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Stress while lacking of control induces ventral hippocampal autophagic flux hyperactivity and a depression-like behavior

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ABSTRACT

Background: Stressed animals may perform depression-like behavior insomuch as stress-provoking blood–brain barrier (BBB) disruption, central immune activation, and autophagic flux changes. This study was undertaken to assess whether adult mice having (executive) vs. lacking (yoke) of behavioral control in otherwise equivalent stress magnitude condition, may display differences in their BBB integrity, ventral hippocampal (VH) interleukin-6 (IL-6) and autophagic flux level and VH-related depression-like behavior. To further understand the causative relation of enhanced autophagic flux and stress-primed depression-like behavior, we assessed the effects of bilateral intra-VH 3-methyladenine (3-MA), an autophagic flux inhibitor, infusion in stressed mice.

Methods: Adult mice used had comparable genetic background and housing condition. Executive/yoke pairs of mice received a 10-day (1 h/day) footshock stressor regimen. Throughout the regimen, the ongoing footshock was terminated immediately contingent on the executive mouse', while irrelevant to the respective yoke mouse' voluntary behavior, or lasting for 7 s. Each dyad's cage-mate receiving no such regimen served as no stressor controls.

Results: Yoke mice displayed disrupted BBB integrity (escalated Evans blue extravasation and decreased VH ZO-1, claudin-5 expression), increases in VH autophagic flux (increased LC3II/LC3I and decreased p62) and immobility duration in forced swimming test. Most of these indices remained unaltered in executive mice. Administration of 3-MA did not affect

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immobility duration in control mice, while prevented the increases in immobility duration in yoke mice.
Conclusions: (1) stress susceptibility may be determined by their differences in stress-coping results; (2) VH autophagic flux increase plays a permissive role in priming the stressed animals susceptible to exhibit depression-like behavior.

At a glance commentary

Scientific background on the subject

Stress has been known to evoke blood-brain barrier integrity disruption, central immune activation, autophagic flux changes and prospective depression-like behavior. It remains unknown whether stressed animals having behavioral control may demonstrate moderate changes in these indices compared to stressed animals without such control. Likewise, it remains argumentative whether stress-enhanced autophagic flux may induce depression-like behavior.

What this study adds to the field

Stress-evoked changes of central BBB protein expressions, autophagic flux and depression-like behavior may be mostly prevented by stressed animals' sense of behavioral control. Moreover, ventral hippocampal autophagic flux increase plays a critical role in priming stressed animals' depression-like behavior.

Blood–brain barrier (BBB) weakness has been suspected to play a prime role in allowing various forms of stimulus-induced central, especially hippocampal, neuroinflammation and -related emotional abnormality [1–4]. Likewise, stress is conjectured to induce central neuroinflammatory responses and depression-like behavior via weakening the BBB [5,6], suggesting that stress may result in disrupted BBB integrity alongside with central neuroinflammatory activation and depression-like proneness. With respect to central immune activation, local interleukin-6 (IL-6) level has been documented for its roles in modulating hippocampal autophagy and the related emotional function in recent literature. For instance, IL-6 activation may protect against depression-like behaviors in rat models, at least in part, by suppressing hippocampal autophagic hyperactivity [7]. Moreover, IL 6-deficient mice have demonstrated transcriptional up-regulation of autophagy-associated genes in hippocampus as compared to wild type mice [8–10]. Furthermore, a recent study has demonstrated that stress-induced increased hippocampal IL-6 expression may play a role in orchestrating stress-induced intensified hippocampal autophagy and subsequent depression-like behavior [11]. The implication of these results is that hippocampal IL-6 level seems to associate local autophagic flux bias and hippocampus-related emotional abnormality. Nonetheless, the causative relationship between stress-primed depression-like behavior and BBB weakness, central IL-6 level and autophagic hyperactivity remained argumentative because concomitant changes of these indices are frequent in animals receiving robust stress regimens. To dissociate the impact of these indices on stress-primed depression-like behavior, it was feasible to exploit stress and stress-attenuating regimens in together.

Reports have revealed that in-born brain structural differences may be used to predict adult animals' stress responses [12,13]. And stress-induced early-life epigenetic modulation also can be used to discriminate adult animals' stress responses [14,15]. These studies support a notion that adult stress responses may be attributed to individual differences in genetic predisposition and/or significant early-life environmental programming experiences. To our knowledge, few study has been done to explore the effects of adult animals' stress-coping experiences on subsequent stress responses. Adults' stress coping experiences and expectations are known to closely associate with one's psychological adaptability [16,17]. Psychological adaptability, in this regard, is a cardinal factor in predicting individual's immune and emotional function [18–21]. One earliest investigation into the psychological adaptability has unveiled the devastating effects of long-term stress and lacking of control-elicited psychological allosteric overload on the severity and fatality of stomach ulcers [22]. Albeit experiencing same stress magnitudes, autonomous behavioral control effectively defuses such stressor-induced psychosomatic loads and pathologies [23,24]. Using a similar "executive-yoke" paradigm as do these pioneering studies, the stressed while having behavioral control (executive) male hamsters are found to display intact intercourse hit rates, while the equivalently stressed but lacking of behavioral control (yoke) hamsters exhibit deteriorating ones [25]. These findings, taken together, prompted us to hypothesize that yoke mice receiving a long-term stress regimen while lacking of behavioral control may be prone to exhibit stress-produced negative impact on the BBB integrity, ventral hippocampal (VH) IL-6 expression and autophagic flux and VH-related depression-like behavior. In contrast, using operant behavior to autonomously terminate the ongoing stressor, the executive mice although receiving same stress regimen may display lesser changes in these indices. Accordingly, this executive-yoke paradigm was used to discriminate the respective impact of stress-altered BBB, IL-6 level and initial process of autophagic flux on stress-primed depression-like behavior. Using the same paradigm, we assessed the modulating effect

of stress-induced ventral hippocampal (VH) autophagic flux hyperactivity on a VH-related depression-like immobility phenotype using forced swimming task.

