Social interaction plays a critical role in neurogenesis and recovery after stroke

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Stoke survivors often experience social isolation. Social interaction improves quality of life and decreases mortality after stroke. Male mice (20–25 g; C57BL/6N), all initially pair housed, were subjected to middle cerebral artery occlusion (MCAO). Mice were subsequently assigned into one of three housing conditions: (1) Isolated (SI); (2) Paired with their original cage mate who was also subjected to stroke (stroke partner (PH-SP)); or (3) Paired with their original cage mate who underwent sham surgery (healthy partner (PH-HP)). Infarct analysis was performed 72 h after stroke and chronic survival was assessed at day 30. Immediate post-stroke isolation led to a significant increase in infarct size and mortality. Interestingly, mice paired with a healthy partner had significantly lower mortality than mice paired with a stroke partner, despite equivalent infarct damage. To control for changes in infarct size induced by immediate post-stroke isolation, additional cohorts were assessed that remained pair housed for three days after stroke prior to randomization. Levels of brain-derived neurotrophic factor (BDNF) were assessed at 90 days and cell proliferation (in cohorts injected with 5-bromo-2′-deoxyuridine, BrdU) was evaluated at 8 and 90 days after stroke. All mice in the delayed housing protocol had equivalent infarct volumes (SI, PH-HP and PH-SP). Mice paired with a healthy partner showed enhanced behavioral recovery compared with either isolated mice or mice paired with a stroke partner. Behavioral improvements paralleled changes in BDNF levels and neurogenesis. These findings suggest that the social environment has an important role in recovery after ischemic brain injury.

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INTRODUCTION
It is increasingly accepted that social interactions are critical for maintaining physical, mental and social well-being. Social isolation has been associated with increased blood pressure, elevated cortisol levels1, enhanced inflammatory and metabolic responses to stress,2,3 and modifications in transcriptional pathways linked with glucocorticoid and inflammatory signaling.4–7 Compared with individuals with social cohesion, isolated individuals have enhanced susceptibility for the development of cardiovascular disorders,8 infectious diseases,9 cognitive decline10 and increased risk of cerebrovascular disorders including ischemic stroke.11 Stroke is now the fourth leading cause of death in the United States.12 Stroke-related mortality has dropped over the past 10 years, primarily due to improvements in medical care and acute stroke treatment, but this has not been accompanied by a decrease in the incidence of ischemic stroke. This is alarming, as stroke is the leading cause of chronic disability in adults, and increases in the number of stroke survivors will further escalate health care costs over the next several decades as our population ages.12,13 Therefore, any potential opportunity to reduce stroke-related disability and improve functional outcome would have tremendous public health impact.

Data from the US census show an increase in the number of people living alone since 1980s; a trend that is expected to continue.14 Individuals who report lack of social support or isolation have an increased incidence of recurrent stroke, poorer recovery, greater functional decline and higher mortality following a stroke compared with individuals with strong social support.15–18 As not all strokes can be prevented, more attention is needed to develop strategies to improve function after a stroke has occurred.

Attesting to the importance of social factors in stroke outcome is that these same effects can be reproducibly demonstrated in animals; social interaction has been shown to reduce histological damage from experimental stroke, whereas pre-stroke social isolation (SI) enhances injury.7,19,20 To date, previous studies have focused on isolation prior to, or at the time of, induction of injury.21–22 However, as most patients are not identified as ‘isolated’ until after their stroke, the examination of the effects of post-stroke isolation is important. In addition, as isolation increases infarct size, prior studies have been confounded by the larger infarcts seen in isolated animals. We hypothesized that social interaction will enhance functional recovery independently of infarct size and that the partner’s health status might have a role. To test this hypothesis, we utilized a novel delayed post-stroke housing paradigm to normalize infarct size between groups. The effect of post-stroke environmental manipulation on histological outcomes, IL-6 levels, chronic behavioral recovery and neurogenesis was assessed.

