Hippocampal BDNF Mediates Recovery From Chronic Stress-Induced Spatial Reference Memory Deficits

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Masters of Arts

Approved October 2013 by the
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ARIZONA STATE UNIVERSITY

December 2013

ABSTRACT

Chronic restraint stress impairs hippocampal-mediated spatial learning and memory, which improves following a post-stress recovery period. Here, we investigated whether brain derived neurotrophic factor (BDNF), a protein important for hippocampal function, would alter the recovery from chronic stress-induced spatial memory deficits. Adult male Sprague-Dawley rats were infused into the hippocampus with adeno-associated viral vectors containing the coding sequence for short interfering (si)RNA directed against BDNF or a scrambled sequence (Scr), with both containing the coding information for green fluorescent protein to aid in anatomical localization. Rats were then chronically restrained (wire mesh, 6h/d/21d) and assessed for spatial learning and memory using a radial arm water maze (RAWM) either immediately after stressor cessation (Str-Imm) or following a 21-day post-stress recovery period (Str-Rec). All groups learned the RAWM task similarly, but differed on the memory retention trial. Rats in the Str-Imm group, regardless of viral vector contents, committed more errors in the spatial reference memory domain than did non-stressed controls. Importantly, the typical improvement in spatial memory following recovery from chronic stress was blocked with the siRNA against BDNF, as Str-Rec-siRNA performed worse on the RAWM compared to the non-stressed controls or Str-Rec-Scr. These effects were specific for the reference memory domain as repeated entry errors that reflect spatial working memory were unaffected by stress condition or viral vector contents. These results demonstrate that hippocampal BDNF is necessary for the recovery from stress-induced hippocampal dependent spatial memory deficits in the reference memory domain.

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Introduction

The stress response allows organisms to adapt to a constantly changing environment, and its importance is emphasized by its conservation across species. The brain is critical for initiating the stress response . When a stressor is perceived, the hypothalamic-pituitary-adrenal (HPA) axis is activated. The hypothalamus releases corticotrophin-releasing hormone and arginine vasopression to stimulate adrenocorticotropin hormone release from the anterior pituitary into the general blood supply. Adrenocorticotropin hormone then acts on the adrenal glands to initiate glucocorticoid synthesis and release to mobilize stored energy. Given the importance on the body's metabolism, glucocorticoids inhibit their own release at multiple levels by direct negative feedback to the hypothalamus and anterior pituitary, as well as targeting other HPA axis afferents (hippocampus, medial prefrontal cortex) to shut-off the stress response. With, prolonged glucocorticoid exposure through chronic stress or from insufficient control of these negative feedback mechanisms, the body and brain can show wear and tear, called allostatic load (citation). Some suggest that cost of this allostatic load may lead to the development and/or exacerbation of neuropsychiatric diseases (citations). It is important to note that many individuals are resilient to the effects of chronic stress. That is, they are able to rebound from the effects of chronic stress or they are able to handle the chronic stress bombardment. On the other hand, some individuals are thought to be vulnerable, or more sensitive to the effects of chronic stress. What makes some individuals resilient and others susceptible to the effects of stress is an important question. The current study set out to investigate the mechanisms behind

recovering from chronic stress in order to better understand factors that render the brain and an individual resilient or vulnerable to the effects of chronic stress.

Chronic stress impacts many structures in the brain with the hippocampus being particularly vulnerable. The hippocampus is well documented to contribute to spatial learning and memory (Devan et al., 1996; Eichenbaum, 1999), which is compromised in response to chronic stress (Conrad et al., 1996; Wright et al., 2006; Kitraki et al., 2004; Song et al., 2006; Luine et al., 1994; Conrad 2010). Interestingly, when chronic stress impairs spatial learning and memory, alterations in the architecture and physiology of the hippocampus often co-occur (reviewed in Conrad 2006). For example, chronic restraint stress impairs hippocampal dependent spatial learning and memory and this corresponds with simplified hippocampal CA3 dendritic architecture (Sousa et al., 2000; McLaughlin et al., 2007; Hoffman et al., 2011; Hutchinson et al., 2012) and blunted long-term potentiation (Radecki et al., 2005), a form of neuronal plasticity thought to represent a model of learning and memory processes (Bliss and Collingridge, 1993). Taken together, these studies support the interpretation that chronic stress disrupts hippocampal function and its corresponding synaptic plasticity.

When chronic stress ends, the stress-induced spatial memory deficits and CA3 dendritic retraction show recovery in the following weeks. Three separate studies using different spatial tasks report that chronic stress hinders spatial learning and memory that recovers within weeks after the stressor has ended (Morris water maze: Sousa et al., 2000; radial arm maze: Luine et al., 1994; and radial arm water maze (RAWM): Hoffman et al., 2011). In parallel with these changes, chronic stress-induced alterations in neuronal dendritic morphology follow a similar time course with chronic stress causing atrophy in

the hippocampal CA3 apical region (Watanabe et al., 1992a; Sousa et al., 2000; Hoffman et al., 2011) that recovers (Sousa et al., 2000; Hoffman et al., 2011). These studies show that even in adulthood, organisms display remarkable behavioral and morphological plasticity when they recover from stress.

Brain derived neurotrophic factor (BDNF) may contribute to the mechanisms mediating the recovery from stress-induced spatial learning and memory deficits. BDNF comes from a family of neurotrophic proteins that influence growth, differentiation, and survival of neurons in developing and adult brains (Lu et al., 2005; Karatsoreos and McEwen, 2013; Numakawa et al., 2013). BDNF and its receptor, tropomyosin-receptor kinase B (TrkB), are highly expressed in the brain, and are especially concentrated in the hippocampus (Conner et al., 1997; Drake et al., 1999; Gray et al., 2013). Importantly, BDNF regulates neuronal morphology and synapse formation, plasticity, and strength (Levine et al., 1995; McAllister et al., 1996, 1999; An et al., 2008; Verpelli et al., 2010), outcomes that are diminished with reduced BDNF expression (Bartoletti et al., 2002; Ninan et al., 2010). Within the hippocampus, chronic stress decreases BDNF expression (reviewed in Duman and Monteggia, 2006), which contributes to poor hippocampal plasticity and function. Specifically, chronic stress alters hippocampal BDNF mRNA and protein levels (Smith et al., 1995; Murakami et al., 2005; Lakshminarasimhan and Chattarji, 2012) and transgenic mice expressing low BDNF levels show simplified dendritic arborization (Margariños et al., 2011). Radecki and colleagues, 2005, found that rats subjected to chronic stress showed spatial learning and memory deficits, which were corrected by infusions of BDNF into the hippocampus before and throughout the stress paradigm (Radecki et al., 2005). Therefore, BDNF may contribute to the effects

generated by the stress response to produce distinct outcomes on brain morphology, especially within the hippocampus, which may hinder spatial learning and memory.

Whether hippocampal BDNF plays a role in the recovery of stress-induced spatial ability deficits remains to be determined. The current study used RNA interference to temporally and spatially down-regulate BDNF within the dorsal hippocampus in adult rats in order to investigate its consequences on spatial learning and memory following chronic stress and after a recovery period. Moreover, we targeted the dorsal CA3 region of the hippocampus because the CA3 dendritic arbors are one of the first hippocampal regions to express apical dendritic retraction to suggest it as being highly sensitive to chronic stress (Conrad, 2006). We hypothesized that hippocampal BDNF expression mediates spatial learning and memory following chronic stress and a recovery period. We predicted that (1) chronic stress would impair spatial learning and memory, which would be exacerbated by low hippocampal BDNF expression and that (2) chronically stressed rats with down-regulated hippocampal BDNF expression would fail to show spatial learning and memory recovery following chronic stress.

Methods

This experiment was conducted in accordance with federal guidelines as described by the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, National Research Council, Institute of Laboratory Animal Resources on Life Science, Washington, D.C., 1996) and institutional guidelines set forth by the Arizona State University Institutional Animal Care and Use Committee.

Subjects

Seventy-two young-adult male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at Arizona State University housing facilities. All rats were paired housed, on a reverse light cycle (12:12, lights off at 0600), for the duration of the study in a colony room that contained animals in the same treatment condition (i.e. unstressed controls were housed with unstressed controls, stressed rats given a post-stress recovery period were housed with other rats given a post-stress recovery period, etc.). Water and food were available *ad libitum*, except during the restraint procedures. After arrival, rats were given one week to acclimate prior to surgery. Rats were weighed weekly throughout the duration of the experiment. All behavioral testing was conducted during the dark phase of the light cycle.