Methods and materials

Animals

Male C57BL/6 mouse weanlings were obtained from the NCKUCM Laboratory Animal Center (Tainan, Taiwan, ROC). Siblings were group housed in plastic cages (3–5 per cage) in a temperature- and humidity-controlled colony room on a 12-h light/dark cycle with lights on at 0700. All mice had access to tap water and Purina Mouse Chow (Richmond, In, USA) *ad libitum*. All experiments were conducted at a temperature (23 ± 1 °C)- and humidity (70%)-controlled laboratory. For each cage, 8-10-week-old, body weight-matched executive, yoke and control mouse cage-mates were dogmatically assigned immediately before the beginning of the 10-day stressor regimen. This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals revised in 2011. All procedures were approved by the local Animal Care Committee at National Cheng Kung University College of Medicine (NCKUCM No. 108160).

Long-term stressor regimen and experimental designs

A 10-day (1-h session/day) footshock delivery regimen was used to serve as a long-term stressor in all executive-yoke dyads. Dyads' cage-mates receiving no such stressor regimen served as no stressor control mice. Footshocks were delivered in a 24-cm long, trough-shape metal chamber as previously described [26]. Mice of each dyad received daily pseudorandomly-scheduled footshock delivery [60 footshocks/day (1-h session), 0.5 mA each, 7 s in the longest duration] for 10 days. Throughout the footshock delivery regimen, the ongoing footshock was subjected to immediate termination contingent upon the executive, not yoke, mice' forward running (or leaping) behavior or a full presentation of 7 s otherwise. Executive and yoke mice in each dyad, accordingly, underwent same magnitudes of stressor (footshock number, onset/offset timing and duration) with the former having behavioral control, while the latter lacking of such control. To reveal reliable impact of such stressor regimen having vs. lacking behavioral control on BBB integrity, ventral hippocampal (VH) BBB junction-related protein levels, VH interleukin 6 (IL-6) level, VH autophagic flux bias and VH-related immobility in forced swimming task, the assays and tasks were done approximately 72 h after the conclusion of the 10-day stress regimen. An experimental timeline is depicted in Fig. 1. To prevent behavioral task confounds on assay results, different batches of mice were used for the object location, forced swimming task, and assays. In brief, 30 mice were used for the BBB integrity (Evan blue extravasation) experiment (10 executive, 10 yoke and the remaining 10 control mice). Eighteen, including executive (N = 6), yoke (N = 6) and control (N = 6), mice were used for VH tight (ZO-1, cludin-5, occludin), and adherens (VE-cadherin) junction component assays. Twenty-one mice (executive, N = 7; yoke, N = 7; control, N = 7) were used for VH IL-6 assays. Twenty-seven (executive, N = 9; yoke, N = 9; control, N = 9) mice were used for the autophagic flux (i.e., LC3II/LC3I and p62) assays. Thirty-six (N = 12 for executive, yoke and control, respectively) mice were used for forced swimming test, while 27 (N = 9 for each group) mice were used for object location test. To reveal reliable adaptation-disrupting effects of stress and lacking of control, mice received forced swimming test at 72 h and 6 weeks (N = 12 for executive, yoke and control) after the conclusion of the stressor regimen. A total of 44 mice were used to empirically reveal the time-dependent inhibitory effects of intra-ventral hippocampal 3-methyladenine (3-MA) infusion on local phosphoinositide 3-kinase class I (N = 20) and III (N = 24) activity. A total of 24 mice (N = 12 for control and N = 12 for yoke group) were used to reveal the efficacy of the intra-VH 3-MA infusion procedure on altering local autophagic flux initiation process. A total of 32 mice (N = 16 for yoke and N = 16 control group) were used for the 3-MA infusion experiment with one half (N = 8 for yoke and N = 8 for control) of mice receiving bilateral intra-VH 3-MA infusions, while the remaining one half (N = 8 for yoke and N = 8 for control) equivalent volume DMSO-containing saline infusions approximately 1 h before the forced swimming test.

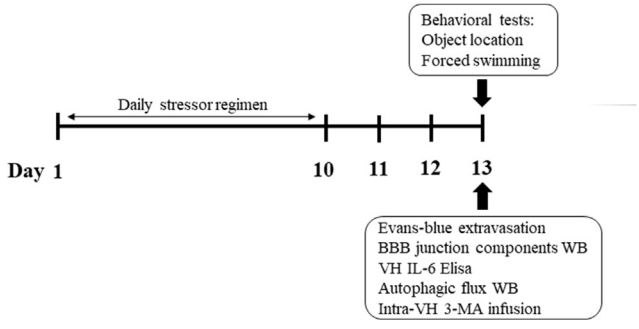

Fig. 1 Experimental timelines for the 10-day stressor regimen and biochemical assays and behavioral tasks. Assays and tasks were done approximately 72 h after the conclusion of the stressor regimen. BBB, WB, VH, IL-6, Elisa, and 3-MA are short forms of blood–brain barrier, Western immunoblotting, ventral hippocampus, interleukin-6, enzyme-linked immunosorbent assay, and 3-methyladenine, respectively.

BBB integrity and Evans blue extravasation

Extravasated Evans blue presence in brain parenchyma has been used to reveal BBB integrity [27,28]. To this end, Evans blue was dissolved in saline (30 mg/ml) and the dye solution was injected into the jugular vein (4 ml/kg) allowed to circulate for 1 h. To assess Evans blue extravasation, mice were perfused through the left ventricle with at least 90 ml of saline solution and such perfusion was terminated as colorless saline was obtained from the right atrium. It has been known that Evans blue may bind to albumin to form the complexes of molecular weight of 67 kDa [29]. Mouse brains were removed and homogenized in PBS. Brain samples were, then, centrifuged at 1000 g for 10 min at 4 °C. Then, 0.3 ml of 100% (w/v) trichloroacetic acid was added to 0.3 ml supernatant and mixed well on a vortex. The mixture was incubated at 4 °C for

18 h and centrifuged at 1000 g for 10 min at 4 °C, the supernatant was collected for electrophotoric assay. Evans blue concentration was measured at 620 nm and calculated according to a standard curve prepared with known concentrations of this dye. Evans blue extravasation was expressed as μg/mg of protein. To qualitatively delineate the BBB integrity differences in executive, yoke and control mice, their coronal brain slices (40 μm in thickness) were used. These brain slice photographs were first loaded on Image J and the red-channel images were then made for the presentation of the BBB integrity differences.

