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Alyssa Espinera 03/27/2012

Enhanced Sensorimotor Functional Recovery and Neurovascular Regeneration after Stroke with Chronic Citalopram Treatment

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Abstract

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Recent clinical trials have reported that selective serotonin reuptake inhibitor treatment after stroke enhances motor functional recovery; however, the underlying mechanisms need to be further elucidated. We hypothesized that daily administration of citalopram would enhance neurovascular repair in the ischemic penumbra while accelerating sensorimotor functional recovery. Focal ischemic stroke was produced in male C57/B6 mice by ligation of the distal middle cerebral artery and 7-minute occlusion of the bilateral common carotid arteries. Citalopram (10mg/kg, i.p.) was injected 24h after stroke and daily thereafter. BrdU was injected daily to mark proliferating cells. An adhesive removal task was used to measure changes in forelimb sensorimotor function after stroke. Immunohistochemical staining was used to assess the presence of newly born neurons and vessels and to illustrate neural migration. Citalopram treatment did not reduce acute infarct volume (72h), but did enhance functional recovery in the adhesive removal behavior task after 14 days. Brain derived neurotrophic factor expression was increased in the peri-infarct region after 7 days of citalopram treatment. Migration of proliferating cells was observed between the sub-ventricular zone neural precursor niche and the peri-infarct area in both control and citalopram-treated mice; however, citalopram-treated animals had more new neurons in the peri-infarct region than did the control stroke animals in both 21 and 28d treatment groups. Additionally, blood vessel cross-sectional area in the peri-infarct region was greater in the citalopram treatment groups. These results suggest that citalopram promotes sensorimotor recovery from stroke while augmenting neurogenesis and total vessel area in the peri-infarct region after stroke.

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TERMINOLOGY and ABBREVIATIONS

- 5-HT Receptor
    - 5-hydroxytryptamine receptors, serotonin receptors, are G-protein coupled receptors found in the central and peripheral nervous system. These receptors mediate both inhibitory and excitatory neurotransmission.
- AKT
    - AKT is another term for protein kinase B (PKB). This protein kinase has role in several cellular processes including cell proliferation, cell migration, transcription, and apoptosis.
- BDNF
    - Brain-Derived Neurotrophic Factor is a neurotrophin and growth factor that acts on neurons in the central nervous system and the peripheral nervous system to enhance growth, differentiation, and survival.
- BrdU
    - Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a thymidine analog that incorporates into DNA acting as a nucleoside. BrdU is used to detect proliferation.
- CCA
    - Common Carotid Arteries
- Col IV
    - Collagen IV is a naturally occurring protein that is abundant in blood vessels.
- Citalopram
    - Selective serotonin reuptake inhibitor commonly used to treat depression and less commonly to treat anxiety.
- CVA
    - Cerebrovascular Accident; Stroke
- DG
    - Dentate Gyrus, part of the hippocampal formation, known to exhibit adult neurogenesis in rats.
- Fluoxetine

  - Selective serotonin reuptake inhibitor commonly used to treat depression and obsessive compulsive disorder.
- FMMS
  - Fugl-Meyer Motor Scale, used to determine degree of disability
- MCA
  - Middle Cerebral Artery
- MCAO
  - Middle Cerebral Artery Occlusion, ischemic stroke
- mRS
  - Modified Rankin Scale
- NeuN
  - Neuronal Nuclei, a neuron specific marker that binds to neuronal DNA.
- NIHSS
  - National Institutes of Health stroke scale
- Penumbra
  - Tissue located immediately outside of the stroke core that is salvageable if re-perfused. Also called peri-infarct region.
- PSD
  - Post-stroke depression
- SSRI
  - Selective Serotonin Reuptake Inhibitor
- SVZ
  - Subventricular Zone
- TrkB
  - TrkB tyrosine kinase is a receptor that binds neurotrophins including brain-derived neurotrophic factor.

INTRODUCTION

Stroke is the fourth leading cause of death and the primary cause of long-term disability in the United States; however, there are limited effective treatments (National Stroke Association, 2010). Tissue plasminogen activator (t-PA) is the sole drug that has been approved to treat CVA (cerebrovascular accident). However, drug efficacy and safety limit patients receiving t-PA to less than 3% of stroke patients ("Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group," 1995). In stroke patients suffering from post-stroke depression, treatment with anti-depressant drugs after stroke decreases depressive symptoms and increases functional recovery (Bilge, Kocer, Kocer, & Turk Boru, 2008; Wiart, Petit, Joseph, Mazaux, & Barat, 2000). The enhanced recovery observed in post-stroke depression patients treated with selective serotonin reuptake inhibitors (SSRI) has prompted a number of clinical trials looking for further therapeutic benefits of anti-depressant administration after stroke in non-depressed as well as depressed patients (Acler, Robol, Fiaschi, & Manganotti, 2009; Chollet et al., 2011; Mikami et al., 2011).

