBI-experienced rats, CQ injection was started at 24 h after TBI. Control rats were injected with the same volume of DMSO. The non-TBI group also had CQ/DMSO or DMSO vehicle only.

Neuron death

Neuronal death was evaluated 1 week after TBI. Rats were intracardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). The brains were post-fixed with 4% PFA for 1 h and then incubated with 30% sucrose for cryo-protection. Thereafter, the entire brain was frozen and sectioned with a cryostat microtome at 30 μm thickness and cryoprotection solution. Brain sections were stained for the Fluoro-Jade B staining (FJB) [39,40]. Degenerating neurons were detected with 450–490 nm excitation and a 515 nm emission filter. To quantify neuronal death, sections were collected from 3.2 mm to 4.5 mm posterior to bregma and five coronal sections were analyzed from each animal. These sections were then coded and given to a blinded experimenter who counted the number of degenerating neurons in the hippocampal CA1 and dentate gyrus (DG).

Detection of live neurons

To identify neuromrophic effects of CQ after TBI, brain sections were immunohistochemically stained by NeuN. Monoclonal anti-NeuN, clone A60 antibody (diluted 1:100, Millipore Co., Billerica, MA, USA) was used as the primary antibody in PBS containing 0.3% Triton X-100 overnight at 4 °C. The sections were washed three times for 10 min with PBS, incubated in biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA) and ABC complex (Vector, Burlingame, CA, USA), diluted 1:250 in the same solution as the primary antiserum. Between the incubations, the tissues were washed with PBS three times for 10 min each. The immune reaction was visualized with 3,3-diaminobenzidine (DAB, Sigma–Aldrich Co., St. Louis, MO, USA) in 0.01 M PBS and the tissues were mounted on the gelatin–coated slides. The immunoreactions were observed under the Olympus IX70 inverted microscope (Olympus Co., Shinjuku, Tokyo, Japan).

BrdU labeling

To test the effects of zinc chelation on neurogenesis, BrdU was injected twice daily for 4 consecutive days starting 24h after TBI [12]. The thymidine analog BrdU was administered intraperitoneally (50 mg/kg; Sigma, St. Louis, MO, USA) to investigate the progenitor cell proliferation. The rats were killed 1 week after TBI.
Coronal sections were immunostained as described [14] using the following reagents: mouse anti-BrdU (Roche, Basel, Switzerland); rabbit anti-Ki67 (recognizing nuclear antigen expressed during all proliferative stages of the cell cycle except G0 [41]; Novocastra, UK); guinea pig anti-doublecortin (DCX) (recognizing immature neurons [42]; Santa Cruz Biotechnology, CA, USA), and ABC solution (Vector Laboratories, Burlingame, CA, USA).

Cell counting

To quantify BrdU, Ki67 and DCX immunoreactivity, sections were collected from 3.2 mm to 4.5 mm posterior to bregma and five coronal sections were analyzed from each animal. These sections were then coded and given to a blinded experimenter who counted the number of BrdU, Ki67, and DCX immunopositive cells in the SGZ and granular cell layer (GCL).

Zinc staining

The N-(6-methoxy-8-quinolyl)-para-toluensulfonamide (TSQ) histochemical method was used as previously described [43,44]. In brief, rats were sacrificed at the designated time points by decapitation under 5% of isoflurane anesthesia and the brains were removed and frozen in powered dry ice. The frozen, unfixed brains were coronally sectioned at 20 μm thickness in a −15 °C cryostat, then thawed on to gelatin-coated slides and dried by gentle air. The
Fig. 2. Clioquinol reduced BrdU-labeled cells in the dentate gyrus. Bromodeoxyuridine binding cells emerged in the dentate gyrus of rats. (A) Brains were harvested at 1 week after TBI and then brain sections were immunohistochemically stained with BrdU. BrdU (+) cells were significantly higher in TBI-induced rats than in the sham-operated rats. TBI-induced BrdU (+) cell production was reduced by CQ. Scale bar = 100 μm. (B) Bar graph represents number of BrdU-immunoreactive cells in the subgranular zone of DG. Data are means ± SE, n = 5 from each group. *P < 0.05.

