THE EFFECTS OF HIPPOCAMPAL LESIONS ON NEUROTROPHINS IN THE RODENT NEOCORTEX

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Dedication

To my late professor, mentor, supervisor and friend Dr. Patrick Jackson who helped me with my grad school application (while on vacation). Here is my "smart stuff" Dr. J.

And to my beautiful daughter Kynlee, who spent many hours playing next to me while I worked on this thesis. I hope this inspires you to achieve greatness in whatever it is you choose to do in life, I love you.

ABSTRACT

Environmental enrichment (EE) upregulates neurotrophin (NT) expression in both the hippocampus (HPC) and neocortex. We expected this effect to be reduced in the NC by hippocampal lesions. To test this, middle-aged female rats with unilateral, bilateral or sham lesions of the HPC, lived in enriched or social housing. After three months, rats freely explored a novel environment for 5 minutes, were immediately perfused and brains processed for brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) protein. Hippocampal lesions had no effect on BDNF in neocortex. Right, but not left, hippocampal lesions had a significant negative impact on behavioral exploration of social rats; and this effect was not present in the enriched group. Enriched rats showed significantly reduced BDNF expression in deep cortex with a similar trend in superficial layers regardless of lesion condition. The role of the HPC in neocortical plasticity processes remains variable and unclear.

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Also, many thanks to: Dr. Michael Eckert for his close mentorship throughout the project, thank-you for all your technical knowledge, Matlab and ImageJ scripts, patience, time and sense of humor throughout this project; thanks to Aubrey Demchuk for mentorship, advice, and teaching me how to perform immunohistochemistry; thanks to both Aubrey and Michael for their help with behavior and perfusions; Leonardo Molina for Matlab script; Valérie Lapointe for help in the wet lab; Maurice Needham for continuous Nanozoomer maintenance; Amanda Mauthe-Kaddoura and Jennifer Tarnowsky for their load-bearing administrative abilities, keeping the Canadian Centre for Behavioural Neuroscience operational.

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BDNF	Brain Derived Neurotrophic Factor
dysRSC	Dysgrandular retrosplenial
EE	Environmental enrichment
grRSC	Granular retrosplenial
HPC	Hippocampus
NC	Neocortex
NGF	Nerve Growth Factor
NT	Neurotrophin
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NMDA	N-methyl-D-aspartate
PFC	Prefrontal cortex
ROI	Region of interest
SC	Social control
TBS	Tris buffered saline
TBST	Tris-buffered saline with 0.05% Tween-20
TrkB	Tyrosine kinase receptor-B
TSA	Tyramide signal amplification

INTRODUCTION

Memory

Neural representations of our daily experience (memories) are "the raw material for intelligence and thought" (B. L. McNaughton, 2010). This type of memory, termed declarative, relies heavily on the medial temporal lobe memory system and is the foundation for semantic knowledge. Semantic knowledge comes from the brain's remarkable ability to store information (episodes and facts in this case) and associate new with previously stored information. How memories are stored, retrieved and gradually transformed into semantic knowledge remains one of the ultimate questions in neuroscience.

The Hippocampus

The hippocampus (HPC), sometimes referred to as the archicortex, represents the 'highest level of association cortex' at the peak of the cortical processing hierarchy (B. L. McNaughton, 2010); it has wide-spread and reciprocal communication with neocortical association regions (Swanson & Kohler, 1986; Van Hoesen, 1982) and is robustly involved in declarative memory. Controversy on hippocampal function arose in 1960 after attempts were made to reproduce a similar pattern of amnesia to that of patient H.M. (Scoville & Milner, 1957) in animal models (Orbach, Milner, & Rasmussen, 1960). Since then, multiple theories have emerged to explain hippocampal involvement in memory. The standard model of systems consolidation theory suggests the HPC initially stores the memory trace and gradually, over time, transfers the memory to the neocortex (NC), ultimately becoming independent of the HPC (Squire & Alvarez, 1995). This standard model came into question following the discovery of 'place cells' in the HPC (O'Keefe & Dostrovsky, 1971); which

are cells that selectively fire in a particular place ('place fields') within a given environment. The discovery of 'place cells' in the HPC naturally led to hippocampal indexing theory (Teyler & DiScenna, 1986) which theorized that the HPC simply provides an address to the memory location in the NC and does not store the memory at all. The characteristics of 'Place cells' make them ideal indexing cells in that they are sparse and orthogonal by nature (McNaughton et al., 1996). The nature of 'place cells' has also elegantly solved the issue of similar events occurring at different places in space (McNaughton et al., 1996) as the two events would be assigned a different index.

More recently, Nadel and Moscovich (1997) proposed the multiple memory trace theory. According to the multiple memory trace theory the HPC is required to create, store and retrieve: spatial, contextual and episodic memories; for the life of the vivid memory" (Nadel, 2018). The HPC is also required to construct semantic knowledge from raw memories, however, its role in this process is transient and once the transformation to semantic knowledge is sufficiently complete, the HPC is no longer required for its retrieval. In this view, the HPC contains spatial and contextual representations in a unique way that is not reproducible elsewhere in the brain (Nadel, 2018; Nadel & Moscovitch, 1997).

Neuroplasticity

As the name implies, neuroplasticity refers to the brain's ability to change and adapt in an experience-dependent manner (learning) or, in response to brain insults or disease. These changes occur on all levels of organization; from chemical and synaptic alterations (Bennett, Diamond, Krech, & Rosenzweig, 1964) to systems level of organization (Merzenich et al., 1984). Storing information in the brain (memory) requires these changes and for memories to persist, these changes must be long-term (Gómez-Palacio-Schjetnan

& Escobar, 2013). Indeed, the HPC is a remarkably plastic region of the brain and one of the few regions that may continue producing new cells throughout life and into old age (Boldrini et al., 2018; Eriksson et al., 1998) but see (Arellano, Harding, & Thomas, 2018; Sorrells et al., 2018).

The entire NC sends highly processed information to the HPC and receives input from the HPC both directly and indirectly (B. L. McNaughton, 2010; Preston & Eichenbaum, 2013). This wide-spread hippocampal-cortical interaction provides a framework for the HPC to drive neuroplasticity in the NC. Given that we know hippocampal activity is correlated with environmental exploration and navigation, it is possible the HPC is responsible, at least in part, for driving neuroplastic change in the NC. The NC is arguably the most sensitive brain structure to experience (Diamond, 2001) and altering the environment, by increasing or decreasing its complexity can yield significant changes to the structure and function of the brain.