Western immunoblotting assays for BBB junction proteins and autophagic flux

Stress is known to disrupt the structure of tight junction, being constituted by a network of transmembrane proteins linking inside to cytosolic protein and cytoskeletal proteins, and adherens junction components [30]. To assess the impact of stressor and behavioral control on tight and adherens junction components and autophagic flux in VH, mice were euthanized and their brains were removed and placed on the dorsal surface on a glass dish sitting on crushed ice. Hippocampal tissues were dissected as previously described [31] and dorsal and ventral hippocampal samples were obtained from the top and bottom halves of bilateral hippocampal tissues. These tissue samples were homogenized in ice-cold lysis buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). The samples were centrifuged at 13500×*g* for 10 min at 4 °C, and the protein concentrations of supernatants were determined by Bradford method (BioRad Laboratories, Hercules, CA, USA). Equal quantities of protein (30 μg) from each sample were re-suspended in loading buffer, denatured at 70 °C for 10 min, and loaded into the wells of the sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred onto polyvinylidene fluoride membranes (SMOBIO, New Taipei City, Taiwan, ROC). The membranes were blocked with nonfat dry milk buffer (5%) for 1 h and subsequently incubated overnight at 4 °C with the following primary antibodies: LC3A/B (1:1000) (Cell Signaling Technology, Danvers, MA, USA), p62 (1:1000) (Abcam, Cambridge, MA, USA), ZO-1 (1:200) (Thermo Fisher Scientific, Waltham, MA, USA), claudin-5 (1:200) (Abcam), occludin (1:200) (Abcam), VE-cadherin (1:200) (Abcam), β-actin (1:10000) (Sigma–Aldrich, St. Louis, MO, USA), and GAPDH (1:10000) (Sigma–Aldrich). The membranes were then processed using the following secondary antibodies: anti-rabbit (1:10000) and anti-mouse (1:10000) (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) for 1.5 h at room temperature. The blots were displayed with ECLTM Western blot detection kit (Amersham, Buckinghamshire, UK). Densitometry was evaluated using MCID image analysis software. Autophagy is one of the intracellular degradation systems, thus autophagic flux, including autophagic induction and a part of degradation process, indexed by LC3II/I ratio and p62 respectively, is frequently used to denote overall autophagic degradation process [32]. It is noted that microtubule-associated protein 1A/1B-light chain 3 (LC3) has two forms, cytosolic (LC3I) and phosphatidylethanolamine conjugate (LC3II) form. And nucleoporin p62 (p62) is known to be a main component of the nuclear pore complex. Accordingly, autophagic flux was indicated by LC3II to LC3I ratio and p62 level.

Interleukin-6 (IL-6) ELISA assay

Brain tissues containing the VH were dissected out as aforementioned and homogenized in icy cold PBS buffer. Samples were, then, centrifuged (5 min at 5000×*g*), the supernatant decanted and was stored at −80 °C until assay. VH IL-6 levels were determined with mouse ELISA kits (Rockville, MD, USA) per the manufacturer's instructions.

Cannula implantation and intra-ventral hippocampal infusion

While 3-MA is frequently used as an inhibitor for autophagic flux, a previous study has suggested that 3-MA may exert inhibitory effects on phosphoinositide 3-kinase class I and III [33]. Inhibition of phosphoinositide 3-kinase class III is expected to inhibit autophagic flux, while inhibition of phosphoinositide 3-kinase class I to enhance autophagic flux. To assess the net modulating effects of our intra-ventral hippocampal 3-MA treatment (50 mM/0.2 μL/side) on local phosphoinositide 3-kinase class I and III activity at 1 h after the infusion, the enzymatic product (phosphatidylinositol 3-phosphate) of class III (Elisa kit, Echelon Biosciences, Salt Lake City, UT, USA) and class I downstream substrate, phospho- and total AKT (primary antibodies, Cell Signaling Technology) were assayed using ventral hippocampal tissues from 3-MA- and vehicle-infused control and yoke mice. To assess the permissive roles of VH autophagic flux bias in mediating stress and lacking of control-induced depression-like phenotype, yoke and control mice received stereotaxic surgery and guide cannula implantation. In brief, mice were subjected to an acrylic glass chamber (14 cm × 11 cm x 11.5 cm) pre-flushed with 3–5% isoflurane (Panion & BF Biotech Inc., Taoyuan, Taiwan, ROC) of 80 ml/min to induce general anesthesia and maintained by 2–2.5% isoflurane of 80 ml/min via a nose mask. Mice were placed on a warming pad throughout the surgical procedure to prevent hypothermia. Stereotaxic surgery, 26-gauge guide cannula implantation (Bregma coordinates: anteroposterior:-2.5 mm; mediolateral:±2.8 mm; dorsoventral:-2.5 mm) and 33-gauge dummy cannula insertion were made. Local anesthetic (0.5 ml xylocaine) was used around the surgical wound closure followed by a subcutaneous nalbuphine hydrochloride (4 mg/kg) injection for treating post-surgical pain. Approximately 72 h after the conclusion of the 10-day stressor regimen, yoke and control mice received bilateral intra-VH infusions with 3-MA (50 mM/0.2 μL/side) (Merck) or equivalent volume of DMSO-containing (10%, v/v) saline using a 33-gauge infusion needle and an automatic pumping system. These mice underwent forced swimming task 1 h after the infusions.

