Intestinal epithelial stem cell transplants as a novel therapy for cerebrovascular stroke

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ABSTRACT

Almost 2/3 of stroke survivors exhibit vascular cognitive impairment and a third of stroke patients will develop dementia 1–3 years after stroke. These dire consequences underscore the need for effective stroke therapies. In addition to its damaging effects on the brain, stroke rapidly dysregulates the intestinal epithelium, resulting in elevated blood levels of inflammatory cytokines and toxic gut metabolites due to a ‘leaky’ gut. We tested whether repairing the gut via intestinal epithelial stem cell (IESC) transplants would also improve stroke recovery. Organoids containing IESCs derived from young rats transplanted into older rats after stroke were incorporated into the gut, restored stroke-induced gut dysmorphology and decreased gut permeability, and reduced circulating levels of endotoxin LPS and the inflammatory cytokine IL-17A. Remarkably, IESC transplants also improved stroke-induced acute (4d) sensory-motor disability and chronic (30d) cognitive-affective function. Moreover, IESCs from older animals displayed senescent features and were not therapeutic for stroke. These data underscore the gut as a critical therapeutic target for stroke and demonstrate the effectiveness of gut stem cell therapy.

1. Introduction

Stroke is the second leading cause of death worldwide with an estimated mortality of 5.5 million per year and chronic disability among 50% of survivors (Donkor, 2018). In addition to sensory and/or motor impairment that occurs immediately after stroke, stroke patients are also at a higher risk for depression, which negatively impacts both functional and cognitive recovery (Hama et al., 2007; Alexopoulos et al., 1997). Although the precise cause of post-stroke depression (PSD) and cognitive impairment (PSCI) is still not well-understood, a commonly accepted mechanism is inflammation, which has been shown to play a role in both diseases (Miller and Raison, 2016). Cognitive impairment after ischemic stroke is associated with elevated levels of pro-inflammatory cytokines in experimental (Silva et al., 2015) and clinical studies (Kulesh et al., 2018; Ader and Cohen, 1993). Relatedly, these neuropsychiatric effects may also occur due to stroke-induced secondary neurodegeneration, a term used to characterize histopathological changes that occur in regions of the brain that are remote from the ischemic area (Zhang et al., 2012; Seitz et al., 1999). In view of the fact that one third of stroke patients will develop dementia and that 2/3rd will develop cognitive impairment (Melkas et al., 2014), there is an urgent need for stroke therapies capable of improving short- and long-term disability.

Recombinant tissue plasminogen activator (tPA), the only FDA-approved drug for treating stroke, is recommended within a narrow treatment window (3 to 4.5 h after stroke) and has a limited success rate (Kilic et al., 1999). Despite identification of many molecular targets and treatments that block these targets, there has been little translational success (Suzuki and Nakano, 2018). Recent studies using neural progenitor cells as transplants have shown some promise in restoring function in stroke patients but also present certain challenges such as sourcing appropriate neural progenitors, limited survival and insufficient neuronal differentiation (Wei et al., 2017; Wang et al., 2020). In view of the emerging evidence of the gut-brain connection in stroke, gut-derived stem cells may present an innovative alternative to neural progenitor cells.
After stroke, up to 50 % of patients develop gastrointestinal complications such as hemorrhage, intestinal paralysis, bowel incontinence, and dysphagia, which may be partially responsible for poor neurological outcomes and increased mortality (Saji et al., 2019; Ali et al., 2020). Preclinical studies have also reported significant gut dysbiosis and permeability of the gut barrier after stroke (Park et al., 2019; Brichacek et al., 2020; Ahnstedt, et al., 2020; El-Hakim et al., 2021). Relatively, diseases such as inflammatory bowel disease, where intestinal barrier properties are affected, have been shown to increase the risk for dementia (Saji et al., 2019). Preservation of the intestinal epithelium and its cellular barriers are critical for containing gut constituents including microbes, inflammatory microbial products such as endotoxin and specialized immune cells whose secretions, such as IL-17A, are critical for maintaining the gut barrier. Release of these substances into circulation, however, can impair neural function. IL-17A, for example, plays a crucial role in promoting the inflammatory response and inducing secondary injury post-stroke (Zhang et al., 2021). Accordingly, therapies that target and ‘repair’ the intestinal epithelium could potentially ameliorate stroke disability.

The intestinal epithelial layer, organized as villus and crypts that directly face the gut lumen, is the fastest self-renewing layer of the gut and is renewed every 3–5 days (Barker et al., 2008; Clevers, 2013; van der Wath et al., 2013; Potten and Loeffler, 1990). This renewal is regulated by Lgr5 + stem cells (IESCs) that reside and proliferate at the base of the crypt. We tested the hypothesis that the IESCs from a healthy donor could be harnessed to repair the intestinal barrier and consequently improve stroke outcomes. Primary IESCs were obtained from healthy adult and middle-aged female and male rats and transplanted to animals of the same sex (but different age) after stroke. IESCs from adult (young) females or males transplanted to sex-matched middle-aged animals repaired gut architecture and decreased gut permeability with a concomitant and persistent decrease in circulating levels of LPS and IL-17A. Furthermore, IESC transplantation prevented both depressive-like behaviors and cognitive impairment in the chronic phase. Organoids from middle-aged animals showed a senescent phenotype and did not improve stroke outcomes when transplanted to adult hosts, indicating that the age of the donor is a critical factor for successful transplantation.

2. Methods

2.1. Animals

Adult (5–7 month old) and middle-aged (12 month old) male and female Sprague Dawley rats were purchased from Envigo Laboratories (IN) and maintained in a constant 12-h dark: 12-h light cycle with ad libitum food and water. All rat procedures were reviewed and approved by the Texas A & M University Institutional Animal Care and Use Committee in accordance with OLAW guidelines for the humane treatment of animals in research. Daily vaginal smears were obtained to confirm that middle-aged females were acyclic (Jezierski and Sohrabji, 2001). Adult/ middle-aged animals of both sexes were used as donor or host for transplantation studies. Rats were randomly assigned to the following groups: Sham (no MCAo), MCAo with vehicle transplant (saline), or MCAo with dispersed organoid (IESC) transplantation. Vehicle or transplant injections were given 4, 24, and 48 h after stroke (Supplementary figure (SF). 1). All animals were fed pelleted food (Harlan 8604 Teklad diet) for at least 4 weeks prior to their assignment to the study. To avoid 30 middle aged females (8 sham, 15 MCAo/Vehicle and 7 MCAo/transplantation), 23 adult females (5 sham, 10 MCAo/Vehicle and 18 transplantation), 29 middle aged males (6 sham, 13 MCAo and 11 transplantation) and 27 adult males (5 sham, 9 MCAo and 12 transplantation) were used for the acute study; 19 middle-aged females (6 sham, 6 MCAo/Vehicle and 7 MCAo/transplantation), 23, middle aged males (7 sham, 7 MCAo and 9 transplantation) were used for the chronic study.