Environmental Enrichment and its Effects on Cortex and Hippocampus

Donald Hebb in 1949 first proposed the idea of environmental enrichment (EE) as an experimental treatment (Simpson & Kelly, 2011; Van Praag, Kempermann, & Gage, 2000). Hebb noticed that rats he took home as pets performed better on behavioral tests than their laboratory-housed littermates (Rosenzweig & Bennett, 1996; Simpson & Kelly, 2011; Van Praag et al., 2000). The notion the brain changes in response to the environment is not a new idea. However, it was not proven until more recently, when groups demonstrated the brain can be morphologically as well as chemically altered by experience (Bennett et al., 1964; Hubel & Wiesel, 1965). There are varying ideas of what constitutes an enriched environment, generally-speaking however, EE is implemented relative to standard

laboratory housing and involves a spectrum of environmental complexity. Multiple factors can be manipulated in EE to increase or decrease the complexity of the environment which typically include: size of housing, number of individuals, number of novel objects, structures to climb in/on, different materials for bedding/nest-building, opportunity to navigate/explore, opportunity for voluntary exercise, different foods and the maintenance of novelty by changing these factors regularly. Importantly, it is the combination and interaction of these factors that is required to elicit the full effects of EE in the brain; for example, neither social interaction nor novel object interaction alone can account for the overall effects of EE (Diamond, 2001; Rosenzweig, Bennett, Hebert, & Morimoto, 1978). Moreover, a complex, enriching environment, as a whole, seems to have greater effects than would be expected by summing the individual factors in the enriched environment (Van Praag et al., 2000).

Morphological Effects

After periods of EE, experience-dependent changes in the NC are shown in brain chemistry, weight, cortical thickness and dendritic arborization (Bennett et al., 1964; Diamond, Krech, & Rosenzweig, 1964; Diamond et al., 1966; D. Krech, Rosenzweig, & Bennett, 1962). Increases in thickness of the NC are found primarily in layers II/III and the change in thickness due to increases in dendritic branching (Diamond et al., 1966). Other changes in the NC include: increases in size and length of dendrites, increases in spine size as well as length of postsynaptic thickening (Diamond, 1988, 2001; Diamond et al., 1964), all of which, probably contribute to the observed increases in cortical thickness following EE.

These morphological changes in the NC in response to an enriched environment regardless of age; and these effects are observed in both parietal and occipital cortex, however, aged rats show less arborization than those of younger rats (Kolb, Gibb, & Gorny, 2003). Dendritic spines are post-synaptic targets of neurons and regions of synaptic communication; thus, it makes sense that experience would alter their characteristics. Indeed, EE leads to a significant increase in spine density in the somatosensory cortex in 3-4-month-old mice (Jung & Herms, 2014). Another chronic imaging study demonstrated that spine elimination is enhanced days after formation enhancement (Yang, Pan, & Gan, 2009); together, these studies suggest spine-turnover is also augmented during EE. Spine number and density increases occur globally and are correlated with learning. Interestingly, after EE, mice trained on a repetitive motor task showed a spatially-clustered topological pattern of spinogenesis (Fu, Yu, Lu, & Zuo, 2012). In addition, age differences in spine density after EE have been shown; spine density decreases from weaning to adulthood but increases after similar housing in adulthood (Kolb et al., 2003). The important point here is that spines are motile, plastic structures, changing constantly with experience via sensory input from the environment. Because there are so few synapses in the brain, these newly formed and rearranged connections almost certainly play a role in the development of semantic knowledge.

Glia also show morphological changes in the NC in response to enrichment, both in cell numbers (Diamond et al., 1966) as well as the size of glial nuclei (Sirevaag & Greenough, 1987). Glia in enriched brains have shown an increase of 14%, and the glia/neuronal ratio has been shown to increase by 12% in enriched over impoverished rats (Diamond et al., 1966). Physical exercise influences gliogenesis in both the NC (Ehninger

& Kempermann, 2003) and the HPC (Steiner et al., 2004). Not only are glia produced through gliogenesis, they also take part in their own experience-dependent plastic change following long-term potentiation. Wenzel and colleagues demonstrated astrocytes in proximity to a potentiated synapse "develop a higher degree of ramification and enlargement of its surface" (Wenzel, Lammert, Meyer, & Krug, 1991). In addition, astrocytes increase the number of contacts with synaptic structures in the visual cortex of rats after enrichment (Jones & Greenough, 1996). Astrocytes have also been shown to control synaptic plasticity, through CA^{2+} signaling, in many thousands of nearby excitatory synapses *in vitro* in region CA1 of the HPC (Henneberger, Papouin, Oliet, & Rusakov, 2010); this has also been shown *in vivo* in mouse barrel cortex (Takata et al., 2011). Microprocesses of astrocytes positioned within relative proximity to a synapse is likely to be important for synaptic plasticity at that synapse. Moreover, signaling cascades that involve CA^{2+} and the dynamics of astrocytic microprocesses may provide a major mechanism for astrocytic involvement of experience-dependent plasticity (Hirase & Shinohara, 2014).

Enrichment has a significant effect on myelination in the prefrontal cortex (PFC) of young (Makinodan, Rosen, Ito, & Corfas, 2012) and adult (Liu et al., 2012) mice. In addition, mice isolated for two weeks immediately after weaning have changes in PFC myelination and function which does not recover after reintroduction to a social environment (Makinodan et al., 2012). In addition, Liu and others in the same year, showed that social isolation in adult mice results in hypomyelination in PFC which subsequently recovers after social reintegration (Liu et al., 2012). These findings show experience-dependent changes in myelination in adults and that social deprivation as juveniles can

negatively and irreversibly impact the normal development of the PFC. Myelination is essential for signal conduction and thus vital for proper neuronal communication.

Experience-dependent, morphological changes in the HPC are less robust than changes in the NC (Eckert & Abraham, 2013) and changes in dendritic arborization likely depend on age (Fiala, Joyce, & Greenough, 1978) as well as sex (Juraska, Fitch, Henderson, & Rivers, 1985) of the animal. Results are variable when it comes to experience-dependent changes in the HPC (Eckert & Abraham, 2013).

The fact that enrichment as well as formal training (learning a task) leads to significant morphological changes in the brain on both a rapid and over-the-lifespan time-scale, leads to the interpretation that these changes are "due to learning" (Rosenzweig & Bennett, 1996).