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MATERIALS AND METHODS

Experimental animals
Six-week-old C57BL/6 mice weighing 20–22 g were purchased from Charles River laboratories (Wilmington, MA, USA) and allowed to acclimate in groups of four for two weeks prior to any housing manipulation. All mice were maintained in a temperature- and humidity-controlled vivarium, with ad libitum access to food and water.

Housing conditions and experimental design
All experimental animals were screened for baseline locomotor activity in the open field. Mice were housed in standard mouse cages (11"L, 6"W, 6"H) with a 12-h light/dark schedule (lights on at 0700 h). Body weights were recorded and mice were pair housed (all mice were housed two per cage with an original cage mate) for 2 weeks before subjecting them to right middle cerebral artery occlusion (MCAO) or sham surgery. Animals were then randomly assigned to one of the three housing conditions—either housed isolated (SI), pair housed with a stroke partner (PH-SP) or pair housed with a healthy partner (PH-HP) either immediately after stroke (cohort-1) or three days after MCAO (cohort-2). In the first cohort, two endpoints were assessed; a three-day survival group was used for neurological deficit scores (NDS), infarct analysis and changes in serum interleukin-6 levels; a 30-day survival group that was used to assess mortality rates (see Figure 1a). A second cohort (cohort-2) was used to study delayed post-stroke housing effects with two different endpoints at 8 and 90 days. Animals were tested in the cylinder, corner and on the tail suspension test (TST) (see Figure 2a for timeline of behavioral testing) and sacrificed at day 90, for BDNF analysis. In the 8-day endpoint, mice were injected with BrdU from day 3 and analysis was performed 24 h after the last injection (day 8), an additional sub-group of mice was allowed to survive for 90 days to confirm neuronal maturation.

All animals were given wet mash for the initial 72 h after stroke or sham surgery. As mortality is higher when chronic endpoints are assessed, all animals that had planned survival endpoints over 3 days were injected with 100 μl of saline subcutaneously for 5 days after surgery. This prevents weight loss and reduces mortality, both stroke and sham mice were administered injections to control for any potential stress of handling or repeated injection. Quantification of infarct volume and all behavioral tests were performed and recorded by an investigator blinded to housing conditions. All behavior tests were performed between 0800 and 1100 h, immediately at the beginning of the light cycle after acclimatizing mice to the testing room lighting conditions. Testing chambers were cleaned with 70% ethanol between mice.

Exclusion criteria
If any mouse did not gain at least one gram of weight during the 2 weeks of pre-stroke pair housing, lost weight during the two weeks of PH, or exhibited signs of fight wounds due to dominant/aggressive behavior of the partner, both mice were excluded from the study as social aversion/stress can also influence stroke outcome.\(^{4,23,24}\) If one mouse of the pair dies at any point during the experiment, that pair is excluded from further study.

Middle cerebral artery occlusion model
Focal transient cerebral ischemia was induced by right MCAO followed by reperfusion as described previously.\(^{7}\) Core body temperature was monitored with a rectal probe connected to a temperature control system (Fine science tools, North Vancouver, BC, Canada). Temperature was maintained with an automatic heating pad at ∼37 °C during surgery and ischemia. Cerebral blood flow measurements by laser Doppler flowmetry confirmed ischemia during MCAO (to <80% of baseline) and restoration of flow during reperfusion in all cohorts.

Infarct analysis
Following 72 h of reperfusion, all animals in cohort 1 were euthanized by pentobarbital overdose, brains were removed and cut into five 2-mm...
coronal sections and stained with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 38 °C. Slices were formalin-fixed (4%) and then digitalized and infarct volumes analyzed (Sigma Scan Pro, San Jose, CA, USA) as previously described. The final infarct volumes are presented as a percentage (percentage of contralateral structures with correction for edema) as in Venna et al. For histology and immunohistochemistry, brains were collected from additional chronic survival cohorts. Animals were deeply anesthetized and perfused transcardially with ice-cold sodium phosphate-buffered saline followed by 4% paraformaldehyde. Brains were deeply anesthetized and perfused transcardially with ice-cold sodium phosphate-buffered saline followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose.