2.2. Organoids culture

Primary IESCs were isolated from adult and middle-aged male and female rats using procedures described in (Booth and O’Shea, 2002). Briefly, animals were given an anesthetic overdose and the small intestine was exteriorized. A 20 mm segment starting from the proximal ileum was dissected and chopped into 2 cm2 pieces. The tissue was washed several times in ice-cold PBS containing 100 U penicillin, 100 µg/ml streptomycin, 25 µg/ml gentamycin and 0.5 mM dithiothreitol. The tissue was passed over a 40 µm mesh filter and washed with growth media containing DMEM, 2.5 % v/v FBS, insulin 0.25 U/ml, streptomycin, 25 µg/ml gentamycin, 5 µg and 10 ng/ml epidermal growth factor. Tissue debris remaining in the filter was discarded, and the effluent containing proliferative crypt structures was centrifuged at 200×g, for 4 min at 4°C. The resulting pellet containing isolated intestinal crypts was used for organoid culture. The pellet was suspended (200–500 crypts/50 µl) in Matrigel with equal volume of organoid basal medium containing 100x α-glutamine (Sigma-Aldrich, Catalog G7513, 20µl/ml), 1 M HEPES (Invitrogen, Catalog 15630-056, 20µl/ml), 50x B27-supplement (Invitrogen, Catalog 17504-044, 40ul/ml) and 100x N2-supplement (Invitrogen, Catalog 175024-048, 20ul/ml) and the crypt-Matrigel suspension was pipetted into a pre-warmed 24 well plate and incubated at 37°C incubator for 15–20 min to solidify the matrigel. The Matrigel was overlaid with 500ul of organoids culture medium (Lukovac et al., 2014) containing the basal medium with growth factors (1000x N-acetylcysteine (Sigma-Aldrich, A9165, 1 mM), 1000x murine EGF (Peprotech, 315-09, 50 µg/ml), 500x murine Noggin (Peprotech, 250–38, 50 µg/ml), 200x murine R-spondin 1 (R&D Systems, 3474, 100 µg/ml), 10 mM/ml of A8301 (Sigma-aldrich 70024–90-7) and 10 mM/ml Y-27632 (ROCK inhibitor, Stem cell Technologies, 72304). Culture medium was refreshed every 4–6 days after seeding.

2.3. IESC labelling and transplantation

Organoid cultures were washed and dispersed into single cells suspension using organoid dissociation reagents (Millipore, SCM300). Single cell suspensions were labeled with cell membrane labeling kit PKH67 according to kit protocols (Green fluorescent cell Linker kit, Sigma Aldrich PKH67GL) to enable detection in vivo. Each animal received 1.5 million dispersed organoid cells injected through the tail vein at 4, 24 and 48hrs after stroke. Approximately 350–400 organoids were used to obtain 1.5 million cells for each injection. This number was selected to approximate a previous study in a gastrointestinal disease model which used ~ 500 organoids for transplantation (Yui et al., 2012). IV injections were used since this represents a translationally tractable delivery route.

Dosing regimen: Pilot studies indicated that a single dose transplant at 24 h after stroke did not improve the infarct volume or sensory motor test (data not shown). Since the normal gut renewal cycle is 3–4 days, the transplant protocol was subsequently increased to 4, 24 and 48 hrs to provide a large supply of stem cells to overlap at least half the period of the gut renewal cycle. Every host received transplants from a unique donor for all of these experiments. The timeline of the acute and chronic transplantation experiment is shown in Supplementary Fig. 1.

2.4. Middle cerebral artery occlusion (MCAo)

Ischemic stroke was induced by intracerebral injection of endothelin-1 (ET-1) to the MCA as previously described (Selvamani and Sohrabji, 2010; Selvamani et al., 2014; Park and Sohrabji, 2016). Animals were observed every 6 h after stroke for the first 24 h and then twice daily until termination.

2.5. Infarct volume

Infarct volume analysis was determined using our previous
procedures (Selvamani and Sohrabji, 2010). Images were coded and infarct volume was measured using image analysis software, Image J (NIH, MD), by an experimenter who was blind to the codes. Total brain infarct was calculated from 3 slices per animal and expressed as the ratio of infarct volume in the ischemic hemisphere to the total volume of the non-ischemic hemisphere.

2.6. Behavioral analysis

All testing was performed between 9 am and 1 pm to minimize the contribution of circadian cycles to behavioral variations, and males and females were tested on separate days to minimize the contribution of odorant cues. All tests were performed and scored by experimenters who were blind to the treatment condition. For assessing sensorimotor function, an adhesive tape removal test was performed before (Pre, – 2 days) and after (2 and 4 days) stroke. The chronic (long-term) behavioral analysis, animals were tested 4 weeks after stroke. The sequence of behavioral testing was Social Interaction, Novel Object Recognition Task, burrowing test and Barnes maze test. In general, all the three group (sham/Vehicle/IESCs treated) of animals were assessed on the same day for a particular behavioral test.

2.7. Sensory-Motor impairment tests

Motor impairment following MCAo was assessed using the vibrissae-evoked forelimb placement task (VIB) and the adhesive-tape removal test (ART) as described previously (El-Hakim et al., 2021; Balden et al., 2012; Selvamani and Sohrabji, 2017). The vibrissae-elicited forelimb placement test was performed prior to and 2 and 4 days after the MCAo surgery and was used to confirm cortico-striatal infarction. For the adhesive tape test, a piece of adhesive backed foam tape was used as tactile stimuli attached to the palmar surface of the paw of each forelimb in succession. For each forelimb, the time it took to remove the stimulus (tape) from the forelimbs was recorded during three trials per day for each forepaw. Animals were allowed to rest for 2 min between sessions, and each test session had a maximum time limit of 120 s.