Biochemical Effects

In addition to morphological changes, biochemical changes have been documented as a result of EE as well. Acetylcholinesterase (an enzyme responsible for breaking down acetylcholine and thus important in the regulation of neuronal activity) activity increases in the NC of enriched rats (Bennett et al., 1964; D. R. Krech, Mark R. ; Bennett, Edward L., 1960). In addition, significant differences in total protein and hexokinase levels were found in enriched rats (Bennett et al., 1964; Diamond et al., 1964). Interestingly, neurons in the enriched NC take up less glucose than those in a standard-housed NC after EE (Diamond, 1988, 2001); implying greater efficiency in an enriched cortex, which may mean two different things. First, efficiency could mean fewer neurons active at any given time (i.e. increased sparsity of coding) or; second, the amount of glucose uptake is altered without a change in neuronal activity.

Physical exercise, an important aspect of the enriched environment, upregulates many important growth factors, including: fibroblast growth factor-2 (Gómez-Pinilla, Dao, & So, 1997), insulin-like growth factor-1 (Carro, Nuñez, Busiguina, & Torres-Aleman, 2000) and brain-derived neurotrophic factor (BDNF) (Jeon & Ha, 2017; Neeper, Góauctemez-Pinilla, Choi, & Cotman, 1995).

BDNF belongs to a family of protein growth factors called neurotrophins (NT) which include nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). NTs are prominent biomarkers for neuroplasticity and regulate the growth, survival, differentiation and maintenance of neurons. NTs, therefore, are good candidates for modulating the neural processes required for memory and learning. At least three members of the NGF family of NT growth factors, NGF, BDNF and NT-3, are highly expressed in the HPC and play roles in neuroplasticity related to learning and memory (Pham, Winblad, Granholm, & Mohammed, 2002). In addition, NTs show differential and regional expression in the HPC and NC following environmental enrichment and voluntary exercise (Falkenberg et al., 1992; Ickes et al., 2000; Neeper et al., 1995; Pham et al., 1999; Pham et al., 2002; Torasdotter, Metsis, Henriksson, Winblad, & Mohammed, 1996).

The most important characteristics of NTs, for the purposes of this study, are two-fold: first, their role in synaptic plasticity mechanisms (Park & Poo, 2013; Zweifel, Kuruvilla, & Ginty, 2005), and thus, processes of memory formation and retention; and second, BDNF expression is directly correlated to neural activity (Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990) translating to experience-dependence (Kolb & Whishaw, 1998; Pham et al., 2002). NTs therefore are likely an important mediator of experience-dependent neuroplasticity in the HPC and NC.

In this study, we enriched rats for three months and subsequently examined BDNF and NT-3 expression in the NC with and without lesions to the HPC. We expected EE to upregulate both BNDF and NT-3 in the NC and that this effect would be attenuated in brains and hemispheres with hippocampal lesions. Ultimately testing if the hippocampus is required for neuroplasticity processes in the NC, indirectly, by measuring BNDF and NT-3, which are prominent neuroplasticity biomarkers, in both the central and peripheral nervous systems.

METHODS

All procedures were performed in accordance with the Canadian Council on Animal Care guidelines and followed protocols approved by the Institutional Animal Care and Use Committee at the University of Lethbridge.

Animals and Experimental groups

Data for a total of 44 female Fischer-344/Brown Norway rats, 6-9 months of age (at the time of surgery), was gathered for the experiment. Due to processing time, 27 rats' data were randomly sampled and analyzed for BDNF, and 12 rats' data was randomly sampled and analyzed for NT-3. Animals were housed under constant humidity and temperature; on a twelve-hour light/dark cycle with food and water available *ad libitum* from birth, throughout the experiment. All procedures, including handling, testing and sacrifice occurred during the light phase of the cycle.

To test our hypothesis that the HPC contributes to neocortical BDNF expression, rats with and without hippocampal lesions lived in enriched or social control (SC) environments (Appendix; Table 1) for approximately 90 days (Figure 1).

Surgery

All surgical procedures were conducted under aseptic conditions and have been adapted from (Sutherland, Gibb, & Kolb, 2010). To reduce stress of human interaction, rats were handled for 30 minutes daily, for two weeks, prior to surgery. Animals set to undergo hippocampal lesions received an injection of sodium phenobarbital (40 mg/kg; intraperitoneally), approximately one hour before surgery, to reduce postoperative seizure activity. Rats received an injection of Metacam (1 mg/kg; subcutaneously) 5-15 minutes prior to surgery for pain control. Surgeries were performed under inhaled isoflurane general

anesthetic with induction at 2.0-4.0% and maintenance at 1.0-2.0% evaporated in 1-1.5 l/min O2. Immediately following induction, rats were placed in a stereotaxic frame, on a heating pad to maintain core body temperature. Body temperature and breathing rate were closely monitored and anesthetic adjusted as needed throughout surgery. An artificial ophthalmic lubricant was applied to the eyes to prevent them from drying out and the top of the head was scrubbed with chlorohexidine and 70% ethanol solutions to prevent infection. A sagittal incision was made in the scalp and skin retracted to expose the top of the scull. Six craniotomy sites were marked over the extent of the hippocampal formation either unilaterally or bilaterally (Figure 2b) and holes were drilled in the scull using a dental drill under saline to prevent heat damage to the underlying cortex. A 30-gauge cannula was attached to a 10μL Hamilton syringe with polyethylene tubing, the syringe was mounted in a KD Scientific syringe pump (KDS LEGATO 111) and the canula was mounted to the stereotaxic frame. The cannula was lowered into the hippocampus, at each site (Figure 2a), and an infusion of N-methyl-D-aspartate (NMDA) at a concentration of 7.5 mg/mL, diluted in 1x phosphate buffered saline (PBS) was performed at an injection-rate of 0.15 μL/min. The cannula was left in place for an additional 2 minutes after each injection to ensure full-volume infusion. A clean, empty, same size needle was lowered strictly into the cortex (not HPC) of all rats, in both hemispheres, to control for cortical damage caused by the cannula itself. All rats were monitored for 2-4 hours following surgery and given a dose of diazepam (4 mg/kg; intraperitoneally), if required, to control for post-operative seizures. All rats received a post-operative dose (1 mg/kg; subcutaneously) of Metacam, every 24 hours for three consecutive days to control pain. All rats recovered in standard plexiglass housing in the colony room for 2-4 weeks prior to environmental assignment.