Neurological deficit scores
Neurological deficit scores were obtained in the intra-ischemic period and at 72 h post-stroke in short-term survival cohort-1. The scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to one side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side and circling immediately when placed on a bench; and 4, no spontaneous locomotor activity or barrel rolling as described previously as in Venna et al.

IL-6 ELISA
Serum was collected from the mice sacrificed at 72 h post-stroke in mice subjected to the three housing conditions (SI, PH-SP or PH-HP) immediately after stroke. Interleukin-6 (IL-6) levels were examined utilizing an enzyme-linked immune absorbent (ELISA) assay (eBiosciences, San Diego, CA, USA) per manufacturer’s instructions.

Cylinder test
The cylinder test was used to assess forelimb use after stroke. The mouse was placed in a transparent Plexiglas cylinder 9-cm diameter and 15 cm in height. A mirror was placed behind the cylinder with an angle to enable the rater to record forelimb movements when the mouse was turned away from the rater. A total of 20 forelimb placements were counted for analysis as previously described.

Corner test
Following the cylinder test animals were tested in the corner test as described in (Venna et al.). The mouse was placed between two pieces of cardboard, each with a dimension of 30 × 20 × 1 cm. The two boards were gradually moved to enclose the mouse from both sides to encourage the mouse to enter into a corner of 30° angle. After vibrissae stimulation, the mouse rears forward and upward, then turns back to face the opening. Twenty trials were performed for each mouse and the percentage of right turns was calculated.

Open field
Mice were placed in an open field chamber (15” × 15”) equipped with 16 infrared beam emitting LEDs. The total number of beam breaks were automatically registered by a computer-operated PAS Open Field system (San Diego Instruments, San Diego, CA, USA). The total number of beam breaks during a 20 min session was analyzed as a measure of spontaneous locomotor activity.

Tail suspension test
Animals were returned to their home cages after open field analysis and allowed free access to food and water at least for 30 min before testing them in TST. TST was performed as in Venna et al. Mice were suspended horizontally by the tail to a metal bar using adhesive scotch tape for a total duration of 6 min. The test was analyzed and the times of active escape behavior (mobility) versus passive behavior with no limb or head movements (immobility) was recorded. Latency to first immobile period was also quantified and is reported as latency. Behavior was scored manually by an experienced rater blinded to housing condition.

BrdU Treatment
Separate cohorts of mice were injected intraperitoneally with 75 mg kg−1 per day of BrdU once daily during days 3–7 after stroke. BrdU (Sigma Aldrich, MO, USA) was dissolved in 0.007 N NaOH in 0.9% NaCl. Mice were either sacrificed on day 8 or on day 90 (n = 4) to confirm neuronal maturation. BrdU+ cell counts were performed on stained sections from perfused brains (n = 8 per group) as detailed below.
Buckinghamshire, UK) was used for signal detection. Densitometry was performed using BDNF (1:200; Millipore) and actin (1:5000; Sigma-loading control) at 4 °C. Tissue sections were incubated with primary antibodies overnight and then incubated for 1 h in blocking solution (10% normal goat serum in PBS). After blocking, sections were incubated with Alexa Fluor 594-conjugated anti-rat and Alexa Fluor 488-conjugated anti-mouse secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 h. Sections were washed in PBS three times and then incubated in DAPI solution (1:1000) for 5 min. Sections were then washed in PBS three times and cover slipped. Sections were visualized using a Nikon Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

Western blot

Brains were rapidly extracted; hippocampi were isolated from brains and were immediately flash frozen. Protein concentration was determined using a Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples were boiled in 1X Laemmli buffer (200 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.01% bromophenol blue). Proteins from 10 µg per well were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% fat-free milk in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween) and incubated with primary antibodies overnight at 4 °C. Membranes were then washed in TBST three times and incubated with HRP-conjugated secondary antibodies (1:5000; General Biological Supplies, Cleveland, OH, USA). Membranes were washed in TBST three times and developed with enhanced chemiluminescence reagents (ECL detection kit, Amersham Biosciences, Buckinghamshire, UK) for 1 minute. Membranes were then washed in TBST three times and exposed to X-ray films. The total numbers of cells were counted using MacBiophotonics ImageJ software (Hamilton, ON, Canada). The total numbers of cells per field were counted using a blinded investigator and the average number of cells was used for statistical analysis.