2.8. Social interaction test

A three-chambered Plexiglass box was used for the assessment of social interaction, using our previous protocols (Panta et al., 2019; Panta et al., 2020). Test animals were habituated in the apparatus for 5 min. Animals were returned to the home cage and were tested after an hour. For testing, an age-matched conspecific, untreated same-sex stranger rat was placed within a plastic mesh cylinder in one of the end chambers, while the test rat was placed back in the middle chamber and allowed to explore for 10 min. The 10 min trials were video recorded for analysis. Sociability was scored as the total time (in seconds) spent by the test rat in the chamber with the stranger rat.

2.9. Novel object recognition task (NORT)

Object recognition memory was tested as described previously (Panta et al., 2019, 2020). The animals were habituated in a plexiglass chamber (16′′ × 16′′) and then trained with two identical objects (odor-free plastic animal toys), (A + A) placed in opposite corners of the chamber, for 10 min. Animals were returned to the home cage and were tested after an hour. For testing, animals were placed in the chamber with two objects in the same location as before, one that was previously available (A) and the other that was novel (B). The 5 min trials were video recorded for the analysis. The amount of time spent exploring the novel object was determined from these recordings by an investigator blind to the experimental condition. Exploration of an object was defined as the animal’s snout directed to the object, sniffing or touching the object with its snout at a distance < 2 cm to the object and/or touching it with the nose. The discrimination index was calculated as follows: DI: [time spent with novel object] - [time spent with familiar object] / [time spent with novel object] + [time spent with familiar object].

2.10. Burrowing test

Burrowing was assessed pre and post MCAo as described previously (Deacon, 2006). Pre-MCAo, the rats were first habituated to the burrowing task, by placing the test animal overnight in a new cage with a tube filled with woodchips. The next morning, the rats were returned to their original double-housed cages with their original cage mates. For testing, the tubes were refilled with wood chips and weighed. Rats were placed individually into the burrowing cages overnight with these tubes, and the tubes were reweighed the following morning. The percent of woodchips burrowed (displaced) was calculated as the difference between the pre and post weight of the tube normalized to the pre-weight. Post-MCAo, the test was performed similarly, except the rats were returned to their original single –housed cages after habituation and testing.

2.11. Barnes maze

This test was performed to assess spatial memory as described previously (Rosenfold and Ferguson, 2014). A circular maze (diameter of 48′′) consisting of 20 holes was used. Each hole consisted of either a small square box (19 total; 4′′ × 4′′ × 2′′) or one bigger escape box (8′′ × 4′′ × 4′′). The test was divided into two phases spanning 8 days: habituation (1 day) and learning (4 days), a 2-day break and then a probe trial (1 day). For habituation, rats were placed in the escape box for 2 min covered with a lid. After 2 min in the escape box, rats were placed inside a dark tube at the center of the Barnes maze. Bright lights (for aversion) were turned on and the center tube gently lifted off allowing the rats to freely explore the maze for a maximum of 5 min to find the escape box. If the rat did not find or enter the escape box in 5 min during the day of habituation, the experimenter manually guided the rat to the escape box. Once the rat entered the escape box, lights were turned off. The next day, during the learning phase, the escape box was placed at a fixed location under the escape hole (goal), and the rats were allowed a maximum of 2 min to find the escape. Each rat underwent three trials at an interval of 15 min, every day for 4 days. If the rat did not find the escape box within the 2 min, they were guided to it and the lights were turned off. After a two-day break, a probe trial was performed where the escape box was replaced with a small square box and the rat explored the maze for 2 min to assess spatial reference memory. Ethovision software (Noldus) was used to analyze the latency to find the escape box as well as velocity and distance traveled during each trial of the learning days. The probe test was analyzed for time spent in the target quadrant, which was where the escape box was located during the 4 learning days.

2.12. ELISA assays

Serum was collected via saphenous draw at baseline and 4 days post-stroke. ELISA assays were used to determine LPS (Mybiosources, MBS268498), MUC-2 (Mybiosources, MBS 2019254) and GFAP (Millipore, M8830). The procedure was performed according to the manufacturer’s directions and our published procedures (El-Hakim et al., 2021; Okoreeh et al., 2017). Plates were read on a microplate reader (450 nm; TECAN, VT) and the concentration of the samples was obtained by interpolation from the standard curve. Levels of a panel of inflammatory cytokine/chemokine in the serum were quantified using a rat cytokine/chemokine panel which detects 27 analytes (Millipore, MA). The procedure was performed according to the manufacturer’s directions and our published procedures (El-Hakim et al., 2021).
2.13. Gut permeability analysis

Gut permeability test was performed at 4d post stroke as described in our previous published protocol (El-Hakim et al., 2021). Size-graded dextrans labeled with either fluorescein isothiocyanate (FITCD, 10 kDa) or rhodamine (RhoD, 70 kDa) were administered by oral gavage (60 mg/100 g of body weight) to animals with Sham/MCAo/IESCs treated rats. Only females were used for this study. All rats received the oral gavage at 4 days post-stroke, and blood was collected from the tail tip at 60, 90, and 120 min later. Blood samples were stored in the dark at 4°C for 4 h. Samples were then centrifuged for 2 min at 1200 rpm, and the supernatant was diluted into 1:1000-fold with 1XPBS. Prepared samples were added to a 96-well microplate to determine the concentration of fluoro-
recently labeled dextran in the serum by spectrophotometer (Tecan, USA) with an excitation frequency of 490 nm and emission of 520 nm for FITCD-10 kDa, and excitation frequency of 540 nm and an emission of 625 nm for RhoD-70 kDa. Each assay plate also had known quantities of serially diluted FITC-dextran and Rho-dextran (5, 10, 100, 200, 300, 400, 500 and 600 ng/ml) standards. The plasma from a naive rat (not administered with labeled dextran) was used to determine the background.