Surgery

One week after acclimation, all rats underwent stereotaxic surgery targeting the dorsal hippocampus. All surgeries were performed under aseptic sterile conditions. In each case, rats were anesthetized with a ketamine cocktail (1ml/kg, i.p., 70 mg/kg ketamine, 6 mg/kg zylazine, 10 mg/kg acepromazine, in 0.9% sodium chloride), received a dose of Marcaine (bupicvicaine, 0.25% Marcaine with sterile 0.9% sodium chloride) under the incision line 30 minutes prior to surgery (subcutaneous, dose of up to 0.4 mL), and a dose of Meloxicam 30 minutes prior to surgery (subcutaneous, 1.0mg/kg of 5mg/mL of Meloxicam). Rats received boosters of the ketamine cocktail (0.5ml/kg) throughout the surgery. When the rats no longer responded to a foot pinch, they were placed on the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and

the head was secured with a nosepiece and ear bars. The scalp was scrubbed with 2% chlorhexidine acetate surgical scrub (Fort Dodge Animal Health, Fort Dodge, IA, USA) and an incision was made along the midline of the scalp. The skin was held with tissue forceps while lambda and bregma were located and then leveled to zero. Glass Hamilton syringes (5 mL, National Scientific Company, Rockwood, TN, USA) were used to infuse viral vectors. Six injections (three in each hemisphere) each consisting of approximately 0.2 μL of viral vectors targeted to the dorsal hippocampus, using coordinates from Paxinos and Watson (1997) as follows: In mm from Bregma: anterior/posterior (A/P): -3.3, Medial/Lateral (M/L): ±3.0, Dorsal/Ventral (D/V): -3.9; A/P: -3.8, M/L: ±3.4, D/V: -4.0; A/P: ±4.3, M/L: +4.0, D/V: -4.2. After the last injection, the incision was sutured using coated vicryl sutures (Ethicon, Inc., Somerville, NJ, USA) and swabbed with triple antibiotic ointment (E. Fougera & Co, Melville, NY, USA). Rats were placed under a heated lamp until they awoke. Rats were then individually housed for approximately three days until the scalp incision healed. During this healing period, rats received post-operative doses of Meloxicam for pain management as well as triple antibiotic ointment over the wound to reduce the risk of infection. Rats were then returned to their original cage mate for the remaining duration of the study.

Viral Vectors

Adeno-associated viral (AAV) vectors containing the coding information for siRNA directed against BDNF and the coding information for enhanced green fluorescent protein (GFP; together, abbreviated throughout the manuscript as ‘siRNA’) were used to knock down hippocampal BDNF levels. Viral vectors composed of a “scrambled” siRNA

coding sequence that is not currently known to correspond to any known rat mRNA and the coding information for GFP (abbreviated throughout the manuscript as 'Scr') served as a control. Vectors were packaged in plasmids that provided AAV2 replicase and AAV9 capsid functions, and a pHelper plasmid (Stratagene, La Jolla, CA, USA) provided adenovirus helper functions. The vectors were co-transfected into AAV-293 cells (Stratagene, La Jolla, CA, USA) with a molar ratio 1:1:1. A Pol III murine U6 promoter controlled the siRNA. The sequence used was ACCATAAGGACGCGGACTTGT (from National Center for Biotechnology Information, reference sequence NM_012513.3). Two days after transfection, the cells were harvested and suspended in Dulbecco's Modified Eagle Medium, and intracellular viral particles were freeze-thawed three times in order to release the virus particles. Afterward, the cells were centrifuged (13,000rpm for 10min), stored at 80 °C, and titered by real-time PCR (ABI Prism 7700 Sequence Detection System, PerkinElmer Applied Biosystems, Foster City, CA, USA). The average titer was approximately 10^{12} DNase Resistant Particles/ml.

Group Assignment

Rats were randomly divided into one of six groups, (n=12/group) based upon stress and viral vector condition: non-stressed controls infused with a scrambled virus (CON-Scr), non-stressed controls with the siRNA against BDNF virus (CON-siRNA), chronically stressed animals given a three-week pre-test recovery period from chronic stress and a scrambled virus (STR-Rec-Scr) or a BDNF siRNA virus (STR-Rec-siRNA), and chronically stressed animals not given weeks to recover (i.e. tested 'immediately'

after the restraint period) and infused with a scrambled virus (Str-Imm-Scr) or infused with the siRNA against BDNF virus (Str-Imm-siRNA).

Chronic Stress Paradigm

Rats were placed in wire mesh restrainers, 18 cm in circumference and 24 cm in length, ends were sealed with grip guard, (Flynn and Enslow, San Francisco, CA, USA). The restrainers were secured at both ends with clips (Black Binder Clips, Staples Inc., Framingham, MA, USA). Rats that outgrew the smaller restrainers were then restrained with larger restrainers (23 cm in circumference and 28 cm in length). The restraint process occurred for 6 hours each day for 21 consecutive days between 09:00 h and 15:00 h. At the same time, the food and water was removed from the controls for 6 hours to keep the food and water access similar across groups.

Radial Arm Water Maze (RAWM)

Apparatus

The RAWM was composed of black polypropylene, with eight symmetrical arms (27.9 cm long x 12.7 cm wide) emanating from a circular center (48 cm diameter). The maze was filled with water and allowed to come room temperature ranging from 20°C - 22°C. The water was rendered opaque with black powder tempera paint. Two testing rooms consisted of several salient extra-maze cues including the door to the room, shelves, heat lamps, and cues made of black and white construction paper located on the walls. Groups were counterbalanced between the testing rooms and were tested by two different experimenters.

Procedure

On the day of testing a platform was placed at the end of one of the arms and positioned 2.5 cm below the surface of the water. Platform location was counterbalanced across and within groups, but was held constant across all trials for a given rat. Testing took place over three consecutive days between 09:00 h and 13:00 h. On day one and two of testing rats were given 8 trials (16 total), and one retention trial was administered on day 3. A trial started as soon as the rat was released into an arm that did not contain the platform (start arm), which varied across trials so that the start arm for a given trial differed from the start arm for the previous trial. Also, the start arm was never directly across from the arm that contained the platform. Once the rat found the platform it was allowed remain on the platform for 15 seconds for spatial localization and was then returned to its testing cage, which was located in the testing room under a heat lamp. If a rat failed to find the platform within three minutes, the rat was gently guided to the platform with a net. After each trial, a net was used to stir the water and collect bedding and feces to prevent rats from using non-spatial cues. In order to avoid exhaustion of the rats during testing, rats were tested in squads. That is, rats from different groups were tested together in squads of six to eight (e.g. two rats from the Con-Scr group were tested with two rats from the Str-Rec-BDNF group and two rats from the Str-Imm-Scr group). Once a rat finished one trial, it was placed back in its testing cage while the other rats in the squad completed that trial. Therefore, the time between each trial for a given rat ranged from 5 minutes to 20 minutes.

Tissue and Brain Processing

Brain extraction

On the third day of RAWM testing, rats received one trial (retention trial) and after the platform was located, were placed back into their testing cage. After 30 minutes had elapsed from when the retention trial was given, rats were transported to a necropsy room and were deeply anesthetized with isoflurane. When the rats no longer responded to a tail pinch (approximately 30 seconds to one minute), they were rapidly decapitated.

Brain sectioning and tissue collection

The unperfused brains were removed, flash frozen in 2-methylbutane kept chilled with dry ice, and stored in a -80°C freezer. The brains were sectioned and processed to determine BDNF levels in one hemisphere using a BDNF sandwich-style ELISA and to identify GFP in the other hemisphere, with each side counter-balanced across subjects. Brains were blocked to target the dorsal hippocampus and cut in a series of two coronal sections (at 20 μm) using a cryostat (Microtome HM 500 OM cryostat, kept between -25 and -30°C). When the dorsal hippocampus was visible, a tissue punch (0.5mm diameter) was taken from one hemisphere (right or left counterbalanced across and within groups) and placed in a cold, pre-weighed and labeled eppendorf tube and stored at -80°C to be later analyzed for levels of BDNF. Tissue punches were taken from the CA1, CA3 and dentate gyrus (DG) subregions of the hippocampus. One set of punches was taken from the anterior dorsal hippocampus and another from the posterior dorsal hippocampus (i.e. a total of two punches per subregion were obtained). Two tissue punches were also taken from the auditory cortex that served as a control for the BDNF assay. After being placed

on subbed slides, one set of a series was stained using a cresyl violet (Cresyl violet acetate, Gibbstown, NJ, USA) to ensure correct placement of the tissue punches. The sections were defatted, stained, and rehydrated through a series of solutions as follows: Hemo-De (Electron Microscopy Sciences, Hatfield, PA, USA), 100% ethanol to 95% ethanol to 70% ethanol to double distilled water, then stained in 0.5% cresyl violet for 15 minutes, then rehydrated in an the reverse order of the solutions, and then coverslipped with permount (Fisher, Fair Lawn, NJ, USA). The second set was coverslipped with Vectashield Hard Set Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and used to visually inspect GFP fluorescence under a fluorescent microscope to confirm accuracy of viral injections as well as to confirm expression of viral vector.