Anti-depressant treatments after stroke enhance motor functional recovery independent of their anti-depressant activity. Three months of treatment with the SSRI fluoxetine after stroke significantly improved patient scores in the Fugl-Meyer motor scale and the motor portion of the National Institutes of Health stroke scale (NIHSS) (Chollet et al., 2011). Similarly, in a smaller study, citalopram administration for four months after stroke improved NIHSS performance, unrelated to mood (Acler et al., 2009). Fluoxetine and the tricyclic anti-depressant nortriptyline increased scores in the

modified Rankin scale, which measures independence in activities of daily life (Chollet et al., 2011; Mikami et al., 2011). These studies provide a foundation supporting the use of anti-depressants for improving stroke recovery, however, the mechanisms behind these therapeutic benefits need to be further elucidated in order to improve treatment and develop more targeted therapeutics.

SSRIs have been noted for their effect on neural progenitor proliferation in the hippocampus, dentate gyrus, and sub-ventricular zone (SVZ). Neuronal progenitor proliferation and migration after stroke to the damaged area may contribute to long-term recovery (Kreuzberg et al., 2010; Lindmark & Hamrin, 1988). Chronic SSRI treatment increases expression of neurotrophic factor BDNF mRNA and protein (Balu et al., 2008). Brain derived neurotrophic factor (BDNF) is physiologically and pathologically important in the control of survival, proliferation, and migration of neural progenitor cells in the SVZ (Kozlov et al., 2007). BDNF activates the tyrosine kinase receptor (TrkB) which mediates down-stream signaling cascades involved in growth, survival, and migration including phosphotidylinositol-3 kinase/ Akt pathways, the c-AMP response element (CREB), and the Ras/Mitogen-activated protein kinase pathways (Russo-Neustadt & Chen, 2005). Enhanced BDNF expression is believed to modulate neuronal plasticity, survival, and cell-signaling pathways during development, as well as axonal plasticity during development and adulthood to contribute to learning, memory, and sensorimotor recovery (Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000; Schabitz et al., 2004).

Protection of the neuro-vasculature after stroke is an important step in restoring brain function (Arai et al., 2011). Thus, vascular regeneration after stroke is also a

potential target for SSRI mediated therapies. BDNF-TrkB receptor activation and downstream Akt activation has been shown to promote endothelial cell survival and neoangiogenesis (Kermani & Hempstead, 2007). Therefore, SSRI treatments may contribute to new vessel formation and promote survival of existing vessels after ischemic insult (Greene, Banasr, Lee, Warner-Schmidt, & Duman, 2009).

The current study tested whether administration of the SSRI citalopram after stroke promotes migration of neural progenitors from the SVZ to the peri-infarct, neurovascular repair in the ischemic penumbra, and sensorimotor functional recovery. As hypothesized, citalopram-treated animals showed neural progenitor migration from the SVZ to the peri-infarct region, increased BDNF protein expression in the peri-infarct cortex, and enhanced sensorimotor recovery combined with increased neurogenesis and vessel area in the stroke penumbra.

EXPERIMENTAL PROCEDURES

Treatment Groups (Figure A)

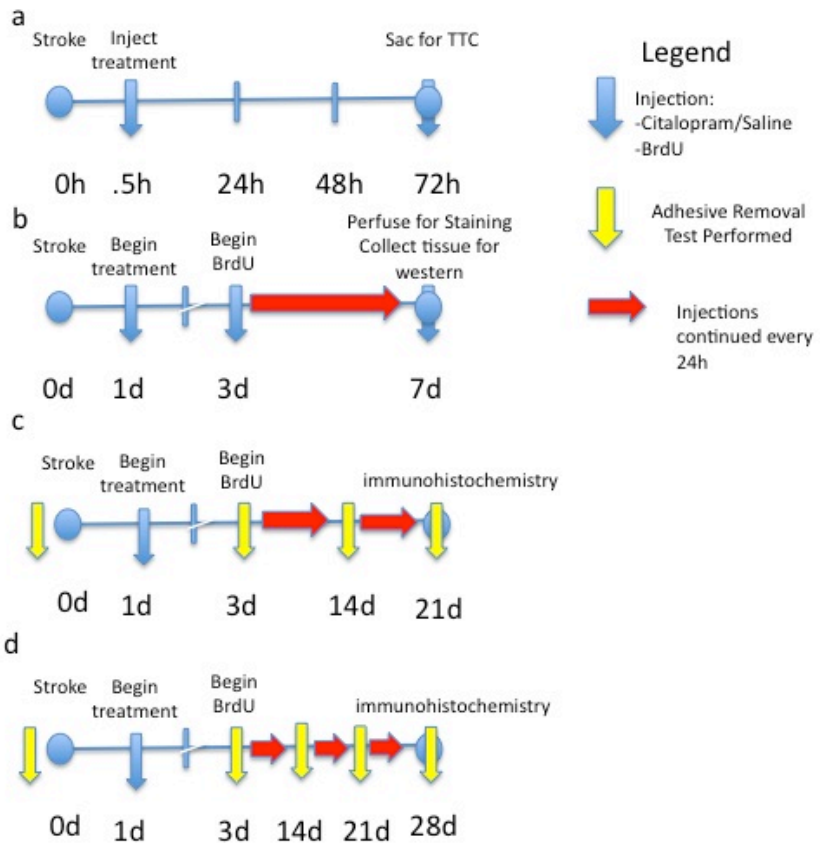
All animals were randomly allocated to either saline or citalopram treatment groups.