2.14. Gut histology

At termination, a portion of the distal ileum was dissected, fixed in 4% PFA, and embedded in Cryo-OCt compound. Cryosections (10 μm) were collected on glass slides and analyzed for immunohistochemistry, hematoxylin and eosin (H&E) staining and periodic acid-Schiff staining as previously described (Kumar et al., 2017). Sections were imaged and coded, and then scored by two blinded investigators on a predefined scale from 1 to 5 according to staining localization and consistency but only one investigator’s scores were used.

2.17. Lgr5+, γH2AX, Wnt3, and DKK1 quantification

Organoid cultures immunostained for γH2AX, Wnt3, and DKK1 were imaged using the FSX100 Cell Imaging System or the FV3000 confocal system. In each case, 100 cells (identified by the nuclear dye DAPI) were randomly selected by an investigator blind to the conditions, and the proportion of these cells that co-localized with γH2AX, Wnt3, and DKK1 were quantified. For Lgr5, all cells in the field of view were counted for DAPI and Lgr5 using Image J, to estimate the proportion of Lgr5 + cells.

2.18. Periodic acid-Schiff (PAS) stain

Cryosections were collected on glass slides and washed and fixed prior to PAS staining protocol (Sigma-Aldrich, 395B) using our established procedures (El-Hakim et al., 2021). Slides were air-dried and cover slipped using DPX media. Sections were imaged on the FSX100 microscope.

2.19. Statistical analyses

Data were primarily analyzed using parametric statistical tests (Prism Graphpad). Survival plots were calculated using the Kaplan-Meier test. For acute behavioral tests, as well as NORT and the burrowing test, data was analyzed by 2 way ANOVA coded for repeated measures. Planned comparisons of pre- and post-stroke scores were analyzed by a paired Student’s t test. For all other comparisons, an unpaired Student’s t test or a one-way ANOVA were used. Group differences were considered significant at p < 0.05 in each case. All data are expressed as mean ± S.E.M. Specific animal numbers used for an assay is described in each figure legend.

3. Results

3.1. Characterization of organoids

Organoids prepared from adult (5–7 month old) or middle-aged (12 month old) female (Fig. 1a–c) and male (Fig. 1d–f) rats were grown in culture for 4–6 days. All organoid cultures showed similar patterns of expression for the epithelial cell plasma membrane marker NaKATPase-α and the proliferation marker Ki67 by 4 days in vitro (Fig. 1b, e, f [male]). In contrast, middle-aged organoids showed low expression of Wnt3a, which is important for maintaining proliferation of Lgr5 + stem cells and elevated expression of DKK1, a potent negative regulator of Wnt signaling (Niida et al., 2004), as compared to adult animals (Fig. 1g, h). In adult organoid cultures, 64.1% of cells expressed Lgr5+, while 43.9% of cells expressed Lgr5+ in middle-aged-derived organoids (Fig. 1j, p < 0.013). Furthermore 36.8% of cells expressed Wnt3a in the young organoid cultures, while <1% of cells expressed this protein in middle-aged-derived organoids (Fig. 1k). In contrast, only 7% of the cells expressed DKK1 in adult-derived cultures as compared to middle-aged-derived cultures, where DKK1 was expressed by > 75% of cells (Fig. 1l). Expression of γH2AX, a marker of DNA damage, was virtually undetectable in organoids derived from adult males and females (Fig. 1m), while 27.5% of cells in organoids from middle-aged rats expressed this marker (Fig. 1m). Collectively, these data indicate a loss of proliferative capacity and a potential senescent phenotype of organoids derived from middle-aged animals as compared to younger adults.

3.2. Adult IESC transplanted to middle-aged hosts home to the gut

Prior to assessing the therapeutic potential of IESC, we determined the location of transplanted IESC when injected into stroke animals. Dispersed organoid cells were labeled with PKH67 and injected into the tail vein iv at 4, 24, and 48hrs after middle cerebral artery occlusion.

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Several tissues, including the brain, liver, spleen and gut, were visualized. No PKH67-labeled cells were detected in the brain, liver and spleen (SF. 2), however, PKH67-labeled IESCs were clearly observed in the ileum. As shown in Figs. 2 and 3, PKH67-labeled IESCs (indicated by red arrows, Fig. 2aiii, bii and Fig. 3aiii, bii) are visible at the base of the crypts in sham and MCAo + IESC transplanted males and females, while vehicle-treated stroke animals exhibit patchy staining of Wnt3a and Lgr5 (Figs. 2 and 3aii, bii). Moreover, IESCs were co-labeled with Wnt3a (Fig. 2aiii, yellow arrows) and Lgr5+ (Fig. 3aiii, bii, Yellow arrows) indicating IESC proliferation potential and homing into the basal part of the crypt as active stem cells (Fig. 2aiii, biiii & Fig. 3aiii, biii). Qualitative assessment of Lgr5+ staining by blind scorers confirmed that the

**Fig. 1.** Characterization of organoid cultures and age differences in IESC phenotype: Organoid cultures derived from adult female (a-c) and male rats (d-f). Representative bright field images (n = 6) of IESC, plated in matrigel and cultured for 4–6 days (a,d). Scale bar: 25 μm. Immunohistochemistry (n = 6) for the intestinal stem cell marker Lgr5+, the epithelial cell marker NaKATPase-α, and the cellular proliferation marker Ki67 all at 6 div in females (b,c) and males (e,f). Scale bar: 22 μm. Representative images (n = 5) for the Wnt signaling marker, Wnt3a (g) and its inhibitor, DKK1 (h), and the senescence marker γH2AX (i) in organoids derived from adult and middle-aged males and females. Scale bar: 32 μm. Histogram depicting mean (±SEM) proportion of cells positive for Wnt3a (n = 5) (j), DKK1 (n = 5) (k) and γH2AX (n = 5) (l). Two-way ANOVA followed by Tukey-multiple comparisons test. *: p < 0.05.
pattern of Lgr5+ staining was improved in stroke animals that received IESCs compared to vehicle group animals (Fig. 3c, d).