Forced swimming, tail suspension and object location tasks

To assess stress and lacking of control-primed depression-like phenotype, forced swimming and tail suspension tasks

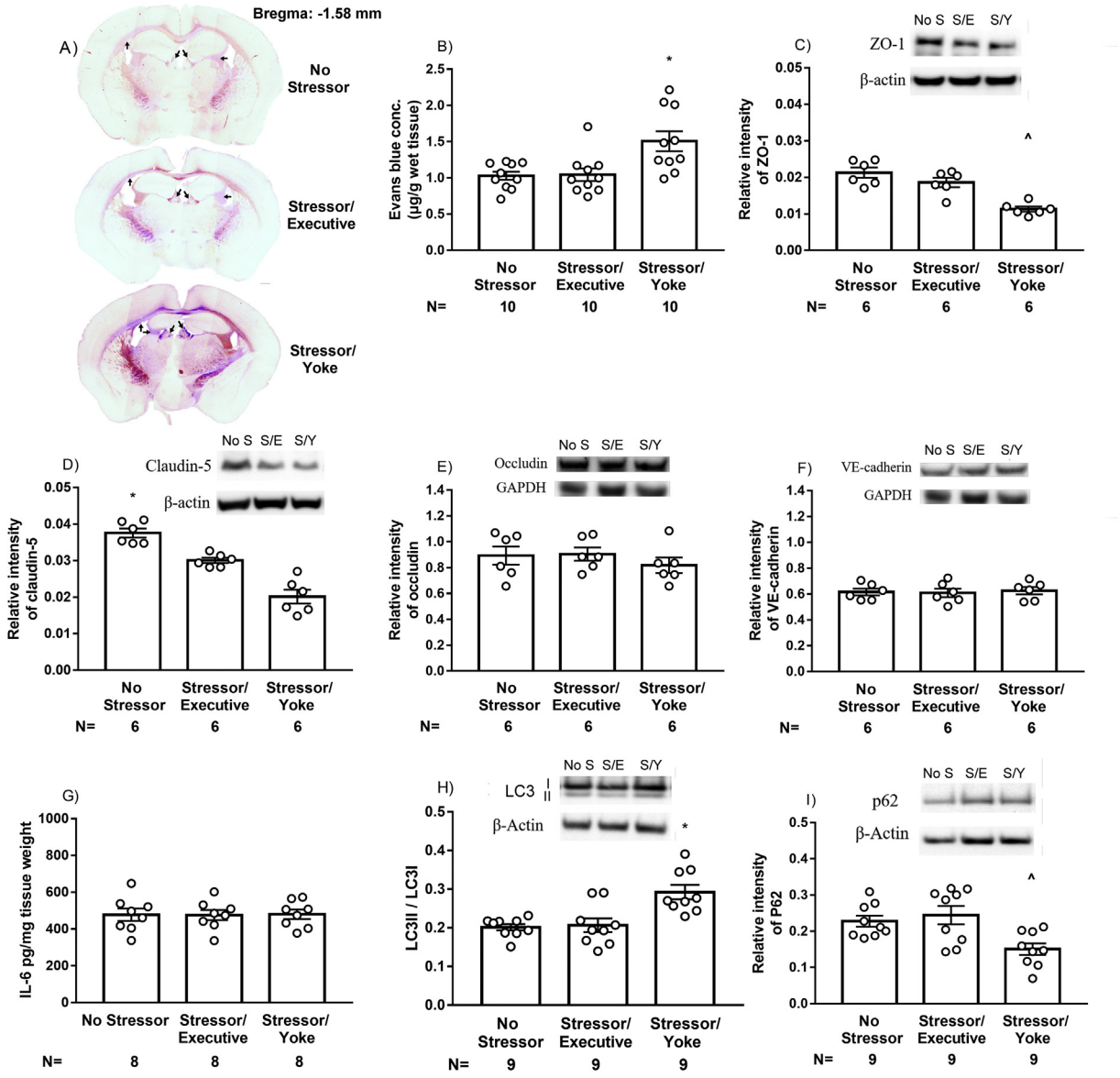

Fig. 2 BBB integrity, junction component protein expressions, IL-6 levels and autophagic flux indices in yoke, executive and control mice. (A)Representative photomicrographs of Evans blue leaking in brain slices (Bregma:-1.58 mm). Dark arrows point to the leaking site. (B)Stressor/Yoke mice receiving the 10-day stressor regimen and lacking of behavioral control exhibited higher Evans blue extravasation as compared to the Stressor/Executive mice receiving same stressors while having behavioral control and the control mice receiving No Stressor. *Significantly greater than the other groups (ps < 0.05). (C)Stressor/Yoke mice demonstrated lower ZO-1 level in VH as compared to executive and control mice. ^Significantly lower than the other groups (ps < 0.05). VH stands for ventral hippocampus. No S, S/E and S/Y are short forms of No stressor, Stressor/Executive and Stressor/Yoke, respectively. (D)The stressor regimen was found to induce decreases in VH claudin-5 level and yoke mice had the lowest VH claudin-5 level among three groups. *Significantly higher than Stressor/Yoke group (p < 0.05). (E)Regardless of the stressor regimen, three groups of mice had comparable occludin levels in VH. (F)Regardless of the stressor regimen, three groups of mice had comparable VE-cadherin levels in VH. (G)Regardless of the stressor regimen, three groups of mice had comparable IL-6 levels in VH. (H)Long-term stress and lacking of behavioral control was found to induce increases in LC3II/LC3I ratio (autophagic flux) in VH, while did not alter such ratios in Stressor/Executive mice. *Significantly higher than the other groups (ps < 0.05). (I)Long-term stress and lacking of behavioral control was found to induce decreases in p62 (autophagosome inclusion) level in VH, while did not alter such levels in Stressor/Executive mice. ^Significantly lower than Stressor/Executive and No Stressor group (ps < 0.05). Group differences are analyzed by Kruskal–Wallis analyses followed by Dunn multiple comparisons if appropriate.