BDNF protein levels

Hippocampal BDNF levels were assessed using a commercially available sandwich-ELISA kit (BDNF Emax ImmunoAssay System Kit, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Tissue punches from the CA1 region of the hippocampus were used to optimize the ELISA. It was determined that shearing of the tissue punches via an automatic tissue lyser was insufficient to extract enough protein for the assays. Thus, Dounce homogenizers (Kimble Chase Life Science and Research Products, Vineland, NJ, USA) were used to manually shear the tissue samples. This method of shearing the tissue led to higher levels of extracted protein and produced ELISA results that were within the detectable range. Due to optimizing the techniques with CA1 punches, there was insufficient CA1 sample data. For the remaining regions, 300 ml of hypotonic lysis buffer (0.05M Tris base in ultra-pure water) containing

a protease inhibitor cocktail (Protease inhibitor cocktail set 1, Calbiochem, EMD Millipore, Billerica, MA, USA) was pipetted into microcentrifuge tubes containing the tissue punches and incubated for at least one hour. After, the lysis buffer and tissue punch were transferred to a Dounce homogenizer and manually sheared. A bicinchoninic acid (BCA) protein assay kit (Micro-BCA assay, Pierce, Rockford, IL, USA) helped determine total protein concentration of the tissue punches. For the ELISA, samples from each region were assayed in duplicate and counterbalanced between and within groups across plates. The BDNF ELISAs had a sensitivity of 15.6 pg/mL and cross-reactivity with other neurotrophins that was less than 3%. Recombinant mature BDNF (supplied by the kit) was used to calculate the standard curve from which the concentration of BDNF in each tissue sample was interpolated. This concentration of BDNF was then divided by total protein (as estimated by the BCA assay) to determine pg BDNF/mg total protein. Data are represented as percent BDNF expression of control rats (Con-Scr).

Viral vector functional assessment

Viral vector expression was confirmed using fluorescent microscopy to visually inspect for GFP location. As the viral vectors contained the coding information for GFP, cells infected with viral vectors expressed GFP protein and fluoresced green. Vectashield Hard-Set mounting medium (Vector Laboratories, Burlingame, CA, USA) was dispensed on slides containing 20μm thick brain sections. Slides were then coverslipped, allowed to dry, and visually inspected for GFP under fluorescent microscopy. To be included in the final analysis, GFP expression must have been visualized in the CA3 region of

hippocampus. If there was no fluorescence in the CA3 region, that rat was excluded from final behavioral analyses.

Synopsis of Experimental Design

At least two weeks after stereotaxic surgery, rats in the Str-Rec group were placed in wire mesh restrainers for 6h/d/21d. Matched control rats had their food and water removed for the same duration as when the Str-Rec rats were in restrainers. The matched controls were also picked up briefly each day in order to be consistent with the daily handling required for the stressed rats. The day after the 21st day of restraint for the Str-Rec rats, the restraint process began for the Str-Imm rats for 6h/d/21d. Again, another subset of control rats had their food and water removed for 6h/d to match the food/water restriction occurring with the Str-Imm rats. The day after the last day of restraint for the Str-Imm rats, RAWM testing began for all conditions. Testing on the RAWM took place over three days, and on the last day animals were sacrificed thirty minutes after their first and only RAWM trial. Trunk blood was collected, as well as the adrenals and brains (Fig. 1).

Statistical Analyses

The statistical software package, SPSS (version 19), the graphing and curve fitting software GraphPad Prism, and a Macintosh computer (OS X 10.7.5) were used for the data analyses. A four-parameter logistic non-linear regression model was used for standard curve fitting in the BCA assays and ELISAs. The unknown concentrations of the samples were interpolated based on the curve. The data were transformed to natural

logarithms to validate the assumption of homoscedasticity. Analyses of variance (ANOVAs) were followed by Fisher's LSD post hoc tests when $p \leq 0.05$. Data are represented by means ± S.E.M.

Results

Viral infection efficacy

The accuracy of tissue punch placement and spread of viral vector infusions was determined by cresyl violet stain and fluorescent microscopy, respectively. Since all viral contents contained the coding sequence for GFP, fluorescent microscopy allowed for visualization of infected cells in all treatment conditions. That is, cells that were infected with either the siRNA or Scr viral vectors fluoresced green. Rats whose brain slices had visible GFP in the CA3 region of the hippocampus were included in the behavioral analyses. The illustration in Figure 2 demonstrates an example of the needle track entering the hippocampus and GFP infected cells within the CA3 region of the hippocampus (Fig. 2). After excluding rats based on this criteria, the final group number per group were: Con-Scr=3, Con-siRNA=8, Str-Rec-Scr=7, Str-Rec-siRNA=9, Str-Imm-Scr=10, Str-Imm-siRNA=10.

Tissue punch assessment

After tissue punches were sampled from the brains of rats, one series of sectioned brain slices were mounted on slides and stained with a cresyl violet to evaluate the accuracy of the punches. Tissue punches were included in the BDNF ELISA if they were localized to stratum pyramidale in the CA3 region for CA3 tissue punches, and the

stratum granulosum of the DG for DG tissue punches. Punches that were outside of these regions were excluded from the BDNF assay (See Figure 2 for examples). Based on these criteria, we achieved an accuracy of 74% for the DG and 76% for the CA3 region. Final group numbers in the BDNF ELISA were: Con-Scr=2, Con-siRNA=4, Str-Rec-Scr=3, Str-Rec-siRNA=7, Str-Imm-Scr=9, Str-Imm-siRNA=10.

Effects of chronic stress and siRNA against BDNF on hippocampal BDNF protein levels

Viral vectors containing the coding information for siRNA directed against BDNF significantly reduced hippocampal BDNF in the CA3 region, as revealed by a 2 X 3 ANOVA for viral vector contents and stress assignment (significant main effect of viral vectors, $F_{(1,29)} = 13.76$, $p < 0.001$. Fig. 3A). No other effects were found to be significant. While not statistically significant, CA3 BDNF expression was approximately 4.3 pg of BDNF per mg of total protein in the Str-Imm-Scr and approximately 6.2 pg of BDNF per mg of total protein in the Con-Scr, which reflected a decrease following chronic stress of approximately 31%. This potential stress-induced decrease is consistent with a pattern noted by other researchers (Taliaz et al., 2011; Lakshminarasimhan and Chattarji, 2012).

BDNF levels in the dentate gyrus and auditory cortex were unaffected by viral vector manipulations. A 2 X 3 ANOVA for viral vector contents and stress condition revealed no significant effects, as all groups had statistically similar BDNF levels in the DG ($p > 0.5$, Fig. 3B), and auditory cortex ($p > 0.5$, Fig. 3C)

Effect of chronic stress and downregulated hippocampal BDNF on spatial reference memory

Prior to running an omnibus ANOVA, the errors across reference memory and working memory trials of the non-stressed control rats were analyzed to determine whether viral vector type influenced performance (compare Con-Scr with Con-siRNA). The two controls were statistically similar for acquisition and retention trials ($p > 0.1$). Consequently, subsequent analyses on the RAWM combined both control groups as one and will be referenced as "Con."

During acquisition on days 1 and 2, all groups decreased the errors made in the reference memory domain. A repeated measures ANOVA for groups across 8 trials on the first and second days of training showed a significant effect of trial on errors in the reference memory domain (Day 1, $F_{(7, 294)} = 8.99$, $p < 0.001$; Day 2, $F_{(7, 294)} = 4.45$, $p < 0.001$). At the start of training on day 1, the average errors in the reference memory domain ranged from 2.4 to 3.3 and decreased to around 0.4 to 1.8 by the end of the last training trial on the same day. There were no other significant effects on either day 1 or 2 (Fig. 4A, 4B).