3.3. IESC transplants and stroke outcomes

The strategy to determine the impact of IESC transplants on stroke outcomes is shown schematically in SF. 1. Six studies were performed: adult-derived IESC transplanted to middle-aged hosts (male and female; acute and chronic), middle-aged-derived IESC transplanted to adult hosts (male and female; acute only). In each case, dispersed organoid cells were injected into the tail vein of animals iv at 4, 24 and 48 h after ET1 induced MCAo. Short-term sensorimotor behavior was assessed pre, 2d and 4d post stroke, while infarct volume was assessed at 4d post stroke. For long term assessment of stroke impairment, animals were tested using several behavioral assays 4 weeks after MCAo. Survival was monitored throughout.

3.4. IESC derived from adult donors transplanted to middle-aged hosts after stroke

Overall, adult donor IESC transplants significantly improved stroke outcomes in middle-aged hosts of either sex. As shown in Fig. 4, MCAo-induced mortality was significantly reduced in middle-aged female rats that received adult female IESCs ($p = 0.0089$) compared to the vehicle-treat stroke group (Fig. 4a). Infarct volume, assessed from TTC-stained coronal sections (Fig. 4b), was significantly smaller in the group that received the transplant (Fig. 4c, $p = 0.0048$). Sensory motor performance was evaluated in the acute phase by the adhesive removal test

![Fig. 2. Localization of IESC transplants after stroke and co-localization with Wnt3a: Dispersed organoid cells labeled with PKH67 (green) were injected into the tail vein of middle-aged female and male rats at 4, 24 and 48 h after ET1 induced MCAo. Representative images (n = 5) of Wnt3a (white arrows) and PKH67 labeled cells were localized to the crypt region of the distal ileum of middle-aged female (a-i & iii) and male (b-i & iii) rats subject to sham and stroke groups. No PKH67 label were seen in sham and vehicle-treated animals (a-ii & ii & b-i & ii). The proliferative marker Lgr5+ was virtually absent in males and females after MCAo + vehicle (a-ii & b-ii) and strongly present in sham and IESC-transplanted MCAo animals (a-i & iii & b-i & ii). Yellow arrows indicate the colocalization of Wnt3a and PKH67 labeling. Scale bar: 10 µm.](image-url)
ART) and the vibrissae-evoked forelimb placement task (VIB). Latency to remove the adhesive tape was similar in both groups prior to stroke but was significantly elevated 2d and 4d after stroke in the vehicle-treated group. In contrast, latency was virtually unchanged from baseline in the group that received IESC transplants (Fig. 4c).

Like middle-aged females, middle-aged males that received IESC transplants derived from adult males showed improvement in stroke outcomes, including reduced infarct volume and improved sensory-motor performance (Fig. 4g-j), although survival was not significantly improved after stroke.

3.5. IESC derived from middle-aged donors transplanted to adult hosts after stroke

In contrast to adult donor/middle-aged host studies, adult animals that received middle-aged organoids after stroke showed little improvement in most measures of acute stroke outcomes. Stroke-induced mortality was not reduced by transplanting middle-aged derived organoids to adults and no improvement was seen in infarct volume or the ART (SF. 3a-h). In females, but not males, there was some improvement noted in the VIB test (SF. 3d).
3.6. IESCs improve gut morphology after stroke

After stroke, the architecture of the gut deteriorates in both adult and middle-aged animals. We next determined whether IESCs transplants would have a restorative effect on the stroke-affected gut, as the brain. Gut morphology was assessed by immunohistochemistry for villin (Fig. 5), a marker for epithelial brush border cells as well as for gut renewal occurring via continual proliferation of crypt cells. In sham animals, the proliferation marker Ki67 is clearly expressed in the basal crypt region (Fig. 5 bi, females). IESC transplanted stroke animals (males and females) (Fig. 5 ai, females; 5bi, males) display clear villin staining in the brush border region (white arrows), indicating that these transplants promote gut repair. Qualitative assessment of villin staining by blind scorers confirmed that the pattern of Ki67 staining was improved in stroke animals that received IESCs (Fig. 5). In contrast to adult donor/middle-aged host studies, adult animals that received middle-aged organoids after stroke showed no improvement in gut morphology (SF. 4a&b).

Villus length to width ratio (**p = 0.00048 females; unpaired t-test; (MCAO + vehicle n = 8; MCAO + Adult IESC n = 8), p = 0.0007 males; (MCAO + vehicle n = 6; MCAO + Adult IESC n = 8)). Sensory motor function was evaluated by the adhesive tape test and vibrisseae evoked forelimb placement task (VIB) (d-j). Histogram depicting mean (±SEM) of the average latency to remove the tape pre and post stroke for the Adhesive removal test and % correct responses on the VIB test. MCAO + vehicle n = 8 (females)/6 (male); MCAO + Adult IESC n = 8 (females)/8 (males). "Main effect of time; "interaction effect. 2-way ANOVA followed by Tukey-multiple comparisons test. *p < 0.01; # main effect of stroke; & main effect of treatment; * interaction effect. one-way ANOVA followed by Tukey-multiple comparisons test. *p < 0.01.
transplanted dispersed adult organoids cells may stimulate gut renewal.

3.7. IESCs reduce gut leakiness after stroke:

Intestinal epithelia form tight junctions (TJs) that are essential to the function of the physical intestinal barrier, regulating the movement of ions, solutes, and water across the intestinal epithelium. TJ dysfunction is associated with metabolic and inflammatory diseases. Immunohistochemistry for the tight junction protein ZO-1 shows continuous expression of the protein at the brush border of the villi in male and female sham animals (Fig. 7a, left panel). This pattern is also well maintained in middle-aged males and females that received adult IESCs (right panel); however, in vehicle-treated animals the villus structure and the brush border was distorted after stroke (Fig. 7a, middle panel). Functional analysis of gut permeability was assessed by measuring the serum levels of two size-graded dextrans (10 kDa, 70 kDa) simultaneously delivered by oral gavage after stroke. Middle-aged female rats were gavaged with dextrans on 4DP, and blood samples were collected 60, 90, and 120 min later. In the case of FITC-D (10 kDa), there was a significant main effect of time and treatment ($F_{(2, 24)} = 8.078, P = 0.0021; F_{(2, 12)} = 5.119, p = 0.0247$). Serum FITC-D was detected at all time points (60 min, 90 min and 120 min) in each group. However, the levels of FITC-D were significantly higher in MCAo + vehicle treated group, compared to the Sham animals or the adult IESCs-treated MCAo group (Fig. 7b). In the case of RhoD (70 kDa), lower amounts of this dextran were detected in the serum as compared to FITC-D, likely due to its larger size. Like FITC-D, serum levels of RhoD were significantly elevated across all time points in MCAo + vehicle treated (interaction effect of time and treatment, $F_{(4, 24)} = 7.504, p = 0.0005$; main effect of time, $F_{(2, 24)} = 13.9, p < 0.0001$; main effect of treatment, $F_{(2, 12)} = 13.17, p = 0.0009$) compared to the Sham group or the MCAo + adult IESCs treated groups (Fig. 7b), indicating significant gut permeability after stroke that was reduced by stem cell treatment.