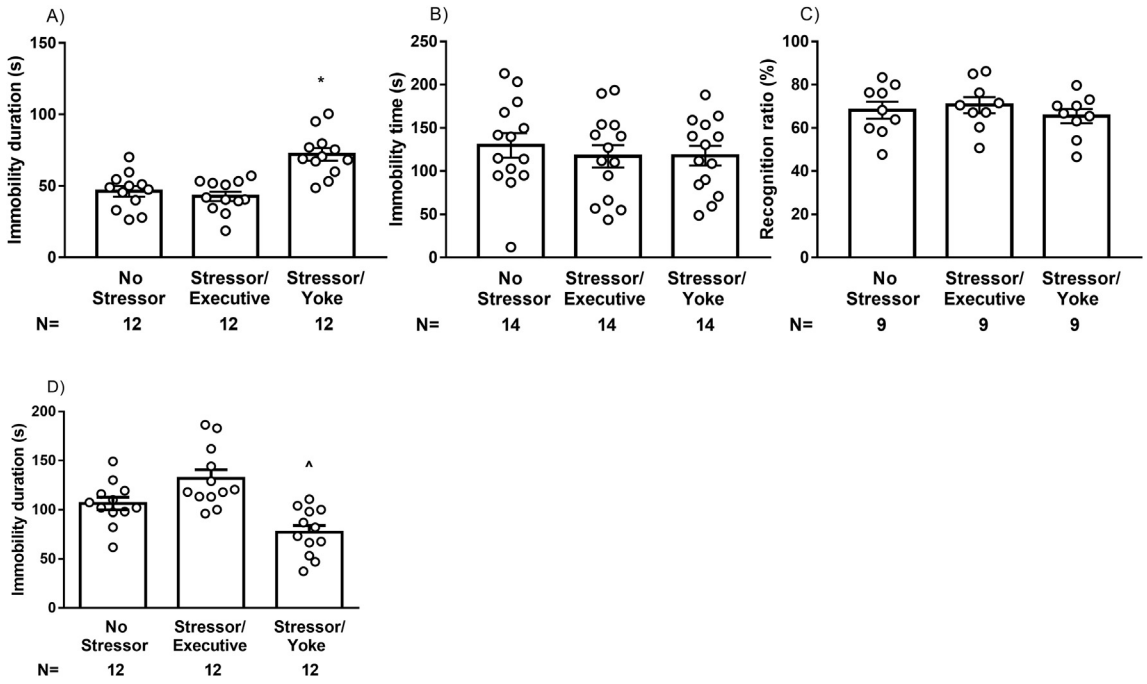

Fig. 3 Long-lasting impact of stress and behavioral control on hippocampus-related functions. (A)Stressor/Yoke mice demonstrated greater immobility durations (in second) as compared to Stressor/Executive and No Stressor control mice in a 6-min forced swimming observation approximately 72 h after the conclusion of the stressor regimen. *Significantly greater than the other groups (ps < 0.05). (B)Tail suspension task was insensitive to the stressor regimen and behavioral control. Three groups of mice demonstrated indistinctive immobility time (in second) in a 6-min tail suspension. (C)Regardless of the stressor regimen or behavioral control, three groups of mice had comparable recognition ratios in object location task. (D)Following the first forced swimming test, mice received a forced swimming retest at 6 weeks after their conclusion of the stressor regimen. Yoke mice displayed shorter immobility duration as compared to executive and control mice. ^Significantly lower than the other groups (ps < 0.05). Group differences in all behavioral task results are analyzed by one-way analysis of variances (ANOVAs), followed by Bonferroni's post hoc tests if appropriate.

were used. Forced swimming task was conducted as previously described [34] with minor modifications. In brief, a home-made transparent Plexiglas cylinder with a 19-cm diameter and 46 cm in depth was used. Water (controlled at 24 ± 1 °C) was filled up to 70% in depth. Mouse was individually lowered down to the cylinder for a 6-min observation. The duration of immobility (remained to be immobile for at least 1 s) were videotaped and analyzed by a rater blind to the groups. For tail suspension task, mice were suspended upside down by the tip of the tail [34]. Immobility referred to the observation that mouse limbs and torso remained immobile for at least 2 s. Likewise, total time of immobility mice spent in this 6-min task were recorded and analyzed by a rater blind to the groups [34]. To reveal the long-lasting effects of the stress and lacking of control regimen on adaptive behavior disruption, a forced swimming test-retest paradigm was used [35]. Accordingly, mice receiving 6-min forced swimming task at the 72 h after the stressor regimen were subjected to forced swimming retest at 6 weeks after such regimen. Hippocampal immune activation has been reported to affect animals' dorsal hippocampus-dependent memory [36]. To assess whether stress and lacking of control may prime animals for dorsal hippocampus-related behavioral impairment, object location task was used. The object location task consisted of habituation, sample and test phases. Three 10-min habituation sessions were first conducted at approximately 2-hr intervals with mice free navigating in an empty Plexiglas square box (40 cm × 40 cm × 30 cm) with black walls and a bright yellow floor in a dimly lit test room. Two similar objects (tea cups with a diameter of 6 cm placed upside down) were then placed in the opposite corners (tea cup border was 6.8 cm away from two adjacent walls) of the box in the same dimly lit test room and mice were allowed to explore in the box for 10 min, serving as the sample phase and the time mice spent in exploring two objects was recorded. As a 5-min retention time elapsed, mice were returned to the box with one tea cup (the one with a lesser time spent in the sample phase trial) removed to a diagonally opposite corner (tea cup border was also 6.8 cm away from two adjacent walls), starting a 5-min test phase. The recognition percentage, ratio of the time spent exploring the tea cup in the new corner over the time spent exploring both tea cups, was used to determine mice' memory performance. Box and the tea cups used were thoroughly cleaned between sessions and phases to prevent the plausible build-up of olfactory cues.