On the single retention trial on the third day, chronic stress significantly impaired reference memory, which was modulated by recovery from chronic stress and viral vector contents. A one-way ANOVA for treatment condition was performed for reference memory errors and revealed a significant effect ($F_{(4,42)} = 4.13$, $p < 0.01$). Post Hoc analyses revealed that chronically stressed rats that were tested soon after restraint ended and infused with either viral vector type (Str-Imm-Scr, Str-Imm-siRNA) made

significantly more errors in the reference memory domain than did Con ($p < 0.01$) or the chronically stressed rats with recovery time and infused with the scrambled viral vector sequence (Str-Rec-Scr, $p < 0.03$). Importantly, downregulation of hippocampal BDNF impaired the recovery from chronic stress-induced spatial memory deficits. Specifically, the Str-Rec-siRNA rats committed statistically more errors in the reference memory domain than did the Str-Rec-Scr and Con rats ($p < 0.03$) and performed similarly as the chronically stressed rats tested immediately (Str-Imm-Scr, Str-Imm-siRNA, Fig. 4C).

Effect of chronic stress and downregulated hippocampal BDNF on spatial working memory

Errors within the working memory domain were analyzed across all trials for the non-stressed control rats (Con-siRNA and Con-Scr) and revealed that the two groups were statistically similar ($p > 0.1$). Therefore, the control groups were combined as one for subsequent analyses for the working memory assessment.

All groups showed improvement in the working memory domain on the RAWM during acquisition on days 1 and 2. A repeated measures ANOVA for groups across trials on day one and day two of training revealed a significant effect of trial on errors in the working memory domain (Day 1, $F_{(7, 294)}=8.39$, $p<0.001$; Day 2, $F_{(7, 294)}=7.42$, $p<0.001$). The average errors in the working memory domain ranged from 1.7 to 2.3 on trial one at the start of day one and dropped to 0 to 0.2 by the last trial of day one, with a similar pattern on day 2 (Fig. 5A, 5B).

On the retention trial on day 3, errors in the working memory domain were not affected by any of the treatments. A one-way ANOVA for group assignment on errors

within the working memory domain during the retention trial failed to show a significant difference between groups (Fig. 5C).

Effects of Chronic Stress and downregulated hippocampal BDNF on physiological measures

Body Weight

Chronic stress significantly altered body weight gain, which was unaltered by the contents of the viral vectors. A 2 X 3 ANOVA for viral vector contents and stress history on body weight gain from week 1 to 4, and on body weight gain from week 4 to 7 revealed a significant main effect of stress history on body weight gain ($F_{(2,41)}$=57.56, $p < 0.001$; and $F_{(2,41)}$=148.21, $p < 0.001$, respectively. Fig. 6A). The effect was further probed to determine the effect of stress history on group assignment. Analysis on body weight gain from week 1 to 4, when the Str-Rec rats underwent restraint, showed that Str-Rec rats gained significantly less weight than did the rats in both the Con and Str-Imm groups ($F_{(5, 41)}$=24.46, $p < 0.001$). Likewise, an analysis on body weight gain from week 4 to 7 across groups demonstrated that the rats in the Str-Imm groups gained significantly less weight during their period of restraint than did both the Con and Str-Rec rats ($F_{(5,41)}$=59.85, $p < 0.001$). Rats in the CON group gained weight continuously throughout the experiment. In contrast, rats in the Str-Rec group lost weight during the three weeks they were subjected to chronic restraint stress, but then gained weight rapidly after restraint ended. For rats in the Str-Imm group, they steadily gained weight during the three weeks they were not placed in restraints, but progressively lost weight during the three weeks that they were chronically restrained. Consequently, restraint slowed body

weight gain when it was implemented and rats showed increases in body weight gain during the weeks that they were not restrained (Fig. 6A).

Adrenal Weights

Restraint stress produced a significant increase in adrenal weights relative to body weights to demonstrate the increased demand on the adrenals. Adrenal weights were analyzed as total adrenal weights per 100 grams of body weight. A 2 X 3 ANOVA for contents of viral vector and stress assignment revealed a significant effect of stress on adrenal weights ($F_{(2, 40)}=5.25$, $p < 0.01$, Fig. 6B). A Post Hoc analysis showed that rats in the Str-Imm group displayed significantly larger adrenal weights that both the Con rats ($p < 0.001$) and Str-Rec rats ($p < 0.03$). Furthermore, it appeared the rats in the Str-Imm-siRNA group had a very large adrenal to body weight ratio. In order to investigate this potential effect, a between subjects ANOVA on group assignment was performed and showed that there was a significant effect of group assignment on adrenal weights ($F_{(5, 40)}=4.28$, $p < 0.01$, Fig. 6B). Post Hoc analysis revealed that rats that were tested immediately after stress ended and infused with siRNA against BDNF (Str-Imm-siRNA) had significantly larger adrenal glands than did all other groups ($p < 0.05$).

Discussion

The current study investigated whether downregulating hippocampal BDNF directed toward the dorsal CA3 region would exacerbate spatial memory deficits produced by chronic stress and hinder the recovery from these deficits after stress has ended. With the latter point, we present new findings that hippocampal BDNF is

necessary for the process of recovery from chronic stress-induced spatial reference memory deficits. Rats with downregulated hippocampal BDNF failed to show improvements in spatial reference memory following a post-stress recovery period. With regard to the first prediction, we failed to find evidence that downregulation of hippocampal BDNF exacerbated spatial learning and memory deficits of chronically stressed rats. Nonetheless, we corroborated past findings to demonstrate that chronic stress impairs spatial reference memory in rats tested soon after the chronic stress manipulation has ended. To our knowledge, this is the first study to show that hippocampal BDNF mediates the recovery from chronic stress-induced spatial reference memory deficits.

Chronic stress results in a number of physiological changes, such as reduced body weight gain and enlarged adrenal glands, reflecting alterations that are commonly observed following chronic stress (Conrad, 2006). Similar to prior studies, chronic restraint in the current study attenuated body weight gain (Watanabe et al., 1992a; Magariños and McEwen 1995; Kleen et al., 2006; Wright and Conrad, 2008; Hoffman et al., 2011; Hutchinson et al., 2012) and increased adrenal gland weights (Watanabe et al., 1992b; Verkuyl et al., 2004; Ulrich-Lai et al., 2006; Kleen et al., 2006; Flak et al., 2011). When restraint ended, body weights and adrenal weights recovered to similar levels as observed in the nonstressed controls. These results demonstrate that our paradigm of restraint was stressful to the rats and that improvement was evident in the rats given a recovery period. Unexpectedly, rats that were infused with the siRNA viral vectors, chronically stressed, and precluded from the recovery period (Str-Imm-siRNA) displayed significantly larger adrenal weights compared to all other groups. The potential

mechanism(s) behind this observation are unknown, but it is possible that BDNF downregulation in the hippocampus blunted the hippocampus' regulation of the HPA axis. Past studies have demonstrated that glucocorticoid receptors (GRs) in the hippocampus are necessary for negative feedback on the hypothalamic-pituitary-adrenal (HPA) axis to attenuate glucocorticoid release (Ratka et al., 1989; Dallman et al., 1992). Following, chronic stress hippocampal GRs are downregulated (Mizoguchi et al., 2003; Kitraki et al., 2004; Wright et al., 2006), which can hinder HPA axis regulation and may lead to enlarged adrenals due to greater demand, as observed with the Str-Imm group. Blunting BDNF expression may exacerbate this further as one study found that the repeated activation of hippocampal GRs suppresses the ability of BDNF to release glutamate *in vitro* (Numakawa et al., 2009). While glucocorticoids, through the activation of GRs, can alter the synthesis and activity of BDNF (reviewed in: Jeanneteau and Chao, 2012), BDNF's actions on glucocorticoids and GRs require further study. Future work will be needed to investigate the relationship between chronic stress and hippocampal BDNF in HPA axis regulation.

One purpose of the current study was to determine whether downregulated hippocampal BDNF would exacerbate the detrimental effects of chronic stress on spatial learning and memory. While, we found chronically stressed rats tested soon after stressor cessation (Str-Imm) displayed the most errors within the reference memory domain than did any other group, we failed to find that BDNF downregulation exacerbated the deficits. Specifically, the Str-Imm-siRNA rats showed statistically similar performance as the Str-Imm-Scr rats on errors within the reference memory domain with regard to acquisition (days 1 and 2) and memory (day 3). Consequently, our prediction that

hippocampal BDNF downregulation would exacerbate chronic stress-induced spatial learning and memory deficits was not supported. Perhaps in the current study, chronic stress may have already lowered BDNF to levels that produced a floor effect that may have rendered any further decreases in BDNF via viral vectors to be ineffective. Another interpretation is that behavioral training in the RAWM may have masked the full extent of stress-induced changes of BDNF expression. Past studies have documented training in the Morris water maze (Kesslak et al., 1998) and radial arm maze (Mizuno et al., 2000) to increase hippocampal BDNF mRNA levels. This latter interpretation is raised because we failed to observe statistically significant decreases in hippocampal BDNF levels following chronic stress, an effect observed by others (Taliaz et al., 2011; Lakshminarasimhan and Chattarji, 2012). While our findings were not statistically significant, the pattern toward a chronic stress-induced decrease in CA3 BDNF levels that reversed during the recovery period was observed. Consequently, the changes in hippocampal BDNF expression in response to chronic stress may be subtle and yet physiologically significant (Mizoguchi et al., 2003). Such relatively small changes have been documented in other systems, such as the small, approximately 10% decreases in hippocampal glucocorticoid receptor mRNA expression that leads to significant effects on spatial memory (Wright et al., 2006). Therefore, there may be valid reasons as to why the current paradigm did not observe downregulated hippocampal CA3 BDNF to exacerbate chronic stress-induced spatial memory.