(a) Animals were injected 30 minutes after middle cerebral artery occlusion (MCAO) and then every 24h till day 3. At 72h post-MCAO, animals were sacrificed.

(b) Animals were injected 24h post MCAO and than every 24h for 7d. At 7d Animals were sacrificed and perfused with formalin. Tissue was collected from a subset of these animals for western blot analysis.

(c) Animals were injected 24h post MCAO and than every day for 21d. Animals were injected with BrdU starting 72h post-MCAO. At 21d animals were sacrificed.

(d) Animals were injected 24h post MCAO and than every day for 28d. Animals were injected with BrdU starting 72h post- MCAO. At 28d animals were sacrificed.



Middle Cerebral Artery Occlusion

The Institutional Animal Care and Use Committee at Emory University approved all *in vivo* experimental procedures. Middle cerebral artery occlusion (MCAO) was conducted as previously described (Ogle, Gu, Espinera, & Wei, 2012) with some modifications. Briefly, adult male C57 mice (Charles River Labs; Wilmington, MA) weighing 20-25g were anesthetized with 4% chloral hydrate by i.p. injection prior to surgery. Once anesthetized, the neurosurgical technician exposed both common carotid arteries (CCA) on the dorsal neck using surgical scissors. She then proceeded to ligate

both common carotid arteries using reversible sutures. Immediately following the ligation, the mouse was placed ventral side up. Using a drill, a small craniotomy was performed exposing the distal lateral branch of the right middle cerebral artery. She then permanently occluded the right middle cerebral artery branch, supplying the whisker barrel cortex, with a 10-0 suture. 7 minutes after CCA ligation the sutures were removed allowing reperfusion of both bilateral common carotid arteries. During the 7 min CCA occlusion, blood flow in the whisker barrel cortex was reduced to less than 20% of the original blood flow as measured by laser doppler blood flow scanner. After surgery, the animals recovered in the incubation chamber until the anesthesia effects dissipated. Animals were either injected 30 minutes post-mCAO (fig. A(a)) or placed in their original cages to recover before being randomly allocated into groups for treatment administration at 24h post-MCAO.

Drug Administration

Citalopram (10mg/kg) was diluted in sterile saline and injected intra-peritoneally (i.p.) 24h after MCAO and then daily for 14, 21, or 28d. In the acute neuroprotection group (refer to figure A(a)), Citalopram (10mg/kg, i.p.) was administered 30 minutes after MCAO and then daily for 3 days until sacrifice at 72h.

BrdU Administration

BrdU (Bromodeoxyuridine) was injected intra-peritoneally (i.p.) beginning 72 hours after MCAO and then daily for 21 or 28d (refer to figure A(c,d).

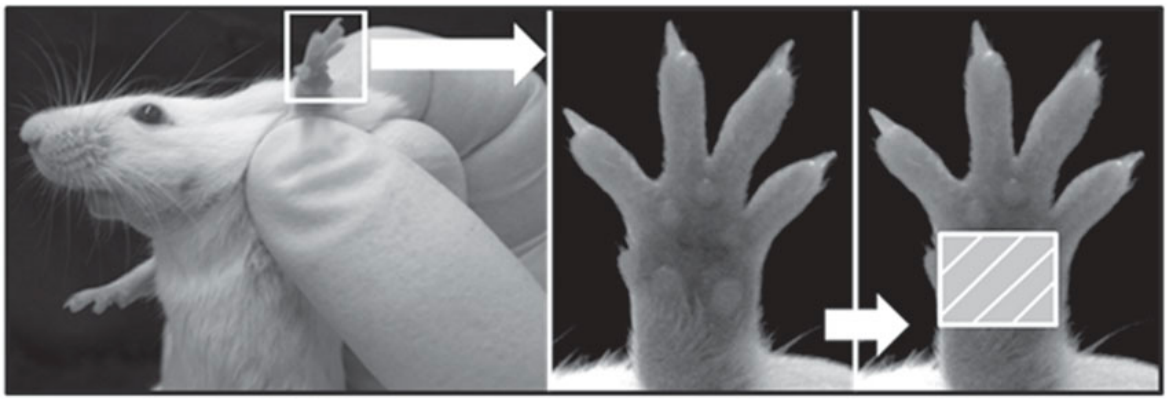
Infarct Volume

Animals (figure A (a)) were sacrificed 72h post-stroke for ischemic infarct size assessment as previously described (Ogle et al., 2012). Animals were anesthetized using 4% chloral hydrate until non-responsive to painful stimuli. Animals were then decapitated using a rodent guillotine (Kent). The complete brain was rapidly, removed, sliced into 1mm coronal sections, and stained in 2% 2,3,5-Triphenyltetrazolium chloride (TTC) solution at 37C for 5 min. Brains were then placed into 10% buffered formalin. 24h later brain sections were scanned and imported into ImageJ software, where the stroke infarct, ipsilateral, and contralateral areas were measured. Direct infarct volume ratio was calculated by dividing the infarct volume by the total brain volume in 6x 1mm slices. Indirect infarct volume ratio was calculated by subtracting the non-infarcted volume of the ipsilateral hemisphere from the volume of the contralateral hemisphere and dividing by total volume. Indirect infarct volume is calculated to account for any inflammation in the ipsilateral hemisphere secondary to recent ischemia (Lin, He, Wu, Khan, & Hsu, 1993).