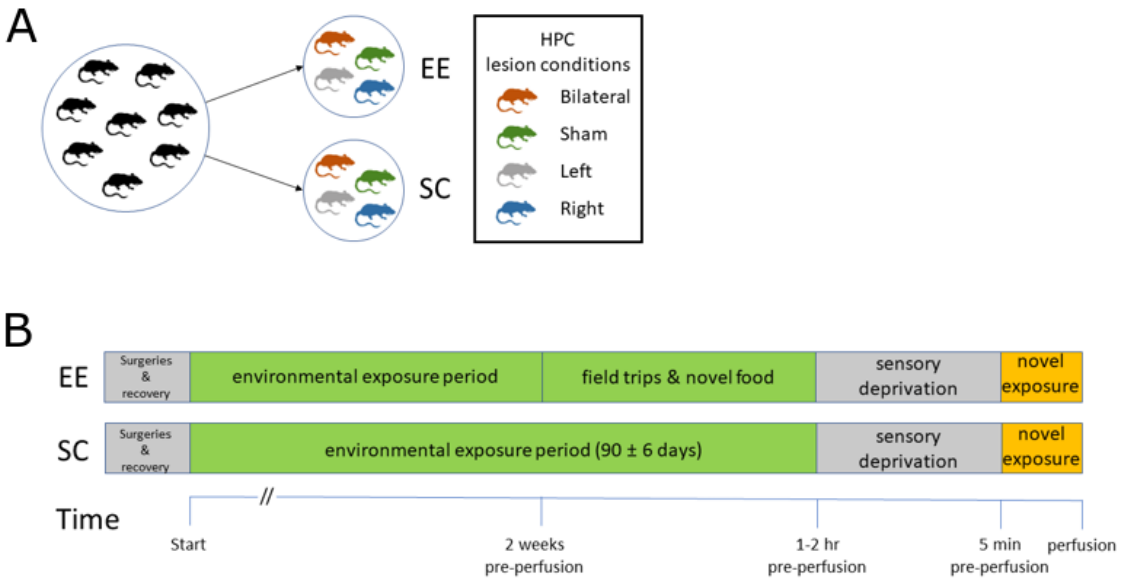

Figure 1: Experimental design and timeline of experiment up to perfusion. Rats were randomly assigned to one of four hippocampal lesion groups (bilateral, sham, unilateral left or unilateral right). After surgery, rats were assigned to either environmental enrichment or to a social control group (A). Rats in the enriched and social control environments underwent the same timeline to perfusion, however, the enriched rats also received field trips to novel rooms in the facility as well as novel food for two weeks prior to perfusion to maximize enrichment (B).

A

Lesion site	Anterior	Lateral	Ventral	Volume (µl)
1	-3	+/- 1.6	-3.6	0.4
2	-3.8	+/- 3.5	-4	0.4
3	-5.2	+/- 4	-4	0.4
4	-4.9	+/- 5.3	-7.2	0.4
5	-5.7	+/- 4.5	-4.4	0.4
6	-5.7	+/- 5.5	-7.3	0.5
7	-5.7	+/- 5.5	-6	0.5

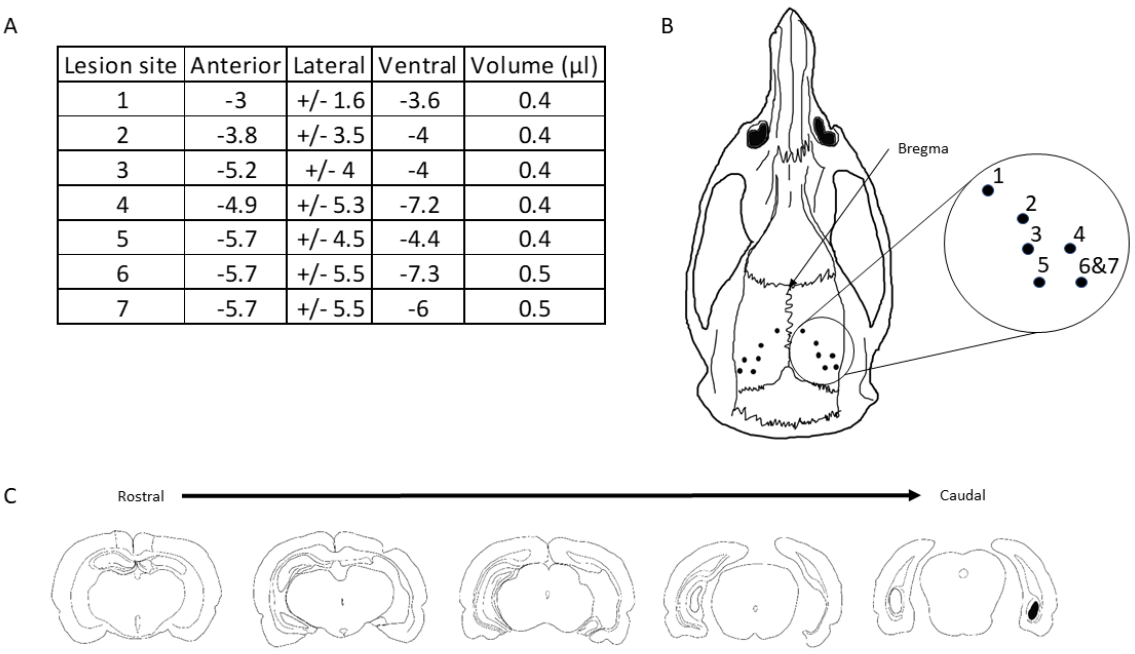

Figure 2: Stereotaxic coordinates with a top-down schematic of the rat scull showing relative locations of the craniotomies and median extent of hippocampal lesions. Panel A is a table of the stereotaxic coordinates used for excitotoxic hippocampal lesions and the volume of NMDA injected at each site. Sites 1-5 received 0.4 µL and sites 6 & 7 received 0.5 µL. Panel B is a schematic line trace of the rat scull showing the locations of the craniotomy sites relative to one another and to bregma (adapted from Paxinos and Watson, 2007). Panel C displays line drawings showing the median extent of hippocampal lesions.

Environmental housing

Approximately half the rats from each lesion group were housed either in large, metal, multilevel enrichment condos (H: 183cm, D: 61.5cm, W: 117cm), while the other half, in extra-wide clear plexiglass standard housing (4-6 per cage) to control for the social aspect of enrichment (Figure 3). All rats remained in their respective environments for 85-97 days continuously in the colony room. EE rats were given excess bedding and copious amounts of paper towel for nest building once per week during condo cleaning. In addition, toys, novel objects and structures (domes and plastic piping) were rotated out and the locations of these, as well as their food was changed once per week to maintain and maximize novelty throughout the enrichment period. For the remaining two weeks of EE, the enriched rats were taken on 'field trips' to novel rooms in the facility and given novel foods (Figure 1b). Rats were transported in clear standard-size plexiglass cages to novel environments which included a large square open field, elevated linear track (~1 m), elevated maze (~1 m), and open tabletops (~1.5 m). Novel foods, EE rats received, included milk chocolate, fruit loops, cheese, granola bars and twizzlers, which were placed around the enrichment condo, for the remaining two weeks before sacrifice to encourage foraging behavior. The enrichment condos as well as the SC housing were cleaned once per week. Other than cage cleaning and tail marking, the SC rats were left alone; while the EE rats were handled and received human interaction regularly.