A novel finding is that hippocampal BDNF mediates the recovery of spatial reference memory following the end of chronic stress. This is supported by the observation that chronically stressed rats infused with siRNA against BDNF within the

dorsal hippocampus failed to recover from stress-induced deficits within the spatial reference memory domain. Specifically, rats in the Str-Rec-siRNA group displayed a high number of first time entry errors (i.e., entries into non-platformed arms) that were statistically similar to the rats that were tested immediately after stress cessation (Str-Imm-siRNA or Str-Imm-Scr). This outcome is consistent with spatial reference memory deficits that follow BDNF downregulation after intracerebroventricular infusions of antisense BDNF oligonucleotides in the hippocampus (Mizuno et al., 2000). Together, these reports support the interpretation that BDNF in the dorsal hippocampus is necessary for spatial reference memory.

The current study also found that chronic stress impaired spatial memory in the reference memory domain without altering the working memory domain. Specifically, chronic stress disrupted stable, long-term information that remained constant over trials (first time entry errors into non-platformed arms) and failed to alter flexible information that varied from trial to trial (repeated entries into non-platformed arms). We had previously found chronic stress to impair both reference and working memory and that both recovered after the end of chronic stress (Hoffman et al., 2011). However, there are reports showing chronic stress disrupts reference memory and not working memory (Srikumar et al., 2006; 2007), which means that it is not uncommon to observe deficits in spatial reference memory and not spatial working memory. Moreover, one study reported that stress-induced working memory deficits are more fleeting than spatial memory deficits (Krugers et al., 1997). Our research group also found that even when conditions are designed to study chronic stress effects on working memory, the deficits can be

transient (Mika et al., 2012). Thus, stress-induced effects in the working memory domain may be more difficult to detect than those in the spatial reference memory domain.

Given that BDNF is required for spatial memory (Mizuno et al., 2000; Cunha et al., 2010), then our finding that hippocampal BDNF downregulation in the unstressed control rats did not significantly affect spatial memory is surprising. Even though hippocampal CA3 BDNF protein levels were significantly lower in the Con-siRNA than the Con-Scr (Fig. 3), both conditions performed similarly on the RAWM (data not shown). Another study revealed similar findings using a lentiviral mediated knockdown of BDNF whereby hippocampal BDNF levels were decreased but without an effect on novel environment exploration or depressive-like behaviors in the non-stressed controls (Taliaz et al., 2011). Perhaps the decreases in hippocampal BDNF play a role in a compromised system, such as providing a vulnerability following exposure to chronic stress.

The mechanism by which BDNF downregulation within the hippocampus disrupts spatial reference memory most likely involves the CA3 region. Viral vector infusions targeted the dorsal CA3 region of the hippocampus, but also included some spread to other regions, such as the dentate gyrus and CA1 regions as well. However, if infusions missed the CA3 region, those animals were excluded from the analysis. Consequently, the common denominator across groups was the targeted infusion of the CA3 region, which was most likely germane for the recovery in spatial memory following the end of chronic stress. Moreover, BDNF levels were statistically similar in the DG region. Given that CA3 apical dendritic arbors recover from stress-induced retraction within the timeframe that spatial memory deficits recover (Hoffman et al.,

2011; Sousa et al., 2000, Luine et al., 1994), perhaps BDNF is involved in mechanisms underlying CA3 dendritic restructuring and corresponding spatial memory. Mechanisms involving BDNF regulating neuronal morphology seems even more plausible given BDNF's role in synaptic plasticity and strengthening, especially in regulating dendritic and spine structure (McAllister et al., 1995; Horch et al., 1999; Yacoubian et al., 2000; Chao, 2003; Cowansage et al., 2010; Koleske, 2013). BDNF's actions might involve tropomyosin receptor kinase B (TrkB), a high affinity receptor for BDNF that is expressed in the CA3 region (Minichiello et al., 2002). TrkB mediates many functions of BDNF, including axonal branching and outgrowth (Jeanneteau et al., 2010), hippocampal LTP (Minichiello, 2009), and learning and memory (Cunha et al., 2010). Importantly, TrkB activation is essential for optimal hippocampal-dependent learning and memory tasks (Minichiello et al., 1999). Taken together, chronic stress-induced spatial reference memory impairments most likely involve hippocampal dendritic architecture in the CA3 region of the dorsal hippocampus and future studies are needed to investigate how a post-stress recovery period and BDNF interact to influence hippocampal CA3 dendritic complexity.

This study demonstrates that BDNF levels in the hippocampus is necessary for the recovery from a chronic stress challenge. In other words, lowered hippocampal BDNF in the CA3 region compromised resilience, or the ability to rebound from adversity. Low hippocampal BDNF levels in non-stressed individuals did not seem to produce any overt detrimental outcomes as measured by spatial memory. So, low hippocampal BDNF levels seemed critical in spatial memory ability when an individual is compromised, such as one exposed to chronic stress. Uncovering factors, such as BDNF, that are important in

mediating plasticity and facilitating recovery may help identify novel mechanisms that can be targeted to enhance resilience in the face of adversity.

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Figure 1: Timeline of Study: Rats were infused with viral vectors containing either the coding information for a siRNA against BDNF or a scrambled sequence with all viral vectors containing the coding information for green fluorescent protein (GFP). After rats recuperated from surgery for two weeks, the stress manipulations started. Rats in the stress recovery (Str-Rec) group were restrained in wire mesh restrainers for 6h/d/21d, which was then followed by a 21-day recovery period without restraint. The opposite was provided for the rats in the stress immediate group (Str-Imm) in which they were left in their home cage for the first half of the study and then restrained for 6h/d/21d during the latter half of the paradigm. The day after the last day of restraint for the Str-Imm group, all rats were tested on the radial arm water maze (RAWM), which took place over three days. After the single retention trial on day 3 of RAWM testing, rats were sacrificed and adrenal glands and brain tissue was processed for analysis.

Figure 2: Verification of Viral Vector Infusion Accuracy and Punch Assessment: Brain tissue was sectioned in the coronal plane and viewed under fluorescent microscopy to determine anatomical location of viral vector infusions. Another set of sectioned brain tissue was stained with cresyl violet to determine accuracy of tissue punches. Neurons that were successfully infected with viral vectors expressed the coding information for GFP. The left hemisphere illustrates photographs of GFP infected cells within the CA3 region of the hippocampus. Examples of an infusion track and correct placement in the CA3 region are represented. For tissue punch assessment, punches were determined to be accurate if they were localized to the stratum pyramidale in the CA3 region for CA3 tissue punches, and stratum granulosum of the DG for DG tissue punches. Tissue

punches that were outside of these regions were excluded from the BDNF ELISA. The right hemisphere shows representative accurate punches in the CA3 and DG regions.

Figure 3: BDNF Protein Levels: Viral vectors containing the coding information for siRNA against BDNF or a scrambled (Scr) sequence were injected bilaterally into the hippocampal CA3 region. An ELISA helped assess BDNF protein levels in the hippocampal CA3 region and DG, as well as the auditory cortex. **(A)** In the CA3 region of the hippocampus, viral vectors coding for siRNA against BDNF decreased BDNF protein levels in all three stress conditions (Con-siRNA, Str-Imm-siRNA Str-Rec-siRNA). In this paradigm, chronic stress did not significantly lower CA3 BDNF levels (compare Str-Imm-Scr with Con-Scr), although the Str-Imm-Scr mean levels were approximately 31% lower than observed for the Con-Scr. **(B)** Viral vectors or chronic stress did not significantly affect BDNF protein expression in the DG **(C)** or the auditory cortex. Data are represented as percent control (Con-Scr). ** $p < 0.001$, relative to Scr counterparts.