Fig. 3. Clioquinol reduced Ki67-labeled cells in the dentate gyrus after TBI. Progenitor cell proliferation emerged in the dentate gyrus of rats. (A) Brains were harvested at 1 week after TBI and then brain sections were immunohistochemically stained with Ki67. Progenitor cell proliferation was significantly higher in TBI-induced rats than in the sham-operated rats. TBI-induced progenitor cell proliferation was reduced by CQ. In the sham operation, Ki67 (+) cells were also reduced by CQ (not statistically significant). Scale bar = 100 μm. (B) Bar graph represents number of Ki67-immunoreactive cells in the subgranular zone of DG. Data are means ± SE, n = 5 from each group. *P < 0.05.
sections were immersed in a solution of 4.5 μM TSQ (Molecular Probes, Eugene, OR) in 140 mM sodium barbital and 140 mM sodium acetate buffer (pH 10.5–11) for 60 s, and then rinsed for 60 s in 0.9% saline. TSQ binding was imaged with a fluorescence microscope (Olympus upright microscope, epi-illuminated with 360 nm UV light) and photographed through a 500 nm long-pass filter using an CCD cooled digital color camera (Hamamatsu Co., Bridgewater, NJ) with Infinity 3 imaging program (Lumenera Co., Ottawa, Canada).

Statistical analysis

All data were expressed as means ± SE. For statistical comparisons between groups, significance was evaluated by two-tailed Student's t-test. P values <0.05 were considered significant.

Results

TBI-induced hippocampal neuronal death is not prevented by CQ

To test whether CQ treatment shows neuroprotective effects after TBI, rats were sacrificed 1 week after insult with or without CQ injection. Neuronal injury was evaluated by FJB staining. Widespread FJB (+) neurons were detected in the hippocampal CA1 and DG area after TBI. Compared with vehicle-treated rats, CQ-treated rats showed a similar number of FJB (+) neurons in the hippocampal CA1 and DG area. Surviving neurons were evaluated by NeuN staining 1 week after TBI. NeuN (+) neurons were disappeared in the hippocampal CA1 and DG area after TBI. Compared with vehicle-treated rats, CQ-treated rats showed a similar number of NeuN (+) neurons in the hippocampal CA1 and DG area, suggesting that neuronal death is not prevented by CQ (Fig. 1).

Progenitor cell proliferation in the subgranular zone of dentate gyrus is reduced by CQ in TBI-experienced rats

To test whether CQ influences progenitor cell proliferation in the adult brain, rats were sacrificed 1 week after continuous CQ treatment without TBI. Rats were injected with BrdU twice per day for 4 consecutive days in both vehicle or CQ-treated groups. Cell proliferation was assessed by BrdU and Ki67 immunohistochemistry. We found decreased number of BrdU labeled cells in CQ-treated rats without TBI compared to vehicle-treated rats. However, the number of Ki67 labeled cells in CQ-treated rats without TBI showed tendency of reduction but it is not statistically significant compared to vehicle-treated rats. To investigate how CQ affects TBI-induced progenitor cell proliferation, rats were injected with BrdU twice per day for 4 consecutive days starting 24 h after TBI. Rats were injected with CQ twice per day for 1 week starting 24 h after TBI. We observed an increase in the number of cells labeled by both BrdU and Ki67 staining in rats that underwent TBI, compared to sham operation. However, CQ-treated rats showed a reduced number of BrdU (Fig. 2) and Ki67 (Fig. 3) labeled cells in the subgranular zone of dentate gyrus after TBI compared to vehicle-treated rats.

Neuroblast production is decreased by CQ in TBI-experienced rats

To investigate how CQ influences an expression in migrating neuroblasts, sham or TBI-experienced rats were continuously injected with CQ. Doublecortin (DCX) is a microtubule-associated
protein expressed by immature neurons. The levels of DCX expression increase in response to TBI, which occurs in parallel with BrdU labeling in measuring neurogenesis. In sham-operated groups (without TBI), CQ or vehicle was injected into intraperitoneal space twice per day for 1 week. In TBI-experienced rats, CQ or vehicle was injected twice per day for 1 week starting 24 h after TBI. Number of neuroblasts was assessed by DCX immunohistochemistry. In the sham-operated group, the number of DCX stained neurons in DG area is lower in CQ-treated rats than vehicle-treated rats. However, this is not statistically significant. The number of DCX immunoreactive cells is significantly increased at 1 week after TBI compared to sham-operated groups. CQ-treated rats showed significantly lower number of DCX immunoreactive cells in the DG of hippocampus compared to vehicle-treated rats after TBI (Fig. 4).

**Hippocampal vesicular zinc level is decreased by CQ in normal or TBI-experienced rats**

To investigate whether CQ treatment decreases vesicular zinc intensity in the mossy fiber of hippocampus, brain sections were stained by TSQ. In the sham-operated rats, CQ-treated rats showed lower intensity of mossy fiber zinc in the rat hippocampus compared to vehicle-treated rats. The vesicular TSQ fluorescent intensity is decreased in the mossy fiber area at 1 week after TBI. CQ treatment further decreased TSQ intensity in the mossy fiber area of TBI-experienced rats (Fig. 5).