In addition to the epithelial barrier, the mucus layer was also assessed by histochemical detection using the PAS stain. Previously considered a lubricant to facilitate the progression of the food bolus, the mucus layer of the gut is now considered one of the first lines of defense of the gastrointestinal tract. In sham animals, dark PAS staining (Fig. 7c) corresponding to robust mucin expression is seen in most of the crypts, villus goblet cells, and enterocytes in the sham group of both females and males. In the vehicle-treated MCAo group (Fig. 7c, middle panel) corresponding to robust mucin expression is seen in most of the crypts, villus goblet cells, and enterocytes in the sham group of both females and males. In the vehicle-treated MCAo group (Fig. 7c, middle panel) weak expression of the PAS stain is seen along the damaged brush border of the villi and goblet cells, as well as the lamina propria and the submucosa, indicating erosion of mucus throughout the gut wall. In

![Image of histological sections showing villin and Ki67 expression](image-url)
transplanted groups, the villus structure is better preserved, and the mucus barrier appears similar to the sham group (Fig. 7c, right panel). Quantitative analysis of goblet cells, that secrete mucin, was lower in both stroke groups as compared to sham, and this was not regulated by IESC transplantation (Fig. 7d). We next tested serum mucin-2 levels, as a further index of gut permeability. Muc-2 (520 kD) proteins were significantly elevated in vehicle-treated MCAo groups (males and females) (Fig. 7e) after stroke as compared to the sham animals, confirming that the mucus barrier deteriorates after MCAo. In contrast, serum muc-2 levels were not elevated in the IESC treated stroke animals, consistent with the data that this treatment preserves the mucus barrier.

IESCs derived from middle-aged hosts transplanted to adult rats showed no consistent improvement in measures of gut permeability (data not shown). The preceding data confirm that gut dysmorphology and permeability is an early response to stroke and show that gut stem cell treatment have the potential to reduce gut permeability, thus decreasing systemic exposure to toxic gut metabolites and the secretions of gut resident immune cells.

We next tested the impact of IESC treatment on the levels of endotoxin LPS, an inflammatory gut metabolite also known to affect blood brain barrier permeability, and IL-17A, an inflammatory cytokine involved in LPS-induced neuroinflammation and cognitive impairment in aged rats, during the acute and chronic phase of stroke. As shown in Fig. 8a, serum LPS, GFAP and IL-17A levels were elevated in the acute (4d) phase of stroke in vehicle-treated female and male rats compared to age and sex matched shams (Fig. 8a). However, females and males that received adult IESC transplants after stroke had significantly lower levels of circulating LPS compared to vehicle treated MCAo animals (Fig. 8a), consistent with decreased gut permeability seen in other assays. Glial fibrillary acidic protein (GFAP), a surrogate marker of BBB permeability resulting from trauma (Nylén et al., 2007; Ren et al., 2016; Lumpkins, 2008), was assessed in the acute phase. GFAP was detected in all samples, however, levels of this protein were significantly elevated in vehicle-treated stroke animals, while adult IESC transplanted animals were no different from sham animals (Fig. 8a). This is consistent with other reports that a ‘leaky’ gut can release inflammatory mediators and immune cells into circulation, which are known to exacerbate neuro-inflammation by acting on the blood brain barrier (Sarkar and Banerjee, 2019; Braniste et al., 2014).

In the chronic phase (4 + weeks after stroke), both LPS and IL-17A were elevated in vehicle-treated MCAo animals, as compared to Sham animals. Furthermore, both analytes were significantly lower in the IESC-treated MCAo animals and were not different from Sham animals. These data suggest a persistent elevation of inflammatory mediators...
resulting from untreated stroke. In view of the evidence that IL-17A is implicated in depressive behaviors and cognitive impairment, we next determined whether IESC treatment would resolve long term affective-cognitive stroke consequences.

Affective changes: Post stroke depressive like behaviors were evaluated by the Burrowing test and the Social Interaction test. Both tests take advantage of species natural behaviors (Langford et al., 2010; Shepherd and Mohapatra, 2018).

Burrowing test: Burrowing is a rewarding activity for rats, hence a decrease in burrowing is interpreted as depressive behavior. This test was performed prior to stroke and 4 weeks after stroke. Removal or displacement of wood chips was estimated as the difference between the weight of chips at the start of the test and then 12 h later. Vehicle-treated animals burrowed significantly less after stroke (main effect of time: female: \( F_{(2, 32)} = 5.835, p = 0.0069 \); male: \( F_{(2, 40)} = 3.311, p = 0.0467 \)), while in sham and adult IESC-treated middle-aged rats, the percentage of wood chips displaced was not significantly different after stroke (Fig. 9a).

3.8 Social interaction

Reduced interaction with a conspecific is indicative of social disinterest and loss of social cognition (Panta et al., 2020). Rats were placed in the central chamber of a 3-chamber apparatus and allowed to freely explore the entire apparatus including the empty chamber and the chamber containing a same sex conspecific. Social behavior, obtained before (pre) and at 4 weeks after (post) stroke, was estimated by the time spent in the chamber containing the same-sex stranger rat (Fig. 9b). Pre-stroke, animals in all groups spent a similar amount of time with the conspecific. After stroke, vehicle-treated rats (males and females) spent significantly less time with the conspecific rat compared to pre-stroke times (\( p < 0.0001 \)), while the sham and adult IESC-treated rats were no different from their pre-stroke times (\( p > 0.98 \) in each case) (Fig. 9b).

Both tests indicate that stroke increases depressive-like behaviors, and that IESC treatment after stroke attenuates the expression of depressive-like behaviors.