Figure 3: Images of environmental housing in which rats lived for duration of EE or SC. After surgery, rats recovered in standard plexiglass cages for 2 to 4 weeks before being placed in either the enriched (top) or social (bottom) environment. Toys and objects in the EE condition were rotated out as well as moved around, within the condo, weekly to maintain novelty. To maximize novelty and enrichment, for the EE condition, regular handling, excess bedding, copious amounts of paper towel (for nest building), toys and other objects, structures to climb, new foods as well as field trips for two weeks before sacrifice were provided to these rats. The SC group receive as little handling as possible other than weekly cage cleaning and tail marking and had one plastic tube to crawl through.

Behavior

To allow pre-expressed immediate early gene expression to dissipate prior to behavior (for a separate experiment); during the last two weeks of the environmental exposure period, rats were gradually habituated to small, covered, opaque transport containers for up to two hours. Habituation took place in the colony room, moreover, the rats were exposed to the same auditory and olfactory stimulation during this time, as they were in their respective home environments. Full habituation was determined to have been reached when rats would quickly enter quiescence and spend most of this time in such a state.

Approximately 1-2 hours before behavior, rats were taken from their respective environments and placed in the small transport containers in the colony room. Rats were then transported individually to a completely novel remote room for behavior. The room was dimly lit and consisted of a grey-painted, wooden, circular track (12.7cm wide with a 5.1cm lip along the edges and a circumference of 342.8cm). Rats were free to explore the track for five minutes and were encouraged (by nudging) to move along the track if stationary for more than 30 seconds. Four novel plastic toy objects were placed on the track at approximately each of the cardinal points (Figure 4). All exploration sessions were video recorded with an infrared camera for later behavioral analysis.

Behavioral Analysis

Three behavioral parameters were quantified: exploratory behavior, object interactions and total distance traveled. Data for all three behavioral measures was gathered using a semi-autonomous, custom MATLAB script. The ‘exploratory behavior’ measure was represented as a positive integer and the sum of dips and rears (for each individual

rat)— each defined as follows. A ‘dip’ was any point in which the nose of the rat was judged to have dipped below the plane of the bottom of the track; and a ‘rear’ was any point in which both front feet were judged to have left the surface of the track. Object interaction was expressed as a positive integer and an interaction was deemed to have occurred if the rat oriented toward one of the objects located on the track. Total distance travelled was measured in pixels, where the rat’s X and Y position on frames throughout the video were tracked by manually scrolling through video frames and clicking the rats center of mass if the rat moved every five to six frames. The X and Y position was automatically populated into a vector of positions for each frame. Interpolation was then used to fill in missing frame data and construct a trace for each rat. Trace data contains location (x and y coordinates) as well as time (frames) data. Pixel values, for total distance travelled, were converted into centimeters using a known distance (width of the track) as a conversion factor.

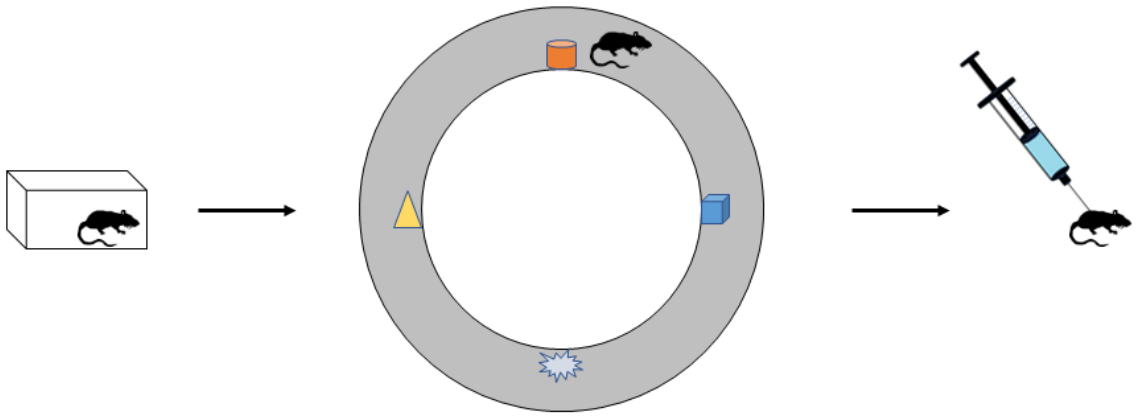

Figure 4: Schematic of experimental behavior protocol. Sensory deprivation followed by 5-minute novel track exploration followed by immediate perfusion and brain extraction. All rats were placed in small opaque transport containers for 1-2 hours of rest (left), in the colony room, prior to being transported to a remote, novel, room for a 5-minute exploration on a circular track (center). Immediately following the 5-minute exploration rats were then immediately and deeply anesthetized with a large dose of sodium pentobarbital (administered intraperitoneally) and transported for immediate perfusion and brain extraction. Note: illustrations are not to scale and objects on track are not representative of the actual objects used.

Sacrifice, tissue sectioning and preparation

Immediately following exploration, each rat was deeply anesthetized with an overdose injection (intraperitoneally) of sodium pentobarbital and immediately transcardially perfused. Tissues were rinsed using phosphate buffered saline, and subsequently fixed using 4% paraformaldehyde. Following fixation, rats were decapitated using a rodent guillotine, brains extracted, post-fixed in 4% paraformaldehyde for 2 hours and subsequently cryoprotected in a 30% sucrose solution and stored at 4 degrees until use.

Coronal sections were taken at 40µm using a modified vibratome (cohort one) or a sliding microtome (cohort two), both setup for block-face imaging. Sections were stored in well plates—free floating—in 1xPBS and 0.02% sodium azide at 4°C until use.

Immunohistochemistry and imaging

Tissue Processing

Prior to processing experimental tissues and to ensure specific binding of mature BDNF and NT-3 antibodies, initial testing involved immunohistochemical processing without addition of primary antibodies or without addition of secondary antibodies. No non-specific labelling was observed.