Statistics

Data were expressed as mean ± s.e.m. except for NDS, which was presented as median (interquartile range). Statistics were performed using SPSS version PASW17. One-way analysis of variance (ANOVA) for analysis involving more than two independent groups, Bonferroni post hoc for multiple comparisons, repeated measures for cylinder and corner tests, a Z-test for proportions was used to analyze survival rates and the Friedman test with Bonferroni correction used for repeated measures ordinal data (i.e., NDS). A probability value, P < 0.05 was considered to be statistically significant. Each dyad was treated as one individual sample for statistical analysis. Investigators performing MCAO, behavioral and infarct size analysis were blind to housing conditions.

RESULTS

Infarct analysis

To examine whether post-stroke housing manipulation influences stroke outcome, all mice were initially pair housed for two weeks prior to a 60-min MCAO and assigned to one of the three ‘immediate post-stroke’ housing conditions: (1) Housed in a cage individually (SI), (2) paired with its original partner also subjected to stroke surgery (PH-SP) (3) paired with its original partner (not subjected to stroke) (PH-HP) (Figure 1a). Regardless of the partner’s health condition, pair-housed mice had significantly smaller infarcts 72 h after stroke compared with SI cohorts in the cortex F(2,26) = 5.12, P < 0.05; striatum F(2,26) = 7.24, P < 0.01 and in the total hemisphere F(2,26) = 13.03, P < 0.01. No significant differences were seen in infarct volumes between PH-HP and PH-SP cohorts; the mean difference (MD) in cortex = 4.3, P = 0.644; MD striatum = 0.344, P = 0.997 and MD total = 1.56, P = 0.93 (Figure 1c).

Neurological deficit scores and serum IL-6 levels

The detrimental effects of SI were also reflected in the NDS, Friedman test for repeated measures revealed no recovery in SI mice χ²(1) = 0.0001; P = 1.0, but a significant recovery in both PH-SP χ²(1) = 10, P = 0.002 and in PH-HP χ²(1) = 9; P = 0.003 (Figure 1d). SI mice had significantly elevated serum IL-6 compared with PH cohorts after stroke F(2,21) = 16.38, P < 0.05. (Figure 1e), post hoc tests revealed no significant difference between PH-SP and PH-HP groups; P>0.05. Sham mice has no significant changes in serum IL-6 levels F(2,13) = 0.64, P = 0.54. These results revealed a similar pattern to that previously seen in mice exposed to pre-stroke SI with isolated animals sustaining larger infarcts, an enhanced inflammatory response and poorer functional recovery than animals housed together.7,19,20

Exclusion and mortality rates

A total of 4% of original mouse pairs were excluded at the beginning of the study due to partner aggression as per pre-set exclusion criteria. In cohort-1 (immediate post-stroke isolation) SI mice had significantly higher mortality (61%) compared with PH-SP (39%); P < 0.05. PH-HP mice had only a 17% mortality (P < 0.01) (Figure 3) at 30 days. These findings suggest that interactions with a healthy partner might offer additional benefit and improve post-stroke survival. These effects appear to be independent of the effects on acute infarct damage, which was equivalent between the two cohorts of PH mice. Survival rates in cohort-2 (delayed post-stroke isolation) were higher as post-stroke care was more aggressive, but SI mice still had significantly higher mortality (20%) compared with PH-SP (10%); (P < 0.05), and in PH-HP (3%); (P < 0.001).