3.9 Cognitive function: cognition was assessed by the novel object recognition test and Barnes maze test, which assess declarative and visuospatial memory respectively

3.9.1 Novel object recognition test

Preference for a novel object over a familiar object, measured by the amount of time spent exploring the object, was assessed before (pre) and 4 weeks after MCAo/sham surgery (Fig. 10a). Increased time spent exploring the novel object indicates retention of the memory of the familiar object and thus the ability to discriminate between the two objects (Panta et al., 2020). Preference was determined by the
discrimination index, where a positive number indicates a preference for the novel object. Initial assessment of Sham animals showed that stable DI values were obtained during a 3 min exploration window for females and a 2 min exploration window for males, hence these times were used throughout the pre and post assessments in all groups. Prior to stroke, all groups showed a similar level of preference for the novel object. Four weeks after stroke, a similar pattern was observed in females and males, such that vehicle-treated MCAo animals had significantly lower DI after stroke (females, \( p = 0.0012 \); males \( p = 0.001 \)), indicating impaired recognition of the novel object. In contrast, MCAo + adult IESC treated animals were no different from their pre-stroke levels (females, \( p = 0.893 \); males \( p = 0.244 \)). Sham animals were also no different when tested 4 weeks later (females, \( p = 0.9246 \); males \( p = 0.3861 \)). These data shows that early treatment with intestinal stem cells preserves long-term non-spatial cognitive memory in animals after stroke.

3.9.2. Barnes maze test

Spatial learning was assessed by latency to find an escape hole in a circular maze over 4-day training period. Latency is expected to decrease over the testing days, indicative of learning, while longer latencies indicate impaired ability to locate the escape hole. Overall there was improvement in latency across the 4 training days (main effect of time (female- \( F(2, 68) = 9.647, p < 0.0001 \); male- \( F(2, 84) = 50.5, p < 0.0001 \) (Fig. 10b), but there were differences due to treatment (main effect of treatment, female- \( F(3, 68) = 14.56, p < 0.0001 \); male- \( F(3, 84) = 5.087, p = 0.0028 \)). Planned comparisons showed that the latency to find the escape hole on the 4th day of training was significantly lower than the latency on the first day in the Sham group (females \( p = 0.0001 \), males \( p = 0.0011 \)), but was virtually unchanged in the MCAo + vehicle group (females \( p = 0.7988 \); males \( p = 0.994 \)), indicating poor...
learning in this group. Following acquisition training, a probe trial was administered 48 h later to assess spatial reference memory. In the probe trial, the escape hole was sealed and the time spent in the quadrant containing the escape hole was recorded. MCAo + vehicle treated animals spent significantly less time in the target quadrant as compared to Sham (females $p = 0.0001$, males $p = 0.0156$) which indicates poor recall of spatial memory after stroke. Vehicle treated animals also performed worse than MCAo + adult IESC-treated animals (females $p = 0.0001$, males $p = 0.0406$), which underscores the efficacy of adult IESC treatment.

Together, these data suggest that, irrespective of biological sex, adult IESCs transplantation therapy results in a profound neuroprotection in the ischemic brain of middle-aged animals, as well as gut repair and long-term behavior outcomes. The reduction of markers of gut leakiness and concomitant decrease in IL-17A further suggests a mechanism by which gut repair may promote brain health.

4. Discussion

Our main findings confirm that MCAo results in an early deficit in sensorimotor skills and long-term impairment in cognition, including spatial and non-spatial memory, as well as signs of depressive behaviors. Repairing stroke-induced gut dysmorphology using intestinal stem cells also improved acute stroke outcomes including reduced stroke-induced mortality, decreased infarct volume, decreased gut leakiness and improved sensory motor performance. Moreover, adult IESCs treatment during the acute stroke phase also abrogated cognitive deficits and depressive phenotypes induced by cerebrovascular ischemia, indicating that this early intervention has the potential for long lasting benefit. It is also worth noting that IESC from young males and females show similar expression of key proteins in organoid cultures and are equally effective in improving acute and and long-term effects of stroke when transplanted to middle-aged, sex matched hosts.

Although stem cell therapy for stroke is under vigorous investigation, as are gut stem cells for intestinal disorders, to our knowledge this is the first study to examine the effect of gut stem cells to repair brain injury. The most common transplants for stroke include mesenchymal stem cells (Sanberg et al., 2012), embryonic stem cells (Tae-Hoon and Yoon-Seok, 2012), neural stem cells (NSC) (Darsalia et al., 2007; Roitberg et al., 2006) and induced pluripotent stem cell-derived NSCs (Baker et al., 2017). Allogenic transplant of fetal porcine cells to humans with basal ganglia stroke showed improvement in a subgroup of patients (Savitz et al., 2005). Cell engraftment in the brain is very limited and while the exact mechanisms are under dispute, recent evidence suggests that rather than cell replacement, transplanted cells may provide growth factor support (De Feo et al., 2012). In the present study, labeled dispersed organoid cells home to the crypt, the site where endogenous stem cells reside, and display the stem cell marker Lgr5, suggesting that they could differentiate and contribute to epithelial repair.

The gut is an early responder to stroke (El-Hakim et al., 2021), occurring simultaneously with stroke-induced hyper permeability of the BBB (Bonfante and Genre, A.J.N.c., 2010). Stroke-induced gut permeability may precede many of the inflammatory events associated with disease (Arrieta et al., 2006), resulting in the transfer of gut resident immune cells into circulation and to nearby nodes, and even the brain (Iadecola et al., 2020; Mowat, 2018). Additionally, gut metabolites such as...
as LPS are also elevated in circulation. Converging evidence from histological analysis of the gut, serum biochemical markers and functional assays from oral gavage studies indicate that IESCs repair the gut and reduces gut permeability. This process may be central to neuro-protection and preserving cognitive function.