A 1 in 24 series (approximately 1mm) for each NT (BDNF and NT-3) were fluorescently labeled using Tyramide Signal Amplification (TSA; PerkinElmer). Free-floating tissue was washed for 25 minutes in Tris-buffered saline with 0.05% Tween-20 (TBST) and 0.3% peroxide, blocked for 1 hour in TBST with 5% goat serum and treated for 48 hours with either rabbit anti-BDNF (1:1000; Millipore) or rabbit anti-NT-3 (1:8000; Abcam) in TBST. To detect primary antibodies, tissue was incubated for 1 hour with goat biotinylated anti-rabbit (1:500; VectorLabs) diluted in TBST, incubated for 2 hours in

streptavidin-HRP (1:200) in TBST, and incubated for 1 hour with fluorescein-tyramide (1:300) diluted in TBST and 0.005% hydrogen peroxide.

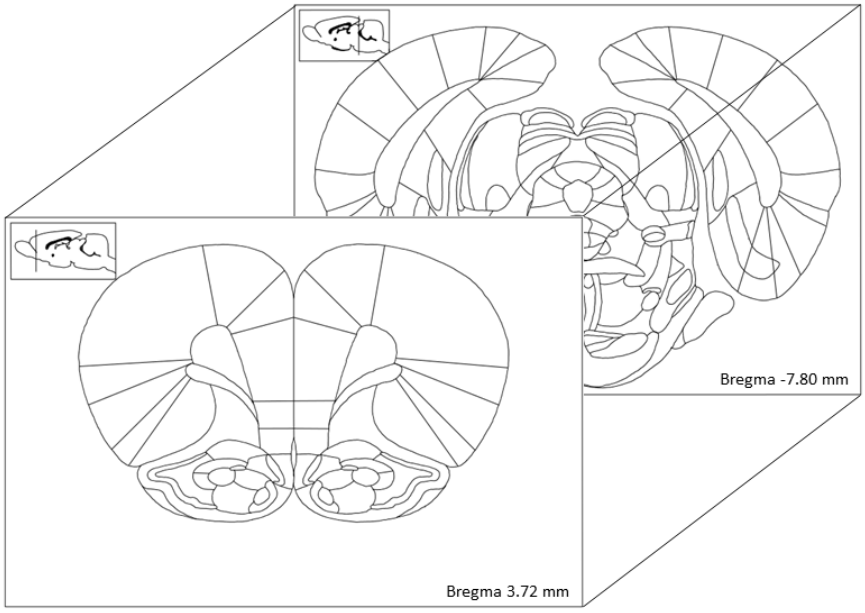

Figure 5: Range of sections, relative to bregma, used for analysis. After taking 40-micron coronal sections across the entire brain and processing the free-floating tissues for BDNF and NT-3, 1 in 24 series (approximately every 1mm) was mounted on microscope slides. More anterior, as well as more posterior sections were discarded due to either difficulty in analysis or poor tissue quality at these extremes. Images are adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2007).

To visualize neurons, tissue was blocked and counter-stained overnight for NeuN (a neuron-specific protein) with anti-NeuN-Cy3 (1:500) diluted in TSA blocking buffer. Sections were manually mounted on Superfrost Plus (Fisher Scientific) microscope slides in a dish of 1xTBS and left to dry completely. Once dry, the slides were incubated in 4′,6-diamidino-2-phenylindole (DAPI; 1:2000) for 1 hour to stain cell nuclei. Slides were subsequently cover slipped with Vectashield (VectorLabs), sealed using nail polish and refrigerated at 4°C until use.

Imaging

Multi-layer, whole-slide, 20x or 40x z-stacks were acquired using a Nanozoomer Digital Pathology Microscope system (Hammamatsu). Scanning parameters were as follows: Color balance; Red = 6, Green = 5 and Blue = 6, exposure time; 2x and spacing of 2 microns between z-stack layers—color balance, exposure time and spacing parameters were held constant across all slides and brains. For each section of each brain, the most in-focus layer was selected, cropped, and exported as a tagged image format at 1.25x for analysis. All sections were selected within the range of about 3.72 mm to -7.80 mm relative to bregma (Paxinos & Watson, 2007) for analysis (Figure 5).

Image processing

Multiple masks were manually drawn using ImageJ (1.47v, National Institutes of Health) for each section in each brain for later use in image processing scripts using Matlab (MathWorks; R2013b, R2015b & R2017a). These masks included three types: Whole-section, cortical and negative masks (to ignore artifacts and blood vessels). Whole-section masks were drawn around the entire section and used to perform subsequent background subtraction and bleed-through correction. Background subtraction was carried out by overlaying the mask with the original image such that only tissue was selected, from this, the histogram of the whole section was obtained and approximately the first 0.05% was manually selecting and discarded as 'background'. The same masked image was also used for bleed-through correction. Cortical masks were manually drawn around cortex which were later used to provide the outer boundaries for our regions of interest (ROI). Negative masks were manually drawn in order to exclude layer I cortex, tissue artifacts and blood vessels from analysis; these were excluded from analysis due to the magnitude of

fluorescent artifacts in these structures which directly affect our main measure (average brightness).

Borders of 18 ROIs were manually drawn and automatically populated with Java plug-ins developed for ImageJ (1.47v, National Institutes of Health) such that 18 distinct cortical regions (each with a superficial and deep component), spanning the entire neocortex, and their corresponding masks could be used to measure average brightness within each ROI. ROIs were drawn based on The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2007) and included the following regions (approximately rostral to caudal): infralimbic, prelimbic, cingulate, motor, sensory, orbital, insular, sensory barrel field, granular retrosplenial, dysgranular retrosplenial, auditory, ectorhinal, perirhinal, entorhinal, parietal, visual, temporal association area, and medial entorhinal cortices. All other automation was performed in Matlab (MathWorks, R2013b, R2015b & R2017a) using a series of custom scripts to perform data acquisition, organization and analysis.

It is important to highlight the difficulty visualizing cytoarchitectural differences between cortical ROIs, thus it is highly probable there was overlap between ROIs in the cortex as a result.

Data analysis

Mean pixel brightness in each ROI was used as the raw measure of BDNF expression. Superficial cortex, deep cortex, ROIs, sections, hemispheres, housing conditions and rats were expressed as dimensions in a matrix, which could either be averaged across or pooled depending on desired analysis (Figure 6). For example, in the unilateral hippocampal lesion group, hemispheres were compared; however, in the bilateral and sham lesion groups, hemispheres were averaged (assuming both hemispheres would be equal in these

conditions). Sections and rats—within each group—were always averaged across using mean.