Functional recovery is independent of infarct size and associated with interactions and health of the partner

One possible explanation for the improved recovery in PH mice is that infarct damage is less due to the acute neuroprotection induced by immediate post-stroke housing. To investigate the specificity of relationship between social interaction, partner’s health and post-stroke recovery, we subjected mice to a ‘delayed post-stroke’ housing paradigm (Figure 2a). As infarct damage is complete in this stroke model by 24 h,31 we allowed the infarct to mature prior to housing in an attempt to normalize infarct size between groups. All mice were kept pair housed for 72 h immediately after stroke. In this way, recovery can be separated from the degree of histological damage, which will be equivalent...
in all cohorts. Mice were then placed back with its previous partner who was either a ‘healthy’ or ‘stroke’ mouse or maintained in isolation (SI). Long-term recovery was assessed with both the cylinder and corner test. Repeated measures ANOVA revealed that there were significant asymmetries (reduced contralateral forelimb use) in the cylinder test in stroke cohorts compared with sham animals regardless of housing condition. There was an overall significant effect of surgery F(1,44) = 309.09, P < 0.01 (Figure 2b), an overall significant effect of housing F(2,44) = 18.3, P < 0.05. There was also significant surgery-by-housing interaction, F(2,44) = 18.83, P < 0.01 suggesting differences in recovery. Independent analyses at each time point (days 3-28) revealed that these observed effects are due to more rapid and complete forelimb motor recovery in PH-HP mice compared with PH-SP; P < 0.05. SI mice had significantly reduced contralateral limb use compared with PH cohorts even at day 28 (*P < 0.01) (Figure 2b). Sham operated mice had no significant differences between groups. Figure 2c illustrates the results of mean non-impaired side turn percentages using the corner test that showed a consistent pattern of recovery. A significant effect of day-by-surgery F(1,44) = 53.73, P < 0.01 was seen, due to the fact that there was an early reduction in turning preference in PH-HP compared with PH-SP; SI mice had a continued turning preference at day 29 compared with PH cohorts. There was also significant surgery F(1,44) = 321.22, P < 0.05 and a housing F(2,44) = 8.87; P < 0.01 for the entire testing period.

Long-term social isolation reduced mobility in tail suspension test and reduced hippocampal BDNF levels. To explore the role of social interaction on depressive phenotypes, mice were subjected to the ‘delayed post-stroke’ housing paradigm detailed above (Figure 2a). When changes in mobility using the TST were evaluated, there was a significant difference between groups F(3,32) = 23.73, P < 0.01. Bonferroni post hoc analysis revealed significant differences between groups F(3,32) = 23.73, P < 0.01. Bonferroni post hoc analysis revealed significant differences in latencies between SI and PH-SP (P = 0.02) and also a significant difference between PH-HP compared with PH-SP groups (P = 0.03). Pair housing significantly reduced the duration of immobility and prolonged the latency to the first bout of immobility compared with SI group (Figure 4b). Independent analysis showed no differences between sham groups, data are presented as pooled for sham groups (Figure 4). Spontaneous locomotor control in open field showed no differences between groups F(3,32) = 0.98; P = 0.42, suggesting that decreased mobility is not due to motor deficits (Figure 4c). Assessment of hippocampal brain-derived neurotrophic factor (BDNF) levels, an important growth factor for neuronal survival and maturation with known antidepressant effects, showed no differences in sham groups F(2,5) = 0.44, P>0.05; but revealed a significant difference between stroke groups F(3,28) = 20.12, P < 0.05. Bonferroni post hoc tests showed that there was a significant reduction of BDNF expression in SI compared with PH-SP (P = 0.008) and PH-HP (P < 0.001). BDNF levels in PH-HP were higher than PH-SP (P = 0.048) but not significantly different from pooled sham group, P = 0.9 (Figure 4d).