Figure 4: Errors Within the Reference Memory Domain on the RAWM: The number of errors committed within the reference memory domain was scored as first time entries into arms that never contained a platform. **(A, B)** All groups showed that they learned the task and were statistically similar to each other, as demonstrated by decreased entry errors across days 1 and 2. **(C)** During the single retention trial on day 3, statistically significant differences emerged, with rats that were chronically stressed and tested immediately after stressor cessation (Str-Imm) committing significantly more reference

memory errors than did the Con and Str-Rec-Scr groups, regardless of contents of viral vectors (Str-Imm-Scr, Str-Imm-siRNA). Importantly, rats given time to recover (Str-Rec) differed in their performance on the retention trial based upon viral vector contents. Rats infused with the siRNA against BDNF (Str-Rec-siRNA) made a high number of errors in the reference reference memory domain and were statistically similar to rats in the Str-Imm group. In contrast, rats in the Str-Rec group infused with the scrambled virus (Str-Rec-Scr) made the fewest errors, on average, in the reference memory domain and were statistically better than Str-Rec-siRNA. *$p<0.05$ compared to controls (Con) and Str-Rec-Scr.

Figure 5: Working Memory Errors on the RAWM: The number of errors committed within the spatial working memory domain were scored as repeated entries into arms that never contained a platform. **(A, B)** All groups showed improved acquisition that was statistically similar to each other. Specifically, spatial working memory errors decreased across the first two days of testing on the RAWM. **(C)** Similarly, on the single retention trial on day 3, all rats committed statistically similar amounts of errors within the working memory domain.

Figure 6: Physiological Data: **(A)** Body Weight Gain: Chronic stress significantly altered body weight gain. All rats weighed the same at the time of surgery (week 0). Rats in the Str-Rec group (Scr and siRNA) gained significantly less weight than did all other groups during the time they were subjected to chronic restraint stress (weeks 1-4). Similarly, when restraint began for the rats in the Str-Imm group (Scr and siRNA), body weight

gain was significantly attenuated (weeks 4-7). ** $p<0.001$ for main effect of stress condition (regardless of viral vector contents) compared to all other groups. **(B)** Adrenal Weights: Chronic stress significantly altered adrenal weights. Rats in the Str-Imm group had a larger adrenal body weight ratio than did both the Con and Str-Rec rats. Furthermore, Rats in the Str-Imm group (Scr and siRNA) had the largest adrenal to body weight ratio at the end of the study, with rats in the Str-Imm-siRNA group having larger adrenal glands than all other groups. Data are represented as adrenal weight per 100 grams of body weight. * $p < 0.03$ compared to Con and Str-Rec, # $p < 0.03$ compared to all other groups.

Figure 1

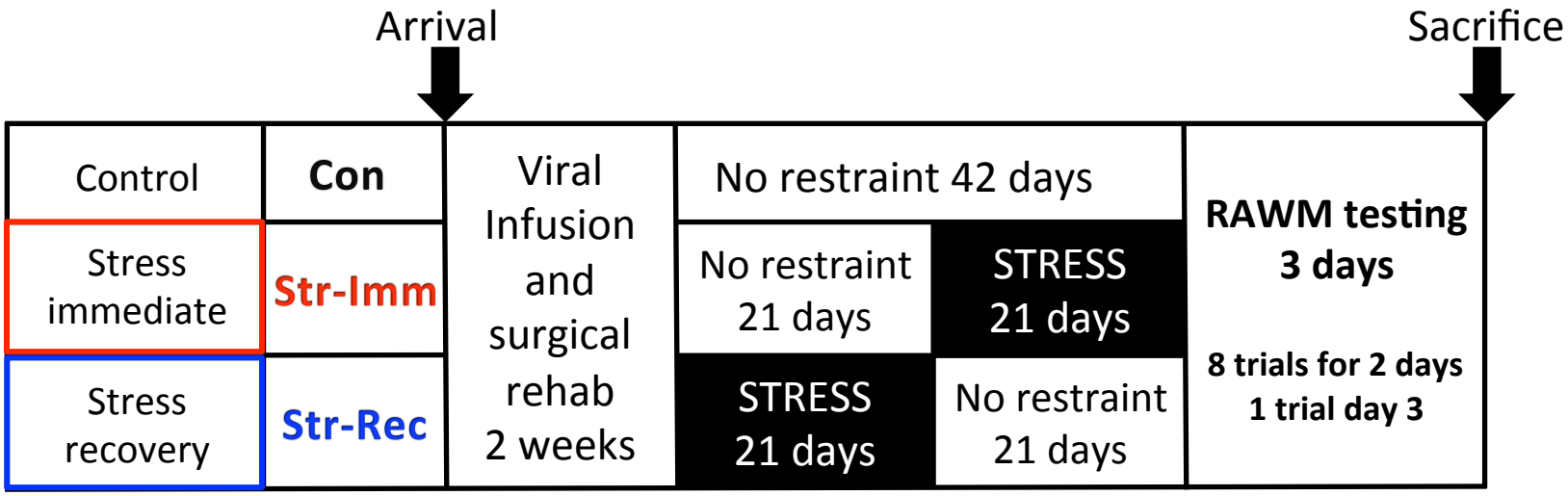

Arrival
Sacrifice
Control
Con
Stress immediate
Str-Imm
Stress recovery
Str-Rec
Viral Infusion and surgical rehab 2 weeks
No restraint 42 days
No restraint 21 days
STRESS 21 days
STRESS 21 days
No restraint 21 days
RAWM testing 3 days
8 trials for 2 days
1 trial day 3