Behavioral Assessment

The adhesive removal behavioral test was employed to measure changes in sensorimotor function as previously reported (Bouet et al., 2009; Freret et al., 2009; Ogle et al., 2012). As shown below, this test is conducted by placing an adhesive tap strip 0.3cm x 0.4cm onto the distal forepaw of the right or left paw alternately. The mouse was then placed into an empty box and the amount of time in seconds it takes for the mouse to notice and remove the adhesive is recorded. Briefly, two training sessions of three trials

each were administered during the week prior to stroke. Baseline adhesive removal time was assessed 1 day prior to stroke with 1 training and 3 removal trials. Removal time was further assessed for each animal (figure A (c,d)) at 3, 14, 21, and 28 days post-stroke. The data was analyzed by comparing fold change (post-stroke removal time (sec)/ baseline removal time (sec)) between treatment groups and over time.



Adhesive Removal Test image from Nature Protocols (Bouet et al., 2009)

Immunohistochemical Staining

Animals (figure A (c,d)) were sacrificed according to the procedure discussed above. Brains were frozen immediately after sacrifice in optimal cutting temperature (OCT) media (Sakura Finetek; Torrance, CA) at -80°C. Sections were cut at 10μm thickness from frozen brains on a cryostat. Slides were fixed for 5 min in 10% buffered formalin, washed in phosphate buffered saline (PBS) three times, then incubated for 15 minutes in -20°C methanol. Sections were air-dried for 5 minutes, rehydrated in PBS for 1 min, and then sections were incubated in 2N HCl at 37°C for 1 hour. Sections were neutralized by washing in borate buffer three times. The slides were then incubated in 0.2% TritonX-100 for 45 minutes, and then washed in PBS three times. After washing,

slides were incubated in 1% fish gelatin (Sigma, St. Louis, MO) for 30-60 min. Neuronal nuclei (NeuN, Millipore; Billerica, MA), Collagen IV (Millipore), and BrdU (AbD Serotec; Raleigh, NC) antibodies were incubated overnight at 4°C. Slides were then washed and incubated with donkey anti-mouse Cy5 (Jackson Immunoresearch; West Grove, PA), donkey anti-rat Cy3 (Jackson immunoresearch; West Grove, PA), and donkey anti-goat 488-conjugated secondary antibody (Invitrogen; Grand Island, NY). Slides were washed three times in PBS and cover-slipped prior to imaging. Staining procedures were modified from standard protocols used Ogle et al. (2012).

To capture proliferating cell migration, sections were stained with doublecortin (DCX) and BrdU (bromodeoxyuridine). 72 hours after MCAO, animals (figure A (b)) were perfused with 10% buffered formalin, brains were removed and placed in formalin for 24 hours, and then placed in sucrose. Brains were frozen at -20°C in OCT and were cut into 14μm thick sections on a cryostat. The slides were placed in formalin to fix for 10 min, washed in PBS 3x, fixed in methanol 2x at 7min, incubated in 0.2% TritonX-100/PBS for 5 min, and 1% fish gel for 30-60 minutes (Sigma, St. Louis, MO). Primary antibody DCX (Santa Cruz Biotechnology, Santa Cruz, CA) was applied overnight followed by secondary anti-goat 488 (Invitrogen; Grand Island, NY). After DCX staining, slides were co-stained with BrdU to reveal the presence of migrating neural progenitors.

Cell counting using design based stereology

Sections were chosen by systematic random sampling with every 20th brain slice across the region of interest being imaged and counted (6 sections per animal, with

sections more than 200μm apart) as previously described (Treins, Giorgetti-Peraldi, Murdaca, Semenza, & Van Obberghen, 2002). For multistage random sampling, six 40X images from the cortical region supplied by the right MCA were captured in each sampled section. The region was identified using a detailed coronal mouse brain atlas. The region of interest was defined as a 1.2 mm region within the perfusion territory of the MCA. Data is represented as the total number of cells per animal in the region of interest.

Vessels were counted and represented by the total number of vessels per animal in the region of interest. Vessel area fraction, the area of collagen IV staining over total area of interest, was measured using ImageJ software.

Western blot analysis

Animals were sacrificed on day 7, after continuous injections with citalopram or saline that began 24h post stroke (figure A (b)). Tissue was collected from the ischemic penumbra, defined as a 1.2 mm region within the perfusion territory of the MCA. The tissue was added to lysis buffer and homogenized. The resulting supernatant was used for BCA assay to determine the protein concentration. Lysis buffer was added in varying amounts to the samples depending on the starting protein concentration. 30μl of dye reagent was then added and the samples were loaded into a 12%-18% gradient gel. The proteins were isolated, electrophoresed, and immunoblotted as previously described (Ogle et al., 2012). Western blots were probed using primary antibodies: anti-BDNF and anti-β-Actin (Sigma Aldrich).