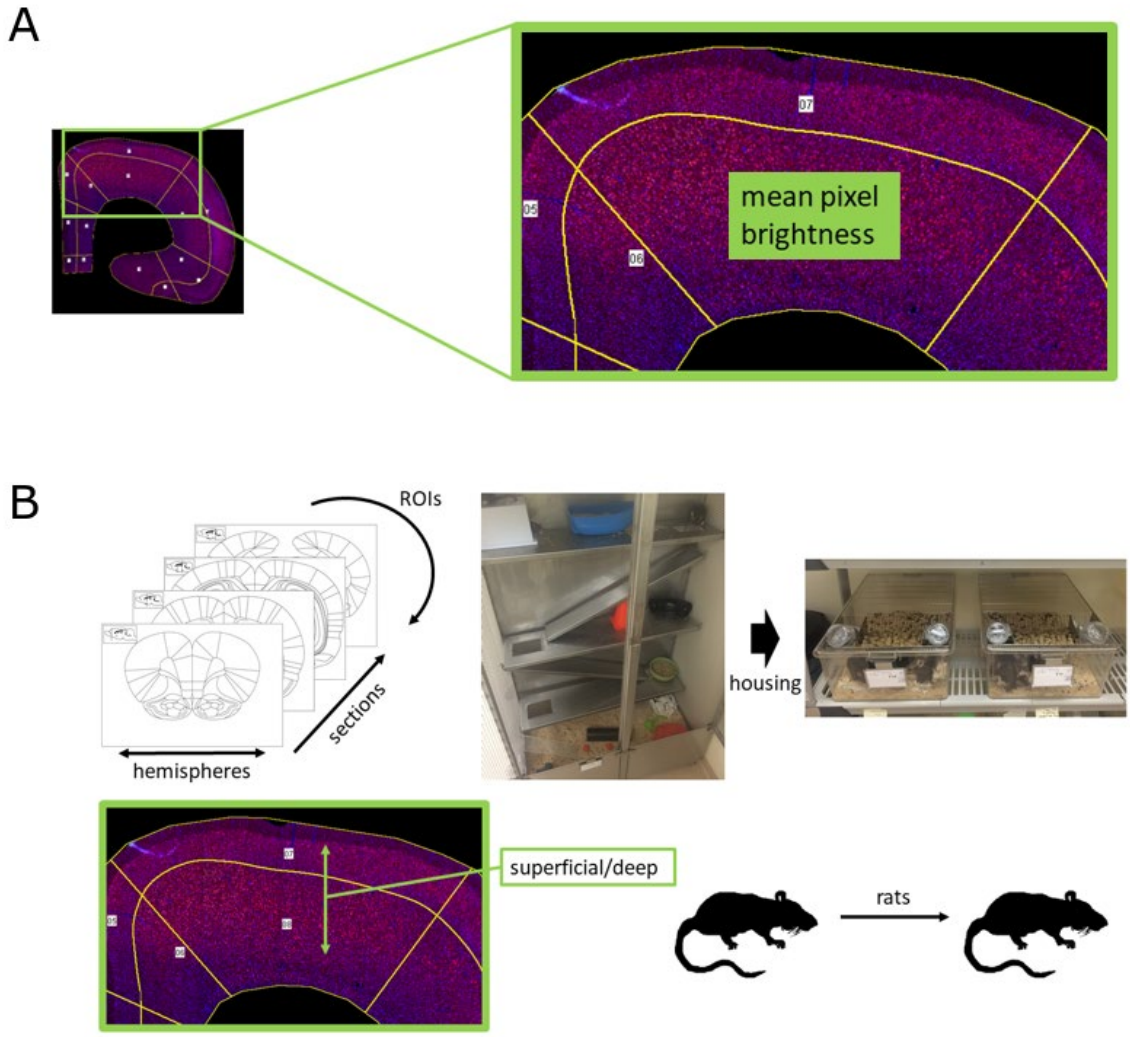

Figure 6: Raw measures for BDNF and NT-3 and the dimensions used to pool, average and make comparisons. Within each ROI mean pixel brightness was used as the basic measurement of NT expression (A) which was always averaged across sections. Since the data in MATLAB was represented as multidimensional matrices, it made sense to refer to the different dimensions as such. These dimensions included: hemispheres, sections, ROIs, housing conditions, superficial/deep cortex and rats (B); section diagrams adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2007). For analytical purposes these dimensions could be averaged across, pooled or compared depending on the analysis desired.

RESULTS

Behavior

Rats were video recorded while they freely explored a circular track for 5 minutes in a dimly lit room immediately before sacrifice. Behaviors were subsequently analyzed, and three behavioral measures were scored: exploratory behavior, total distance travelled and number of object interactions. First, we investigated if there was an effect of hippocampal lesion on behavior. Individual measures were z-scored (to remove highly differential scales of measurement) and then averaged together yielding a single behavioral score for each rat. We then compared all conditions, using socially-housed sham rats as the control. Interestingly, right, but not left or bilateral hippocampal lesions had a marked negative effect on general exploration; however, only for the social rats in this group (Figure 7a). The same effect is observed when looking at a simple measure, such as total distance travelled (Figure 7b). Enriched rats with right hippocampal lesions performed like social rats with sham lesions. Moreover, a housing effect was only observed in the right hippocampal lesioned rats with social rats exploring significantly less than the enriched rats with the same lesion.

Next, we analyzed the correlation between BDNF expression and behavior. To accomplish this, we took an overall correlation between average cortical BDNF expression and the z-scored behavioral index. No detectable correlation was found between BDNF expression and general exploration behavior (correlation coefficient, $r = 0.338$; $p > 0.05$; $n = 27$; Figure 8).

BDNF expression in the neocortex

To observe global expression patterns of BDNF in the NC, heatmaps of rostral to caudal ROIs were created (Figures 9 & 10). It was evident BDNF exhibited regional expression patterns in the NC which was generally unaffected by the removal of the HPC.

Superficial layers of NC generally appeared brighter than in deep layers in the heatmaps. To test if BDNF expression was higher in superficial cortex, bilateral and sham lesion brains were pooled and an overall superficial to deep ratio was obtained after normalizing the expression in each to cell density to account for a higher cell density in superficial layers of NC. We found there was a strong trend toward higher BDNF expression in superficial cortex, however, it remained insignificant after density correction (one-sample t-test; $p = 0.055$; $n = 19$; Appendix; Figure 16).

Enrichment led to reduced BDNF in the neocortex

Enrichment led to a downregulation of BDNF, in deep layers of the NC, regardless of lesion condition. This main effect of EE reached significance in deep layers (two-sample t-test; $p < 0.05$; enriched $n = 13$; social $n = 14$; Figure 13b), however, a similar trend remained in superficial layers of NC (two-sample t-test; $p = 0.115$ enriched $n = 13$; social $n = 14$; Figure 13a).