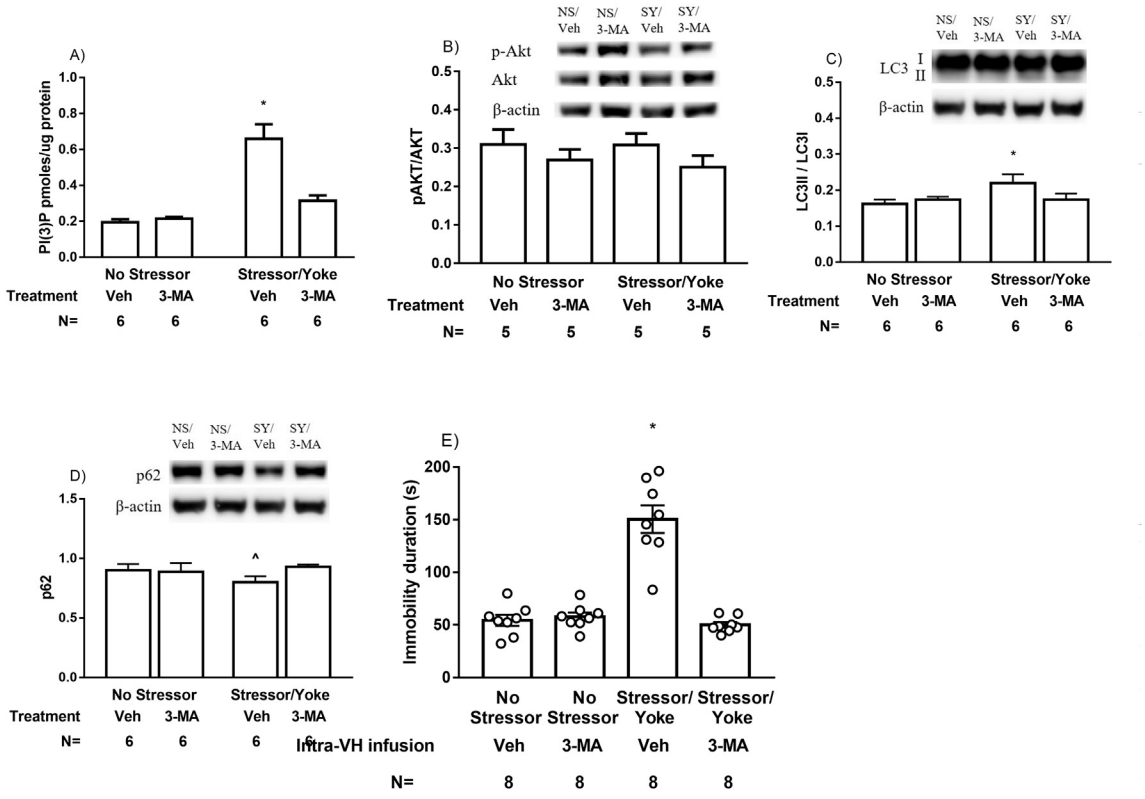

Fig. 4 Effects of intra-VH 3-MA (50 mM/0.2 μL/side) infusions on VH autophagic flux indices (LC3II/I and p62), related class I and III activity and forced swimming immobility duration in control and yoke mice. (A)Using PI(3)P gauge as a phosphoinositide-3 kinase activity index, yoke mice displayed higher phosphoinositide-3 kinase activity in their ventral hippocampal tissues as compared to the control mice. Bilateral intra-VH 3-MA (50 mM/0.2 μL/side) infusions reliably prevented such enhanced phospohoinositide 3-kinase class III activity. PI(3)P is a short form of phosphatidylinositol 3-phosphate. *Significantly higher than the remaining three groups (ps < 0.05). (B)Neither the stressor regimen or 3-MA treatment affected class I downstream target protein phosphorylation in mice' ventral hippocampal tissues. (C)While intra-VH 3-MA infusions did not affect LC3II/I ratio in No Stressor controls, such treatment prevented the stress and lacking of control-primed LC3II/I increase in Stressor/Yoke mice. *Significantly greater than the other groups (ps < 0.05). (D)Intra-VH 3-MA infusions did not affect p62 in No Stressor controls, while such treatment prevented the stress and lacking of control-primed p62 decrease in Stressor/Yoke mice. ^Significantly lower than the other groups (ps < 0.05). (E)While intra-VH 3-MA treatment did not affect recognition ratio in No Stressor control mice, such treatment prevented the stress and lacking of control-primed increases in immobility duration in forced swimming test. *Significantly greater than the other groups (ps < 0.05). Group differences are analyzed by Kruskal–Wallis analyses followed by Dunn multiple comparisons if appropriate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA, USA). Kruskal–Wallis tests were employed to reveal group differences in vans blue extravasation, junction component proteins, IL-6 level, autophagic indices followed by Dunn multiple comparisons if appropriate. One-way analysis of variances (ANOVAs) were used to reveal the group differences in behavioral task results followed by Bonferroni's post hoc tests if appropriate. In an attempt to test whether intra-ventral hippocampal 3-MA infusion may affect phosphoinositide 3-kinase class I and III activity and thus effectively prevent stress and lacking of control-primed increases in autophagic flux indices (including LC3II/I and p62) and immobility duration, Kruskal–Wallis tests were first employed to reveal group differences and then followed by Dunn multiple comparisons if appropriate. All the level of statistical significance was set at $p < 0.05$.

Results

Long-term stress and lacking control, BBB integrity, and ventral hippocampal junction proteins

Stressed mice having (executive) and lacking (yoke) of control and mice receiving no such stress (No Stressor) were used using same intra-jugular Evans blue infusion procedures. We found that executive and control mice demonstrated comparable spectrophotometric readouts, while both lower than

those of yoke mice ($X^2 = 10.97$, $p = 0.0041$) [Fig. 2A, B]. These results indicated that stress and lacking of control disrupted BBB integrity, while same stress while having control did not seem to affect mice' BBB integrity.

Yoke mice had lower VH ZO-1 expressions as compared with executive and control mice ($X^2 = 11.4$, $p = 0.0005$) [Fig. 2C]. Likewise, stressed mice lacking of control (yoke mice) displayed lower claudin-5 level as compared to No Stressor control mice ($X^2 = 15.16$, $p < 0.0001$) [Fig. 2D]. Nonetheless, three groups of mice had indistinctive occludin and VE-cadherin expressions in VH [Fig. 2E and F]. These results, taken together, suggest that long-term stress and lacking of control may render reliable BBB weakness and ZO-1 and claudin-5 down-regulation in ventral hippocampus.