Statistical analyses

All analyses were performed using Graphpad Prism 4.0 statistical software (GraphPad Software, Inc., La Jolla, CA). Multiple comparisons were performed by one-way or two-way analyses of variance followed by Bonferroni's post hoc analysis. Single comparisons were performed using Student's t-test. Changes were identified as significant if the p-value was less than 0.05. Mean values are reported with the standard error of the mean (SEM).

RESULTS

Acute citalopram administration after stroke does not affect infarct volume

To determine whether citalopram administration post-stroke could impact ischemic infarct volume, animals (fig. A(a)) were treated 30 min after MCAO with citalopram (10mg/kg, i.p.) and every 24h until sacrifice. Infarct volume was measured 72h after stroke. Saline-injected, control animals had an ischemic volume of 3.86±0.74% of total brain volume. Citalopram-treated animals had an infarct volume of 3.15±0.41% of total brain volume. There was no statistical difference between the two groups indicating that citalopram administration beginning 30 min post-stroke does not significantly affect stroke infarct volume (Fig. 1).

Figure 1

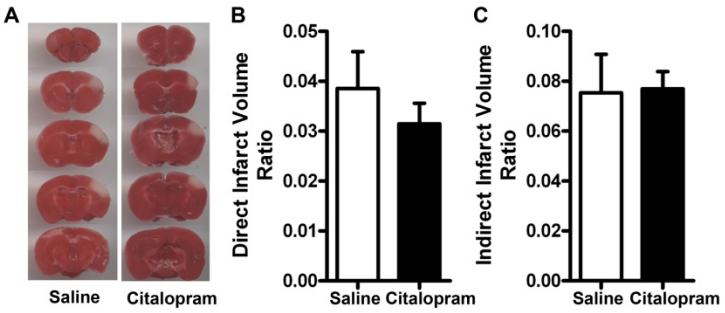


Figure1. Acute Citalopram administration does not attenuate infarct volume. Citalopram was administered 10mg/kg i.p. 30 minutes after MCAO and again at 24 and 48h. Animals were sacrificed at 72h and sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC). Direct and indirect volume analyses revealed no significant difference in infarct volume with acute Citalopram treatment. (n=9/group, Mean ± SEM, $p>0.05$).

Chronic citalopram treatment after stroke enhances sensorimotor functional recovery

The distal MCA focal stroke model targets the somatosensory cortex, primarily affecting whisker and forelimb function. The adhesive removal task is a sensitive behavior test to measure sensorimotor differences following stroke (Bouet et al., 2009; Freret et al., 2009). After pre-stroke training, mice (fig. A (c,d)) were tested at days 3 and 14 after stroke and fold change from baseline was calculated. Three days after stroke, control animals had a significant deficit in adhesive removal time compared to baseline, where as citalopram treated animals did not show this deficit ($t(23)=2.837$, #$p=0.0093$; $t(24)=1.653$, $p=0.1114$). Fold change in removal time at14d post-stroke was significantly

less in citalopram-treated animals compared to control, indicating that citalopram enhanced sensorimotor improvement after stroke (Fig. 2: t(17)=2.352, *p=0.0310).

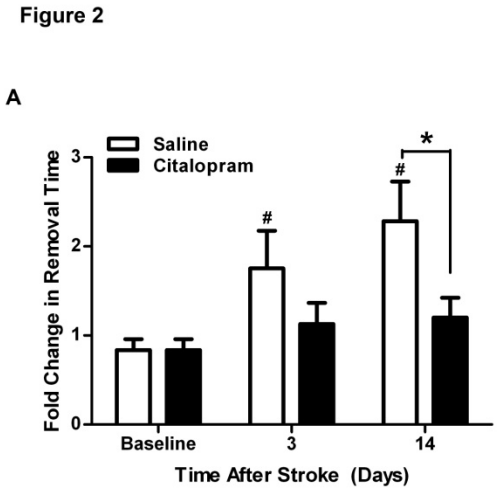


Figure 2. Citalopram treatment after stroke significantly enhances sensorimotor functional recovery. Citalopram or Saline treatment was administered starting 24h after MCAO and then daily for 14 days. Sensorimotor function was assessed by the forelimb adhesive removal behavioral test. Three days after MCAO significant deficits in adhesive removal time were observed in saline-treated mice but not in citalopram-treated mice (n= 6-10/group, Mean ± SEM, #p>0.05 compared to baseline). After 14 days, citalopram-treated animals displayed improved performance of the sensorimotor task, significantly lower than saline-treated animals and not significantly different from baseline. (n=6-10/group, Mean ± SEM, *p<0.05).

Chronic citalopram treatment after stroke increases neurogenesis

DCX signifies new neurons migrating from the SVZ. DCX and BrdU co-staining, 7d after stroke, demonstrates neural progenitor migration from the SVZ toward the peri-infarct region (Figure 3A, B).