Figure 2

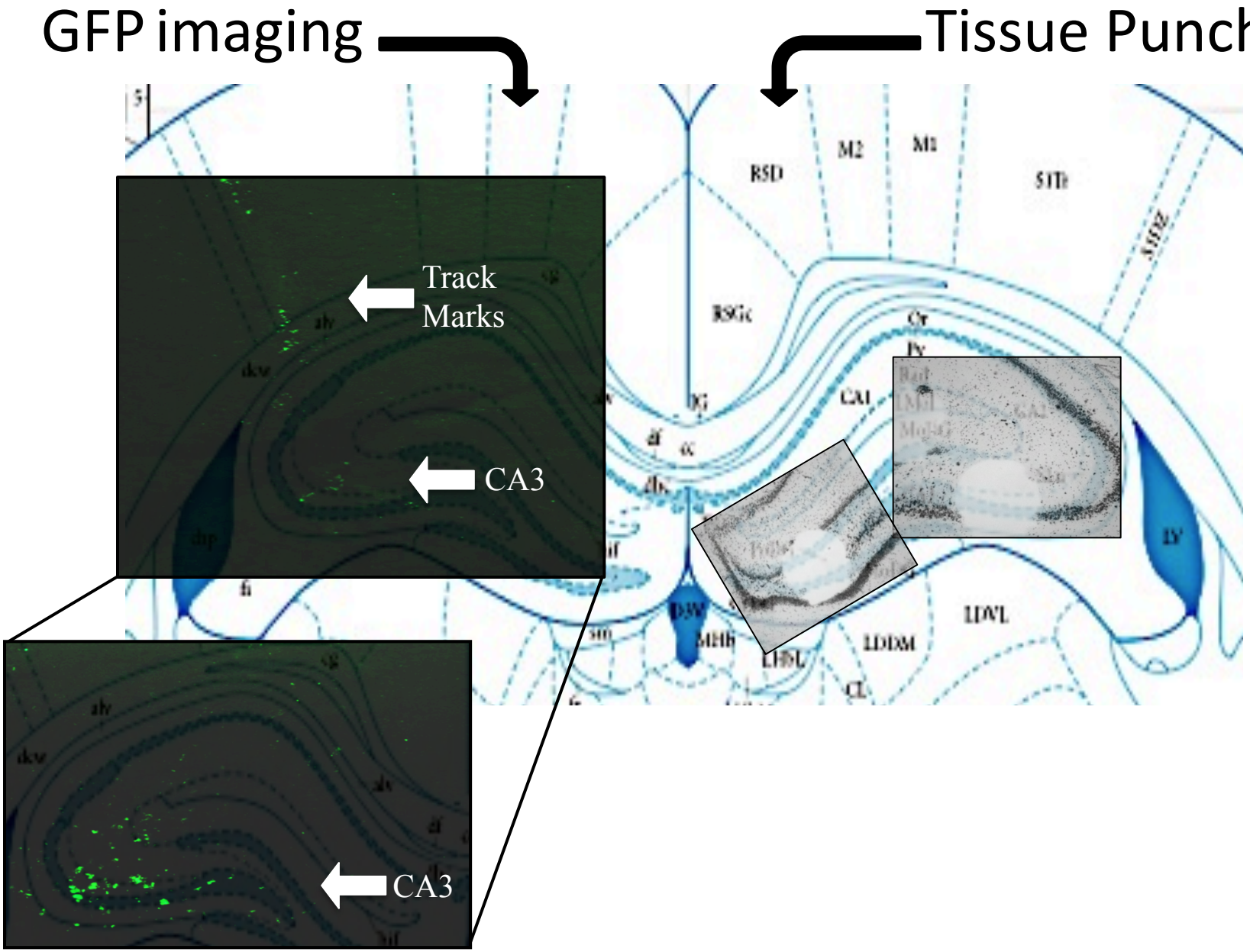

GFP imaging
Tissue Punches
Track Marks
CA3
CA3
M2
M1
RSD
RSGc
CA1
LDVL
LDDM

Figure 3

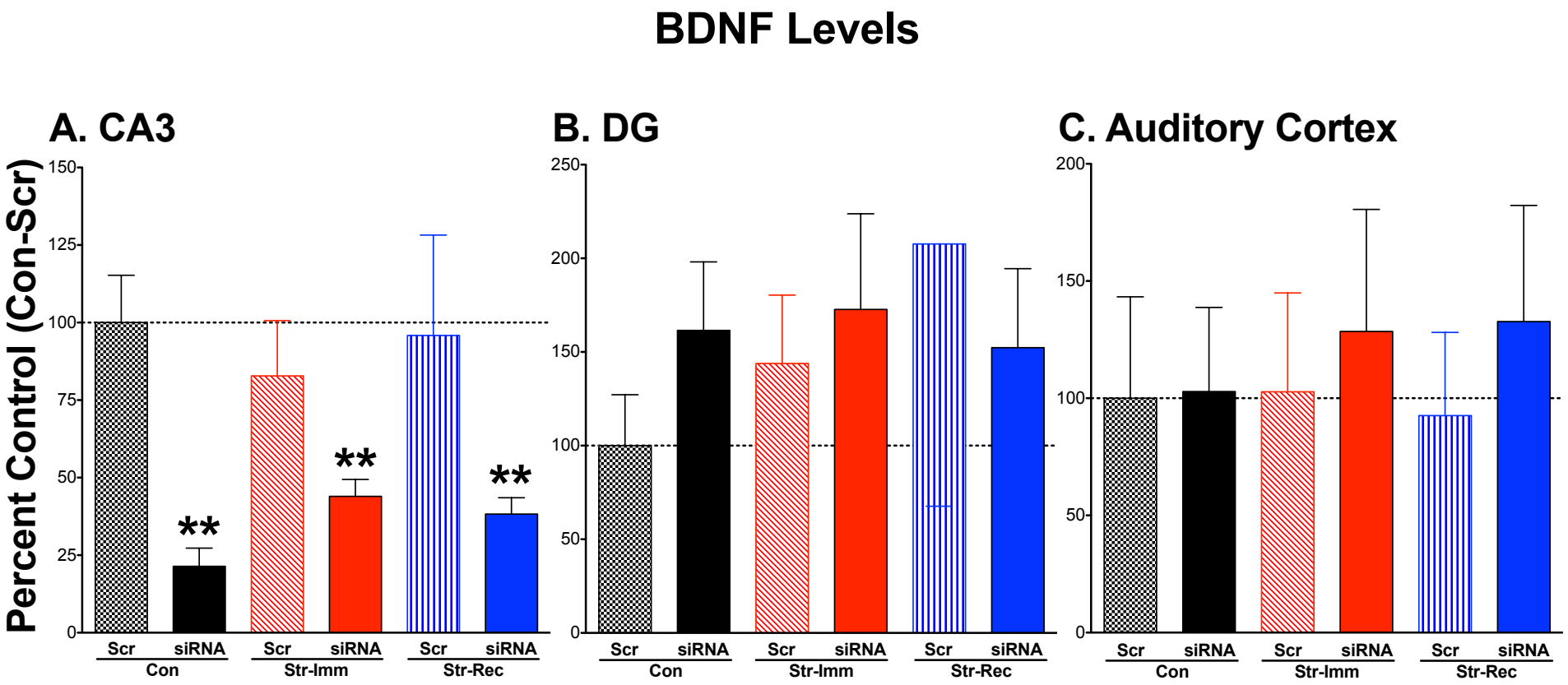

BDNF Levels
A. CA3
B. DG
C. Auditory Cortex
Percent Control (Con-Scr)
150
125
100
75
50
25
0
250
200
150
100
50
0
200
150
100
50
0
**
**
**
Scr
siRNA
Con
Str-Imm
Str-Rec

Figure 4

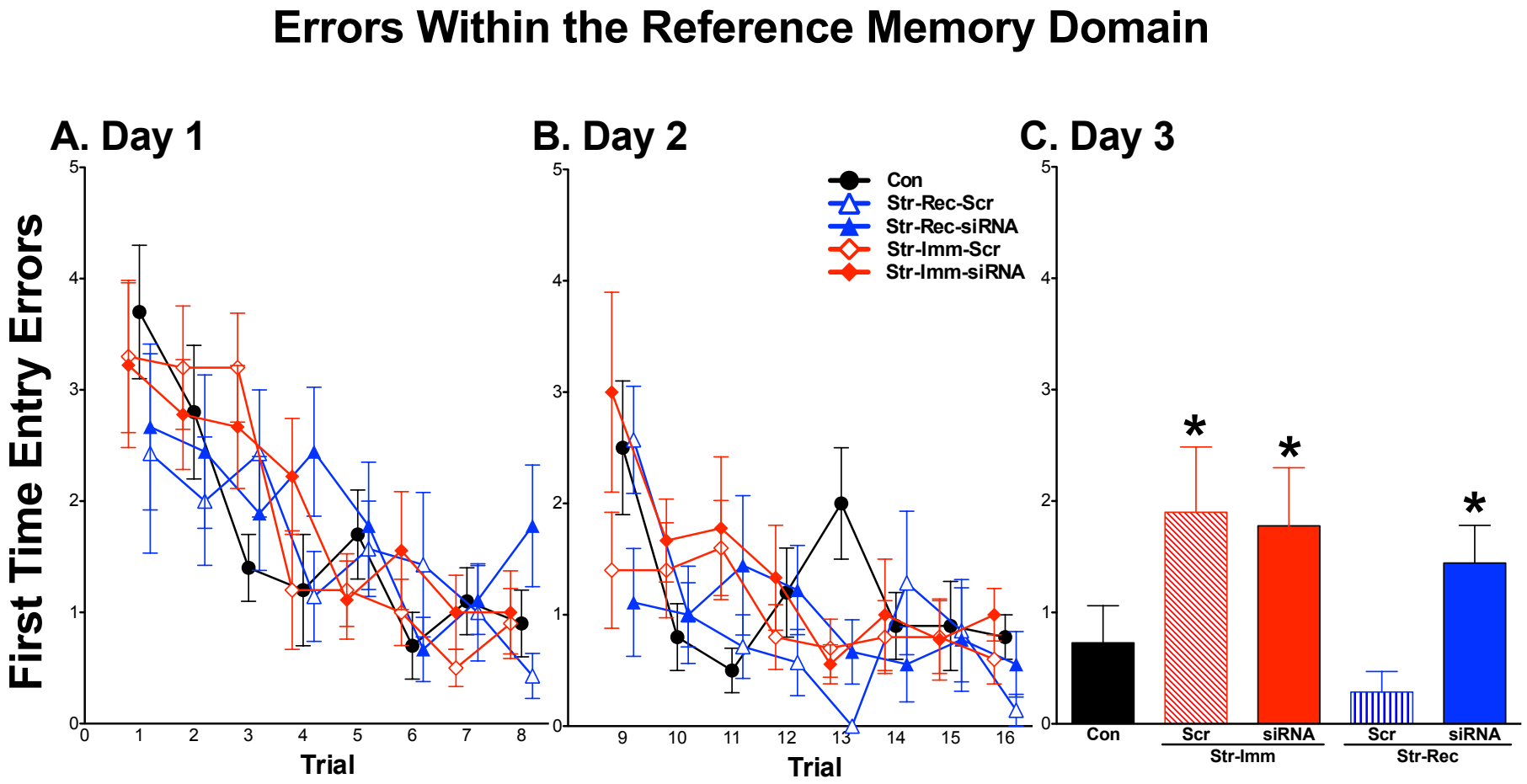

Errors Within the Reference Memory Domain
A. Day 1
B. Day 2
C. Day 3
First Time Entry Errors
Trial
Trial
Con
Str-Rec-Scr
Str-Rec-siRNA
Str-Imm-Scr
Str-Imm-siRNA
Con
Scr
siRNA
Scr
siRNA
Str-Imm
Str-Rec