Functional recovery paralleled enhanced neurogenesis. Brains were analyzed for histological assessment at 8 days post stroke, no significant differences in infarct size between groups was seen (Figure 5a). Equivalent behavioral deficits were seen at day 3 post stroke prior to housing randomization. Groups (SI vs PH) significantly diverged in their recovery by post-stroke day 7. As ischemia is known to induce neurogenesis, which may be an important factor in functional recovery, we assessed stroke-induced cell proliferation on day 8 after stroke in the

![Figure 4](image-url)

**Figure 4.** Housing with a healthy partner significantly improved long-term behavior and enhanced BDNF levels compared with isolated mice or mice housed with a stroke partner. (a) SI mice showed a depression-like phenotype, expressing less mobility when tested at day 90 using the tail suspension test compared with PH-HP and shams. (b) SI mice also expressed a reduced latency to the first bout of immobility compared with PH-HP and shams. (c) All stroke mice expressed similar spontaneous locomotor activity when tested prior to TST. (d) BDNF levels were significantly reduced at day 90 in whole brain homogenates of SI mice. β-actin was used as loading control and densitometry data are presented as normalized ratio. *P < 0.05 compared with SI and **P < 0.05 compared with PH-SP. Error bars denote s.e.m. HP, healthy partner; PH, pair housed; SI, social isolation; SP, stroke partner.
subventricular zone in additional cohorts of animals injected with BrdU at days 3–7 after stroke. SI mice had significantly fewer BrdU+ cells compared with either PH cohorts F(2,96) = 60.98, P < 0.001 (Figure 5c). There was an additional effect in mice housed with a healthy partner (PH-HP) at day 8; MD 16.6, P < 0.01 (Figure 5c). Quantification revealed that SI mice had significantly fewer BrdU+ cells compared with either PH cohorts F(2,96) = 66.07, P < 0.001 in the infarct area. There was an additional effect in mice housed with a healthy partner (PH-HP) at day 8; MD 22.8, P < 0.01 (Figure 5d), these changes are consistent with the enhanced recovery seen in that cohort. When assessed for neuronal maturation on confocal brain sections obtained at day 90, we found that a number of BrdU+ cells exhibited markers of mature neurons (NeuN) (Figure 5f).

**DISCUSSION**

This study has established that early post-stroke interactions can reduce histological damage after ischemic challenge. These findings are consistent with the beneficial effects of pair housing observed in pre-stroke isolation models, and suggest that this may have important translational relevance for treatment of stroke patients. More importantly, this work shows that pair housing, even if delayed for days after stroke, leads to enhanced functional recovery after injury, even in the absence of a significant reduction in brain damage.
of histological damage. This novel finding suggests that there is considerable brain plasticity and capacity for repair, even days after injury, enhancing the translational significance of this work. Our results also demonstrate that housing stroke mice with a healthy partner significantly hastens functional recovery and reduces mortality compared with mice housed with a stroke partner, whereas isolated mice demonstrated the poorest recovery. These effects parallel increased cell proliferation and higher hippocampal BDNF levels.

In previous work, we have demonstrated that pre-stroke social isolation is detrimental. However, as stroke is an unpredictable event, manipulating social environments prior to an event in patients at risk is likely to be ineffective from a therapeutic standpoint. Therefore, it is initially wanted to determine if these same manipulations performed after injury would have similar effects at a time when patients would be presenting to medical care. We found that immediate post-stroke pair housing significantly decreased infarct volume, and this was seen in animals paired with either a healthy partner or a partner that also had been subjected to stroke. We further hypothesized that housing with a sick partner would reduce the benefit of PH while a healthy partner may further ameliorate beneficial effects of PH. When tested, no significant differences were observed between PH-HP and PH-SP in either the amount of histological injury or serum IL-6 levels. We also found a similar improvement in functional outcome, as measured by the NDS, regardless of the health of the cage partner. This suggests that the detrimental functional outcome, as measured by the NDS, regardless of the serum IL-6 levels. We also found a similar improvement in functional outcome, as measured by the NDS, regardless of the health of the cage partner. This suggests that the detrimental functional outcome, as measured by the NDS, regardless of the

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Confl cts of Interest
The authors declare no conflict of interest.
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