New neurons were visualized in the peri-infarct cortex 21 and 28 days after stroke by BrdU and NeuN co-immunohistochemical staining (Fig 3C-E). Both control and citalopram-treated mice (fig. A (c,d)) had new neurons in the peri-infarct region (Fig 3 D, E); however, citalopram-treated animals had significantly more BrdU/NeuN co-labeled cells as a fraction of total NeuN-positive cells compared to saline treated animals at 21 and 28d (Fig 3F: $t(12)=3.037$, $p=0.0103$; $t(11)=3.665$, $p=0.0037$).

Figure 3

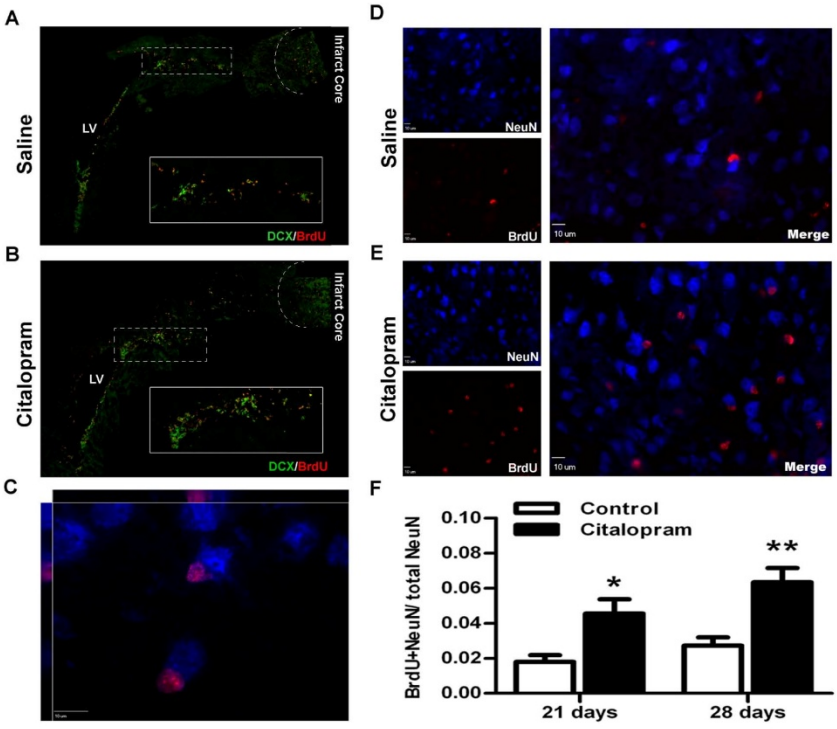


Figure 3. **Citalopram treatment increases new neurons in the peri-infarct region**. Migration of neural progenitors was visualized using Doublecortin (DCX, green) and Bromodeoxyuridine (BrdU, red). (A, B) In the SVZ, BrdU + cells are co-labeled with DCX+ neural progenitors. These cells were found along the ventricle and between the

ventricle and the stroke cortex, suggesting an apparent migration toward the peri-infarct region in both treatment groups. Inset is enlargement of migrating neural progenitors. Neurogenesis was visualized using neuronal marker NeuN (blue) and BrdU (red). (C) High power image in the peri-infarct cortex shows co-localization of NeuN and BrdU. (D) Saline treatment and (E) Citalopram treated animals have new neurons 28d after stroke. (F) Quantification of the number of co-labeled NeuN and BrdU labeled cells (new neurons) in the peri-infarct region. Both 21d and 28d Citalopram treatment groups had significantly more new neurons in the peri-infarct region compared to the saline treated animals. (n=7, Mean ± SEM, *$p<0.05$)

Chronic citalopram treatment after stroke increases peri-infarct vessel area.

To determine the effect of citalopram on the vasculature of the penumbra after stroke, brain sections (fig. A (c,d)) were stained with collagen IV to mark the basal lamina of vessels and for BrdU to mark proliferating cells (Fig 4 A, B). Citalopram-treated animals trended toward a higher percentage of BrdU/Collagen IV co-labeled cells in the vessels, although these data did not achieve statistical significance at 21 or 28d (Fig. 4C: $t(12)=1.420$, $p=0.1810$; $t(12)=1.612$, $p=0.1330$). Vessel area fraction (vessel area relative to area of the region of interest) was also quantified by measuring collagen IV expression. Citalopram-treated animals had a significant increase in vessel area after 21 and 28d treatment compared to saline-treated animals (Fig. 4D: $t(12)=4.667$, **$p=0.0005$; $t(12)=2.637$, *$p=0.0231$).