Figure 5

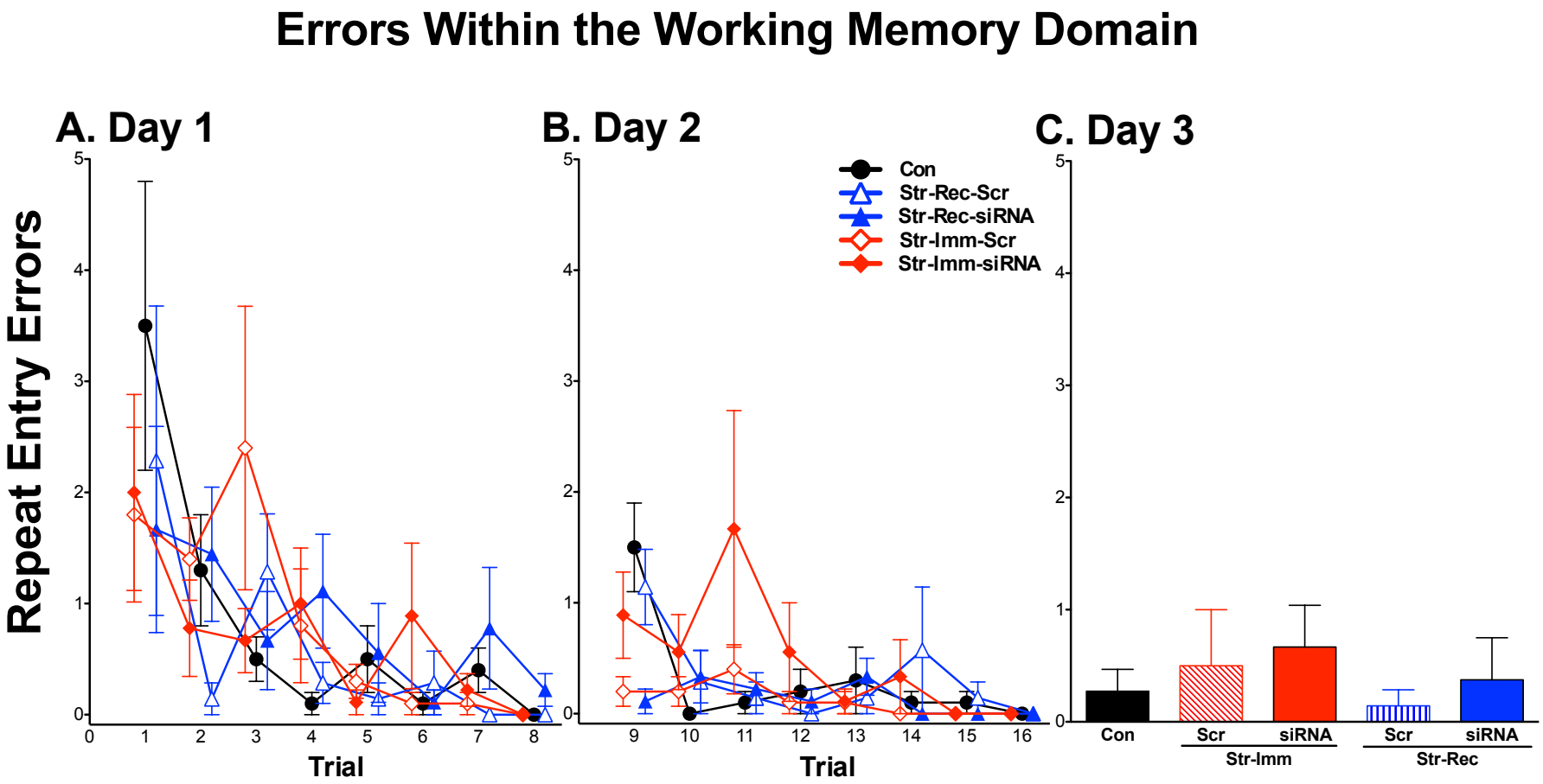
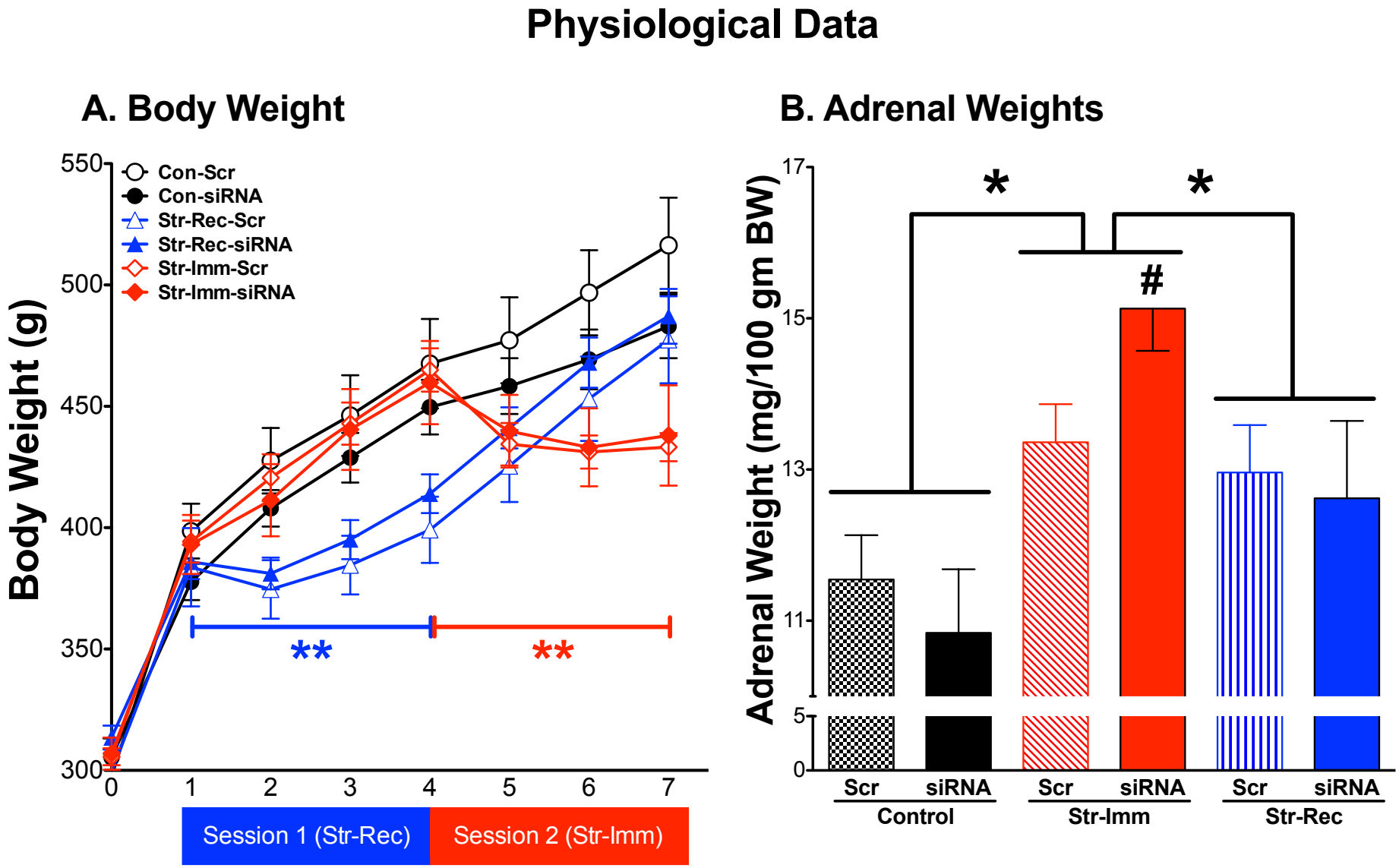

Errors Within the Working Memory Domain
A. Day 1
B. Day 2
C. Day 3
Repeat Entry Errors
Trial
Trial
Con
Str-Rec-Scr
Str-Rec-siRNA
Str-Imm-Scr
Str-Imm-siRNA
Con
Scr
siRNA
Scr
siRNA
Str-Imm
Str-Rec

Figure 6


Physiological Data
A. Body Weight
B. Adrenal Weights
Body Weight (g)
550
500
450
400
350
300
0
1
2
3
4
5
6
7
Con-Scr
Con-siRNA
Str-Rec-Scr
Str-Rec-siRNA
Str-Imm-Scr
Str-Imm-siRNA
**
**
Session 1 (Str-Rec)
Session 2 (Str-Imm)
Adrenal Weight (mg/100 gm BW)
17
15
13
11
5
0
*
*
#
Scr
siRNA
Scr
siRNA
Scr
siRNA
Control
Str-Imm
Str-Rec