Stress and lacking of control and IL-6, autophagic flux in ventral hippocampus

Regardless of behavioral control, stressed mice had indistinctive IL-6 levels in VH. Likewise, stressed mice exhibited comparable VH IL-6 levels as No Stressor control mice [Fig. 2G], suggesting that the stressor regimen does not induce reliable changes in VH IL-6 level.

Control and executive mice exhibited indistinctive LC3II/I ratio and p62 level in VH. However, yoke mice were found to demonstrate higher magnitudes in autophagic flux, i.e., higher LC3II/LC3I ratio and lower p62 level, as compared to the executive and control mice ($X^2 = 12.29$, $p = 0.0021$; $X^2 = 8.074$, $p = 0.0176$) [Fig. 2H and I].

Stress and lacking of control and hippocampus-related behavior

In comparison with dorsal hippocampus, ventral hippocampus is more susceptible to chronic stress pre-exposure and subsequent acute stress challenge [36]. To dissociate the modulating effects of stress and lacking of control on ventral and dorsal hippocampus, three behavioral tasks (ventral hippocampus-related forced swimming and tail suspension and dorsal hippocampus-related object location tasks) were used. We found that yoke mice exhibited longer immobility duration in forced swimming task as compared to executive and control mice, while the latter two groups comparable immobility durations [$F(2,33) = 17.73$, $p < 0.0001$] [Fig. 3A]. However, mice exhibited indistinctive immobility time in the 6-min tail suspension task regardless of the stressor regimen and behavioral control [Fig. 3B], suggesting that the tail suspension task used is insensitive to the present stress and lacking of control protocol. Moreover, indistinctive recognition ratios were noticed in dorsal hippocampus-dependent object location memory among these three groups [Fig. 3C]. Following the first forced swimming test, executive, yoke and control mice received a forced swimming retest at 6 weeks after the conclusion of the stressor regimen. Yoke mice were found to display shorter, rather than longer, immobility duration as compared to executive and control mice [$F(2,33) = 13.6$, $p < 0.0001$], while the latter two groups demonstrated comparable durations [Fig. 3D].

Intra-ventral hippocampal 3-MA infusion and forced swimming immobility

To decide whether bilateral intra-ventral hippocampal 3-MA infusions may inhibit phosphoinositide 3-kinase class III activity and class I targeting AKT protein phosphorylation, yoke and control mice were used. Kruskal–Wallis test revealed that yoke mice did display higher phosphoinositide-3 kinase activity in their ventral hippocampal tissues as compared to the control mice ($X^2 = 18.41$, $p = 0.0004$). Likewise, bilateral intra-VH 3-MA (50 mM/0.2 μL/side) infusions reliably inhibited phospohoinositide 3-kinase class III activity in ventral hippocampal samples in yoke mice (Fig. 4A). However, neither our stressor regimen or 3-MA treatment affected class I downstream target protein (AKT) phosphorylation in mice' ventral hippocampal tissues (Fig. 4B). Importantly, we thereby repeated previous findings that the stressor regimen produced reliable VH LC3II/I ratio increase ($X^2 = 14.17$, $p = 0.0027$) [Fig. 4C] and p62 decrease ($X^2 = 11.1$, $p = 0.0112$) in yoke mice [Fig. 4D]. While bilateral intra-VH 3-MA (50 mM/0.2 μL/side) infusions did not alter these autophagic flux indices in control mice' VH, yoke mice' LC3II/I ratio and p62 changes were prevented by such 3-MA infusions in VH [Fig. 4C and D]. Approximately 72 h after the conclusion of the stressor regimen, yoke and control mice were used for intra-3-MA infusion and behavioral experiment. Intra-VH infusion with 3-MA (50 mM/0.2 μL/side) did not affect the immobility duration in control mice, while such 3-MA treatment reliably prevented the elevation in immobility duration in yoke mice ($X^2 = 18.84$, $p = 0.0003$) [Fig. 4E].

Discussion

Since male siblings, also cage-mates, were used in this study, executive, yoke and control animals' genetic predisposition and early-life environmental programming differences were, to some extent, parceled out. Thus, their structural and functional differences may be, at large, attributed to mice' stress and stress-coping results/expectations at adulthood. Except for stress-elicited slightly low ventral hippocampal claudin-5 level, stressed mice having behavioral control (i.e. executive mice) and control mice receiving no such stress demonstrated comparable BBB integrity, ventral hippocampal IL-6 level, autophagic flux and forced swimming immobility duration. Equivalently stressed mice while lacking of autonomous behavioral control, in contrast, were found to have disrupted BBB integrity, drastic decreases in ventral hippocampal BBB tight junction proteins (ZO-1 and claudin-5), significant increases in autophagic flux and a depression-like behavior. These results, taken together, suggest that stress, itself, is not sufficient to induce brain structural and functional plasticity. In addition to stress exposure, passive stress-coping experiences play a permissive role in priming animals' brain plasticity and subsequent stress susceptibility.

To date, many studies have shown that stress may render disrupted BBB integrity and depression-like behavioral phenotype [5–8]. In parallel with these results, we found that

stressed mice lacking of control (i.e. yoke) mice also displayed disrupted BBB integrity and increases in a depression-like behavior in this study. Using the executive-yoke paradigm, the executive mice received equivalent footshocks as did the yoke mice in this study. Paradoxically, the executive mice were found to have unaltered BBB integrity and depression-like behavior magnitude. While both executive and yoke mice received footshock-delivering stressor regimen, the former seemed to prevail soon (data not shown) in their spontaneous operant behavior contingent upon the termination of on-going footshocks across the stress regimen. Such autonomous behavioral control and outcome expectation are suspected to enhance implicit psychological aspect of control. That is, such psychological aspect of control is thought to not only discriminate executive from yoke mice in their stress-produced devastating effects upon brain structure and function but to provide significant buffering effects against the footshock stressor. In fact, a recent study has provided evidence to support this notion by showing that animals' stress response magnitudes may be predicted by their previous active vs passive stress coping results [38].