Figure 4

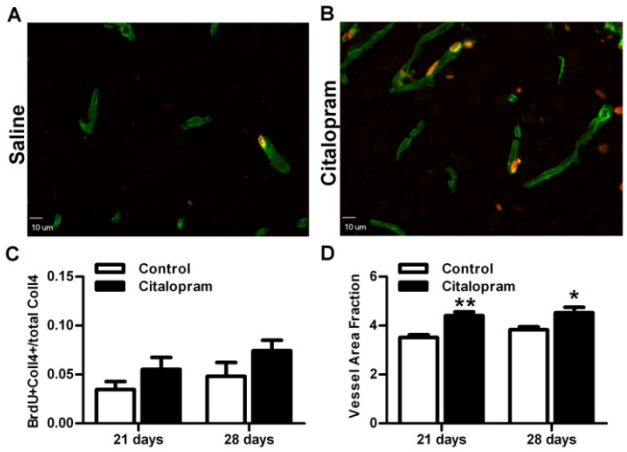


Figure 4. Citalopram treatment enhances vessel representation in the peri-infarct region. Vessels were visualized and counted using vessel basal lamina marker Collagen IV and dividing cells were marked using BrdU. (A) Immuno-staining for vessels (collagen IV, green) and newly divided cells (BrdU, red) after 28d saline treatment and (B) citalopram treatment. (C) Quantification of the number of co-labeled Collagen IV and BrdU vessels signifying proliferating vessels in the peri-infarct region (n=7, Mean ± SEM, $p>0.05$). (D) Vessel area fraction (vessel area/ area of interest) in the peri-infarct. Citalopram treated animals had significantly greater vessel area fraction compared to saline treated groups. (n=7, Mean ± SEM, $*p<0.05$).

Chronic citalopram treatment after stroke increases BDNF in the peri-infarct cortex.

Peri-infarct protein BDNF expression was assessed by western blot analysis 7d after stroke with or without daily citalopram treatment (fig. A (b)). Citalopram treatment lead to up-regulated BDNF protein in the peri-infarct region compared to vehicle-treated animals (Fig. 5: $t(7)=2.705$, $p=0.0304$).

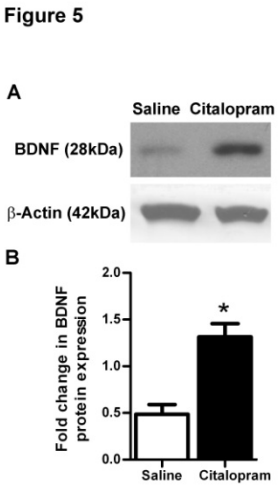


Figure 5. Citalopram induces BDNF expression in the peri-infarct region. A. Protein analysis of the peri-infarct region was performed 7 days after MCAO. Western blot demonstrates an increase in BDNF protein expression in the peri-infarct with Citalopram treatment. (n=5, Mean ± SEM, * $p<0.05$).

DISCUSSION

Recent clinical trials have found that SSRI treatment enhanced motor recovery after stroke (Acler et al., 2009; Chollet et al., 2011; Mikami et al., 2011); however, the mechanisms behind this clinical functional recovery are not characterized. In treating depression, SSRIs increase neurotrophic factors and subsequently stimulate neurogenesis and synaptogenesis (Castren, 2009; Rantamaki et al., 2007). . We hypothesize that SSRIs may function in a similar manner after stroke by enhancing neurotrophic factors during the recovery process, promoting neurovascular repair. Consistent with SSRI depression treatment, this study suggests that acute treatment with SSRIs does not attenuate stroke core. The current study demonstrates that chronic citalopram treatment after ischemic stroke enhances sensorimotor recovery in parallel with enhanced cortical BDNF expression, increased number of new neurons, and increased vessel area fraction in the peri-infarct region.

The stroke model utilized in the current study produces a small focal ischemic stroke in the mouse whisker barrel cortex and forelimb motor cortex. The stroke model mimics the most prevalent human stroke, a small infarct involving 4.5-14% of the ipsilateral hemisphere (Carmichael, 2005). The adhesive removal behavioral task is a sensitive indicator of sensorimotor deficits caused by the mouse MCAO model used in the present study (Mohajerani, Aminoltejari, & Murphy, 2011; Schabitz et al., 2007), as illustrated by the deficits seen in the control group at 3 days after stroke. At 3 days citalopram-treated animals did not show significant deficits from baseline; however, the citalopram and saline animal removal times were not significantly different. Enhanced sensorimotor function was observed in citalopram-treated animals at 14 days. In the SSRI post-stroke clinical trials, patients were treated with citalopram for three months and then monitored for up to a year. The subjects treated daily with SSRIs for three months had better scores for motor recovery at three months and at the one year evaluation, suggesting that SSRIs may foster long-lasting recovery (Mikami et al., 2011). Taken together, these findings suggest that citalopram treatment promotes the restoration of motor function and may provide sustained recovery after stroke.

Improved functional recovery could indicate either neuroprotection in the acute phase or an enhanced regenerative process. Hoever, infarct volume was not significantly different between citalopram-treated animals and vehicle-treated animals after 72 hours. Thus, citalopram acute neuroprotection is unlikely to be the underlying mechanism for the observed accelerated functional recovery. These results are consistent with SSRI studies indicating that anti-depressants function through delayed mechanisms (Rantamaki et al., 2007).