**Discussion**

Based upon considerable evidence that zinc is crucial for cellular proliferation and migration and the fact that the hippocampus contains an unusually high concentration of chelatable zinc, we hypothesized that brain zinc might play a modulatory role in hippocampal neurogenesis after traumatic brain injury (TBI). Accordingly, this study found that zinc chelation by clioquinol (CQ) substantially reduced TBI-induced progenitor cell proliferation. This suggests that chelatable zinc is an important mediator of neurogenesis in the hippocampus after brain injury and may be a key observation in understanding post-TBI hippocampal pathology, as well as offering a potential therapeutic route of intervention.

It has been well-established that zinc accumulation in postsynaptic neurons results in neuronal death in several disease conditions, including prolonged seizure [45,46], ischemia [47,48], brain trauma [35,36] and hypoglycemia [49,50]. This contrasts with other studies that have shown that zinc has several beneficial roles in the brain [25]. Zinc is an essential component of enzymatic functions that influence cell division and proliferation and studies have shown that zinc deficiency impairs brain development [51]. Taken together, the evidence suggests that zinc is an essential transition element for neurogenesis.

Hippocampal neurogenesis persists through adulthood in rodents and in humans [52–55]. Neuronal precursor cells reside in the SGZ of the dentate gyrus, where they proliferate continuously into the granule cell layer [2,55,56] and chelatable zinc is highly concentrated in the mossy fiber of dentate granule cell of the hippocampus [57,58].

To test our hypothesis that zinc is essential for hippocampal neurogenesis, we used the zinc chelator, CQ. Previous studies demonstrated a transient increase of progenitor cells after hypoglycemia and increased susceptibility to seizure lasting 2 weeks post-insult [14,33]. The reason for an increase in neurogenic activity at early time points after hypoglycemia and seizure is uncertain, however we speculate that this transient increase in neurogenesis after hypoglycemia and seizure is related to synaptic release of zinc.

![Fig. 5. Clioquinol reduced TSQ intensity after TBI. (A) TSQ fluorescence images in the mossy fiber area 1 week post-insult in sham-operated or TBI-experienced rats. Vesicular TSQ intensity is high in mossy fiber area of dentate granule cell layer in sham-operated rats. However, the vesicular TSQ fluorescent intensity is decreased in the mossy fiber area at 1 week after TBI. CQ treatment decreased TSQ intensity of mossy fiber area either in sham-operated rats or in TBI-experienced rats. Scale bar = 200 μm. (B) A graph represents quantitated intensity of TSQ fluorescent in the hilar area. CQ-treated rats show significantly lower TSQ intensity than vehicle-treated rats either in sham-operated or in TBI-experienced rats. Data are means ± SE, n = 3 from each group. *p < 0.05.](image-url)
and cytology after hippocampal neuronal death. Continuous liberation of free zinc from the degenerating neurons or from mossy fiber may continuously stimulate progenitor cell proliferation and support survival of neuroblasts after hypoglycemia and seizure. By providing continuous treatment with CQ for 1 week after TBI, we tested the effects of zinc chelation on basal neurogenesis as well as on TBI-induced transient neurogenesis. In the present study, we found decreased basal progenitor cell proliferations and a reduced number of neuroblasts compared to the vehicle-treated group in uninjured animals that received CQ. However, only the number of BrdU (+) cells was reduced in the basal condition in a statistically significant manner. The reason why such a difference may occur is not clear from the present study. We speculate that concentration or duration of CQ treatment may affect this discrepancy.

However, 1 week of continuous injection with CQ after TBI substantially reduced the number of BrdU (+), Ki67 (+) and DCX (+) cells in the hippocampus. In this condition, CQ may effectively act in the extracellular synaptic space as a zinc chelator. Alternatively, CQ may also act as a zinc ionophore to maintain metal ion homeostasis and to thereby maintain a "normal" brain zinc state [59,60]. Thus, if the increased neurogenesis seen after TBI can be considered to be "abnormal" or "pathogenic", then the reduction in vesicular zinc seen with CQ treatment could prevent or reverse a transition from normal to abnormal brain zinc state, which may normalize neurogenesis after TBI.

Taken together, the present study suggests that zinc plays a key role in hippocampal neurogenesis and zinc modulation by CQ reduces TBI-induced hippocampal neurogenesis. Thus, our present study demonstrates that vesicular zinc in the hippocampus may be involved in promoting neurogenesis in the adult brain following pathological events.

Conflict of interest

The authors declare no conflict of interest.

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