A previous study has revealed that a 5-wk chronic stressor regimen enhances blood IL-6 level and concomitantly decreases hippocampus-related task performances [39]. Moreover, acute resistance exercise may enhance serum IL-6 levels at least lasting for 24 h after conclusion of the exercise [40]. Furthermore, stress-induced increased hippocampal IL-6 expression has been suspected to prime stressed animals to display intensified hippocampal autophagy and hippocampus-related behavioral deficits [8,39]. In parallel with these reports, we found that long-term stress produced reliable escalations in both ventral hippocampal autophagic flux and depression-like immobility in yoke mice. However, yoke mice' ventral hippocampal IL-6 level seemed to be unaltered. Likewise, stressed mice having behavioral control (executive) also displayed unaltered ventral hippocampal IL-6 level. The discrepancies of post-stress IL-6 levels could arise insomuch as a few differences between other studies and ours. First, our 10-day stressor regimen may produce minor and short-lasting increases in plasma IL-6 level. After all, a 5-wk chronic stressor regimen is used in Li et al.s' study (2008) and an acute injection-related stress is obvious prior to their IL-6 assays [39]. Second, footshock stressors may exert lesser potent effects as multiple doses of LPS treatment on provoking immune responses and escalating plasma IL-6 level [8]. Third, concomitant changes in hippocampal IL-6 levels and depression-like behavior performance could be due to within-subject design of choice [8,39]. In their experimental designs [8,39], animals are subjected to novel stressor challenges, i.e., stress-provoked emotion-related tasks, prior to their hippocampal IL-6 assays [8]. However, we attempted to avoid such confounding impact of stress-related behavior task on subsequent bioassays by using between-subject design. Per our results, we conclude that ventral hippocampal IL-6 level, at best, play a minor role in mediating stress-primed long-lasting escalations in local autophagic flux or depression-like immobility. Although ventral hippocampal IL-1, another neuro-inflammatory marker, level [41] could be critical in this regard, our previous findings have indicated that brain IL-1 levels seem to be more resilient to stressor challenges as compared to IL-6 levels [42].

Stressed mice lacking of behavioral control (yoke) had disrupted BBB, increases in VH autophagic flux (increased LC3II/LC3I and decreased p62) and immobility duration in the forced swimming test. In an attempt to test the likely causative relationship between VH autophagic flux escalation and VH-related depression-like behavioral performance, intra-VH administration with 3-methyladenine (3-MA), an autophagic flux inhibitor, was used in yoke and control mice. At least 1 h before the tissue autophagic flux assays, intra-VH 3-MA (50 mM/0.2 μL/side) infusions effectively prevented the VH autophagic flux escalation in yoke mice. Accordingly, approximately an hour prior to the forced swimming test, bilateral intra-VH administration of 3-MA (50 mM/0.2 μL/side) was given. We found such 3-MA treatment effectively prevented the increases in immobility duration in yoke mice, while such administration did not affect the immobility duration in control mice. The implications of these results are two-fold. First, stress and lacking of behavioral control-primed autophagic flux changes are more likely involved in animals' depression-like behavior performance. Second, the depression-like behavior-preventing effects associated with intra-VH 3-MA administration could be due to the quick toll-like receptor 9 (TLR9) recruitment-inhibiting and/or oxidative stress-relieving character of 3-MA. After all, a previous report has indicated LC3II may reciprocally activate the TLR9 in a fast mode at the early stage of mitochondrial autophagy [43]. And TLR9 activation has been lately revealed to significantly induce cellular oxidative stress [44]. Hippocampal oxidative stress escalation may cause local neuronal viability inhibition and compromised neuron-glial integrity [45]. Thus, intra-VH 3-MA administration is suspected to prevent the stress-caused depression-like phenotype by inhibiting autophagic initiation and oxidative stress in ventral hippocampus.

When rodents receive forced swimming test-retest paradigm, rodents frequently demonstrate fast-onset immobility in the retest [46]. Such fast-onset frequent immobility in retest is absent by pre-test adrenalectomy, while rescued by corticosterone and its analog supplement [46]. These findings suggest that learning/memory consolidation and HPA axis activation may be involved in this fast and frequent immobility performance in the retest [46]. In addition to the implication of forced swim immobility to depression-like genotypes and phenotypes, investigators maintain that animals' immobility, especially in the forced swimming retest, also can be attributed to a learning and memory-based adaptive behavior [46–49]. And limbic circuits, their forebrain and ventral hippocampal inputs and striatal outputs, are proven to be involved in this adaptive immobility in this regard [35,49,50]. To demonstrate the reliable impact of the present stress and lacking of control regimen on limbic plasticity and adaptive behavior of choice [46], forced swimming retest was introduced at 6 weeks after the conclusion of the stress regimen. While yoke mice demonstrated depression-like phenotype by displaying the longest immobility duration in the forced swimming test, same yoke mice exhibited shortest immobility duration among three groups at the

retest. In contrast, control and executive mice displayed long and comparable immobility durations in the retest. These results, taken together, suggest that learning/memory-based adaptive behavior of choice is impaired exclusively in mice receiving stressor regimen and lacking of control. Moreover, those mice receiving stressor and lacking of control may also have compromised limbic plasticity and function.

In this study, we found that stress and having control did not affect BBB integrity, ventral hippocampal autophagic flux and depression-like behavior magnitudes. In contrast, stress while lacking of control resulted in decreases in ventral hippocampal BBB component proteins while increases in autophagic flux and ventral hippocampus-related depression-like phenotype. Among these changes, ventral hippocampal autophagic flux increases seemed to be a critical factor in priming the stressed while lacking control animals susceptible to subsequent stress-induced depression-like behavior.

Conclusions

These results, taken together, prompt us to conclude that stress-primed long-lasting autophagic flux increase in VH, at least, play a role in priming the stressed animals lacking of behavioral control susceptible to a depression-like behavioral phenotype.

Conflicts of interest

None.

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