SSRIs such as citalopram are blood-brain barrier permeable and are designed to allow for enhanced serotonin activity at synaptic clefts (Gardier, Malagie, Trillat, Jacquot, & Artigas, 1996; Russo-Neustadt & Chen, 2005). Increase in serotonin receptor binding leads to a time-dependent upregulation in neurotrophic factors, namely BDNF (Balu et al., 2008; Castren, 2009). Citalopram dosage was chosen based on previous evidence that 10mg/kg citalopram, delivered by i.p. injection to mice, increased BDNF levels in the brain cortex over long-term treatment (Balu et al., 2008). According to the neurotrophin depression theory, downstream activation of BDNF-regulated signaling pathways is the primary mediator of the SSRI anti-depressant effect (Russo-Neustadt & Chen, 2005). Enhanced serotonin and BDNF levels stimulate the formation of new neurons in the dentate gyrus and the SVZ, which are areas known to undergo adult neurogenesis (Jacobs, van Praag, & Gage, 2000; Schabitz et al., 2007). BDNF may also play a positive role in the survival and migration of SVZ-derived neural progenitors (Kozlov et al., 2007; Schabitz et al., 2007). The observed migration of progenitors to the peri-infarct region is a natural response to focal MCAO and this response may be enhanced by SSRI treatment (Li, Yu, Ogle, Ding, & Wei, 2008; Lindmark & Hamrin, 1988). In the present study. citalopram administration after ischemia also up-regulated BDNF protein expression in the peri-infarct cortex and enhanced the number of new neurons present in the peri-infarct region after 21 and 28 days treatment. Increased neurogenesis after MCAO may explain the sensorimotor improvement observed in mice and the functional recovery documented by several clinical trials with chronic citalopram treatment (Acler et al., 2009; Chollet et al., 2011; Mikami et al., 2011).

Neurogenesis and neuroblast migration are highly associated with the vasculature after stroke. It has been shown that stroke actually enhanced neurogenesis and neuroblast migration from the SVZ (Wang et al., 2012). Remodeling of microvessels in the peri-infarct region after stroke may play a role in the observed recruitment of newly born neurons from the SVZ to the peri-infarct region (Ohab, Fleming, Blesch, & Carmichael, 2006). Endothelial cell survival and vascular proliferation are enhanced by BDNF (Chen et al., 2003; Greene et al., 2009), which was increased with citalopram treatment. Vessel regeneration and protection are very important for the overall stroke outcome. In a recent clinical trial, pro-angiogenic plasma factors after stroke correlated with a better NIHSS outcome, where the presence of anti-angiogenic factors in patient plasma predicted a worse long-term recovery (Navarro-Sobrino et al., 2011). Vessel area in the peri-infarct region was significantly increased by citalopram. Increased vessel area may imply that there was less vessel damage in the citalopram treatment group. Previous studies have indicated that enhanced angiogensis promotes new neuron survival (Sun et al., 2003). Counting of BrdU-labeled vessels did not produce a statistical difference; however, there was a strong trend for more angiogenesis in the citalopram group. Augmented proliferation of new neurons and survival of these neurons may be due to larger vessel area in the peri-infarct region. This in turn, may contribute to regeneration of normal brain function and therefore improved sensorimotor function with citalopram treatment.

The current study tested whether administration of the SSRI citalopram after stroke would enhance neurovascular repair in the ischemic penumbra and accelerate sensorimotor functional recovery. We found that citalopram (10mg/kg, i.p.) enhances BDNF protein in the peri-infarct cortex and sensorimotor recovery in correlation with

increased neurogenesis and vessel representation in the stroke penumbra. Since SSRIs are FDA-approved, inexpensive, accessible, and safe for use by both depressed and non-depressed stroke patients (Gainotti, Antonucci, Marra, & Paolucci, 2001; Wiart et al., 2000), this treatment paradigm could potentially offer a new, safe avenue for stroke therapy.

LIMITATIONS

This study suggests that citalopram given 24h post-MCAO can enhance neurogenesis and angiogenesis with statistically significant sensorimotor recovery. However, there are limitations to this study, which may prevent the generalization of such results to humans. In humans, strokes are heterogeneous in nature and the damage can cause a variety of neurological deficits. The stroke model employed in this study produces a highly consistent, small infarct in the whisker barrel cortex and part of the motor cortex. The behavioral tests conducted were specific to these areas. Thus, the observed recovery was limited to deficits in these brain regions. Whisker deficits were not tested due to the high variability found when using behavioral tests, such as the corner test (Zhang et al., 2002).

While no acute changes to infarct size were observed with citalopram treatment, the effects of chronic treatment on infarct size were not tested. The TTC experimental procedure used in this study, does not accurately portray infarct size after 7d due to the increase in inflammatory cells that metabolize TTC (Dirnagl, 2010). Although not tested in the present study, nestin can be used as an indicator for infarct size

(Kronenberg et al., 2005). Thus, as a part of a future study, nestin staining could be utilized to visualize the ischemic border in animals chronically treated with citalopram.

Additionally, this study did not seek to isolate the effects of neurogenesis and angiogenesis; thus, these results cannot exclude the possibility that new neurons and enhanced vascularization are not both necessary for the observed behavioral recovery. Further testing is necessary to elucidate the specific mechanism responsible for the observed recovery.

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