An intriguing observation from these studies is that IESC from ‘youthful’ organoid cultures were effective in improving stroke outcomes, while organoids from older animals were ineffective. This may be related to cellular senescence, which is the declining capacity of cells to proliferate after multiple divisions (Hayflick and Moorhead, 1961). Cellular senescence plays a crucial role in embryonic development (Storer et al., 2013; Muñoz-Espín et al., 2013), wound healing (Demaria et al., 2014) and tissue repair (Serrano, 2014; Fairley and Barraclough, 1966). Senescent cells accumulate in aging tissues and are associated with tissue inflammation and impaired tissue function. Stem cells are also susceptible to cellular senescence, as noted during in vitro expansion of mesenchymal stem cells (Squillaro et al., 2016). Aging decreases the functionality of stem cells, including the capacity for self-renewal, which impairs the balance between stem and differentiated cells (Wang et al., 2019). Many stimuli can elicit a senescence response including DNA damage, expression of oncogenes, dysfunctional telomeres, and chromatin disorganization (when bad things happen to good cells, 2007). While cellular senescence may occasionally be advantageous, it also reduces regenerative capacity and repair in aging. In the present study, organoids from middle-aged animals (irrespective of sex) had a lower proportion of Lgr5+ cells in the organoid cultures, and a disproportionately elevated levels of the DNA damage marker γH2AX, as well as reduced proliferative capacity indicated by Wnt3a and high DKK1 expression. Thus while Lgr5+ cells differed by ~20% in adult and middle-aged organoids, Wnt3a, and DKK1 expression was 36-fold and 10-fold different between the 2 ages, and γH2AX was only seen in the middle-aged group. Collectively, these data suggest that gut organoids display age-associated senescence, consistent with the lack of repair seen in animals that received those transplants. While a more systematic assessment of senescent markers in gut IESCs is needed, it is worth noting that brain astrocytes derived from the same middle-aged animal model also show senescence traits, such as an increased inflammatory phenotype and reduced growth factor synthesis (Lewis et al., 2008).

Renewal of the gut epithelium is driven by active Lgr5+ stem cells in the basal crypts. Freshly extracted crypts from a healthy donor can differentiate into the full complement of crypt and villus cells (Barker et al., 2007). Aging decreases the functionality of stem cells, including the capacity for self-renewal, which impairs the balance between stem and differentiated cells (Wang et al., 2019). Many stimuli can elicit a senescence response including DNA damage, expression of oncogenes, dysfunctional telomeres, and chromatin disorganization (when bad things happen to good cells, 2007). While cellular senescence may occasionally be advantageous, it also reduces regenerative capacity and repair in aging. In the present study, organoids from middle-aged animals (irrespective of sex) had a lower

![Fig. 10. Effect of adult IESCs on post-stroke cognitive impairment. Cognitive function was assessed by the Novel Object Recognition Test (NORT) and the Barnes Maze. (a) Histogram depicting the mean ± SEM of the discrimination index (DI) score pre-stroke and 30 days later in sham, MCAo + Vehicle and MCAo + Adult IESC groups. A positive DI indicates preference for the novel object. (b) Histogram depicting the mean ± SEM of the latency to find the escape hole on the Barnes maze over the 4 day training period. (c) Histogram depicting the mean ± SEM of the time spent in the target quadrant during the probe trial. The probe trial was performed 48 h after the last training day. Key: *: Main effect of time, #: main effect of treatment, ½: interaction effect. Planned comparisons indicated by brackets, #: p < 0.05. N = 7-8/group.](image-url)
menopause (or reproductive senescence in rodents) can potentially increase epithelial permeability and deterioration of the mucosal health (Grishina et al., 2014). Moreover, young IESCs from mice showed reduced organoid forming capacity when cultured together with the old Paneth cells, resulting partly from alterations in signals emanating from the aging Paneth cells (Pentimikko et al., 2019). The present study suggests that for stroke-induced gut repair, the ‘age’ of the donor cell, rather than the host, is the critical determinant. Part of this may be related to the regenerative capacity of these stem cells. Wnt signaling is critical for gut epithelial renewal, and age-related changes in this pathway are implicated in the reduced regenerative capacity of aging epithelium (Nalapareddy et al., 2017; Ijiri and Potten, C.S.J.T.B.j.o.c.S., 1986; Ashton et al., 2010). Wnt signaling is also critical for intestinal regeneration in injury models (Saha et al., 2016). Wnt3a maintains the proliferative capacity of Lgr5 + stem cells in various organs such as the small intestine, large intestine, stomach, pancreas and liver (Willert, 2008). Reciprocally, Lgr receptors potentiate Wnt signaling within IESCs following binding to the ligand, R-spondin. In this study, Wnt3a expression was profoundly reduced in organoids from middle-aged animals, and may explain the success of the younger IESC transplants where Wnt expression was higher and DKK1, a Wnt signaling inhibitor, was reduced.

Both human and preclinical studies have implicated IL-17A, the signature cytokine in a subset of CD4 T cells, in the progression of cognitive impairment and dementia (Chen et al., 2020). IL-17A is elevated in plasma (Chen et al., 2014), CSF (Hu et al., 2010) and mononuclear cells (Beharci et al., 2015) of patients with dementia. In the 3xTg-AD mouse, IL17A + cells were elevated in the brain, meninges and cervical lymph nodes at the onset of memory deficits (5-6 months), IL-17A infusion to wildtype mice induced short term memory deficits, while anti-IL-17A treatment improved cognitive performance in 3xTg-AD mice (Brigas et al., 2021). In the present study, IL-17A levels were elevated by stroke with a corresponding impairment in declarative and episodic performance, it is plausible that repairing the gut blood barrier with IESCs, could reduce extravasation of gut resident IL-17A + cells and metabolites such as LPS that stimulate IL-17A.

5. Conclusion

These data underscore the importance of early intervention after ischemia to prevent or alleviate stroke-induced cognitive impairment. It may be cautiously inferred that adult IESC treatment in the acute phase may preserve cognitive resilience, as IESC-treated rats are similar to sham rats (SF, 6, Graphical abstract). While these outcomes need to be assessed systematically, this preclinical study suggests that IESCs transplantation may be a viable alternative source for stem cells for stroke therapies. Future studies should include refinement of the dosing protocol, such as delaying the IESC treatment to achieve the desired effect. In addition, a systematic study of stem cell senescence would be valuable as a mechanistic explanation for the pathophysiology of severe stroke in older demographics. In view of the evidence that having a stroke increases the likelihood of subsequent strokes, it would also be informative to understand the impact of stroke on stem cell pools.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2022.10.015.

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