Hippocampal lesions did not affect BDNF expression in the neocortex

Lesion and control hemispheres were compared in brains with unilateral lesions of the HPC. No detectable effect on BDNF expression in the NC, in any environmental condition was found (paired-sample repeated measures t-test; $p > 0.05$; $n = 4$; Figure 11).

Interestingly, bilateral HPC lesions also had no effect on overall BDNF expression in the NC when compared with sham lesion (two-sample t-test, $p > 0.05$; sham enriched n = 4, bilateral enriched n = 5; sham social n = 4, bilateral social n = 6; Figure 12).

Next, we looked at retrosplenial cortex (RSC) specifically, as RSC is "a major relay" of hippocampal output to NC and is required for spatial and other forms of learning (Mao et al., 2018). Hippocampal lesions had no detectable effect on BDNF expression in RSC (two-sample t-test; $p > 0.05$; bilateral n = 11; sham n = 8 Figure 14a), however, a significant down-regulation of BDNF, was observed, in RSC as a result of EE (two-sample t-test; $p < 0.05$; enriched n = 9; social n = 10; Figure 14b). Since RSC can be separated into two differentially connected subregions, granular which receives strong direct hippocampal input (Sugar, Witter, van Strien, & Cappaert, 2011) and dysgranular which receives weak direct hippocampal input (van Groen & Wyss, 1992), we compared these two subregions. Interestingly, granular RSC had significantly reduced BDNF expression, in both bilateral and sham lesion conditions (paired-sample t-test; $p < 0.01$; bilateral n = 11, sham n = 8; Figure 15).

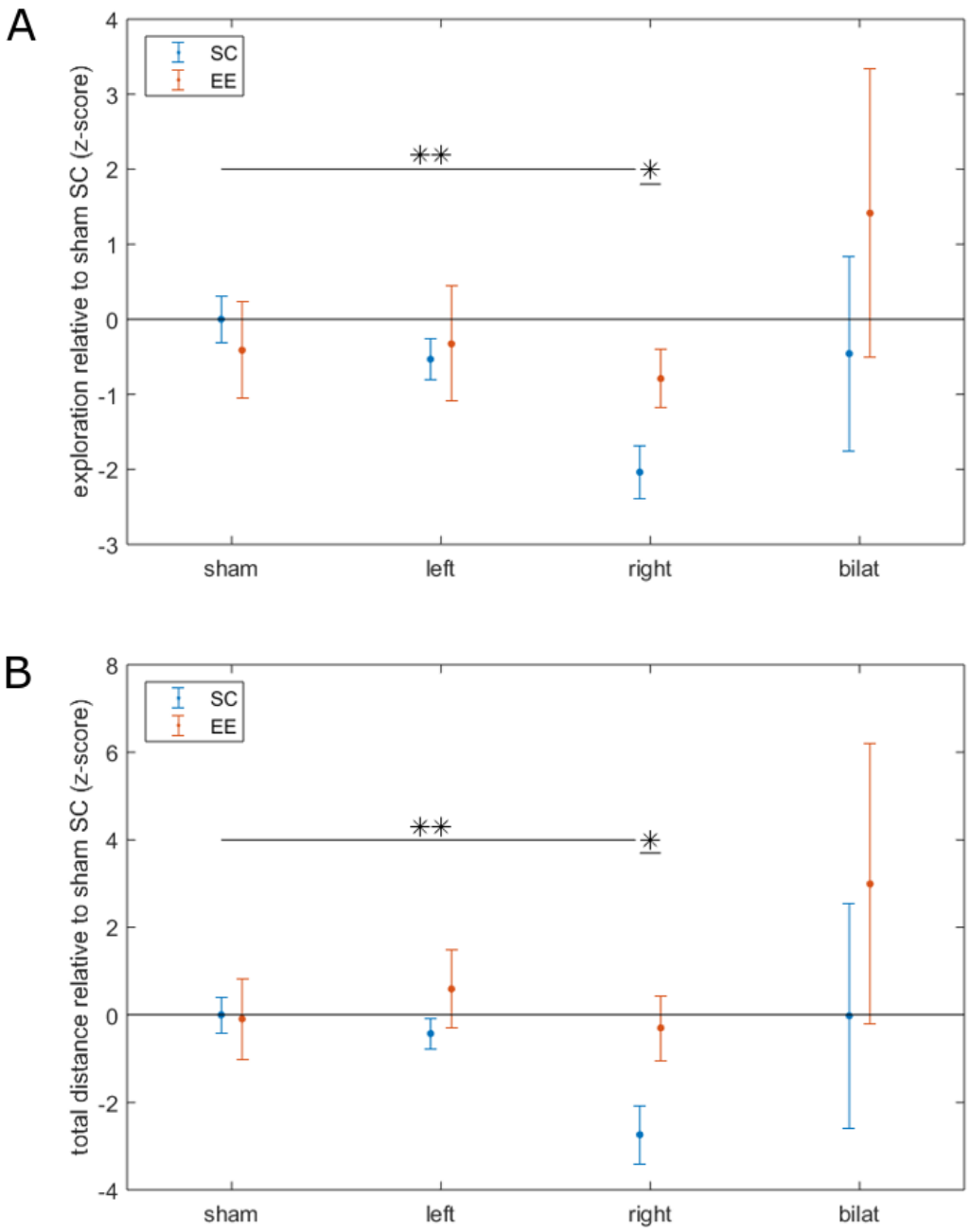

Figure 7: Right hippocampal lesions significantly and negatively impacted SC rat exploratory behavior. Panel A: An exploration index (z-score) was obtained for each rat by averaging z-scores obtained for each measure for each rat. This index was plotted for each experimental condition relative to SC shams. SC rats with right hippocampal lesions explored significantly less than EE rats with the same lesion (two-sample t-test; $p < 0.05$; right lesion; SC n = 5 and EE n = 5). In addition, SC rats with right hippocampal lesions showed significantly less exploratory behavior than SC rats with sham lesions (two-sample t-test; $p < 0.005$; SC right lesion n = 5 and SC sham lesion n = 6). Panel B: Total distance of each rat was z-scored and plotted relative to SC shams. SC rats with right hippocampal lesions travelled significantly less distance than EE rats with the same lesion (two-sample t-test; $p < 0.05$; right lesion; SC n = 5 and EE n = 5). SC rats with a right lesion of the HPC travelled significantly less distance than SC rats with sham lesions (two-sample t-test; $p < 0.01$; SC right lesion n = 5 and SC sham lesion n = 6).

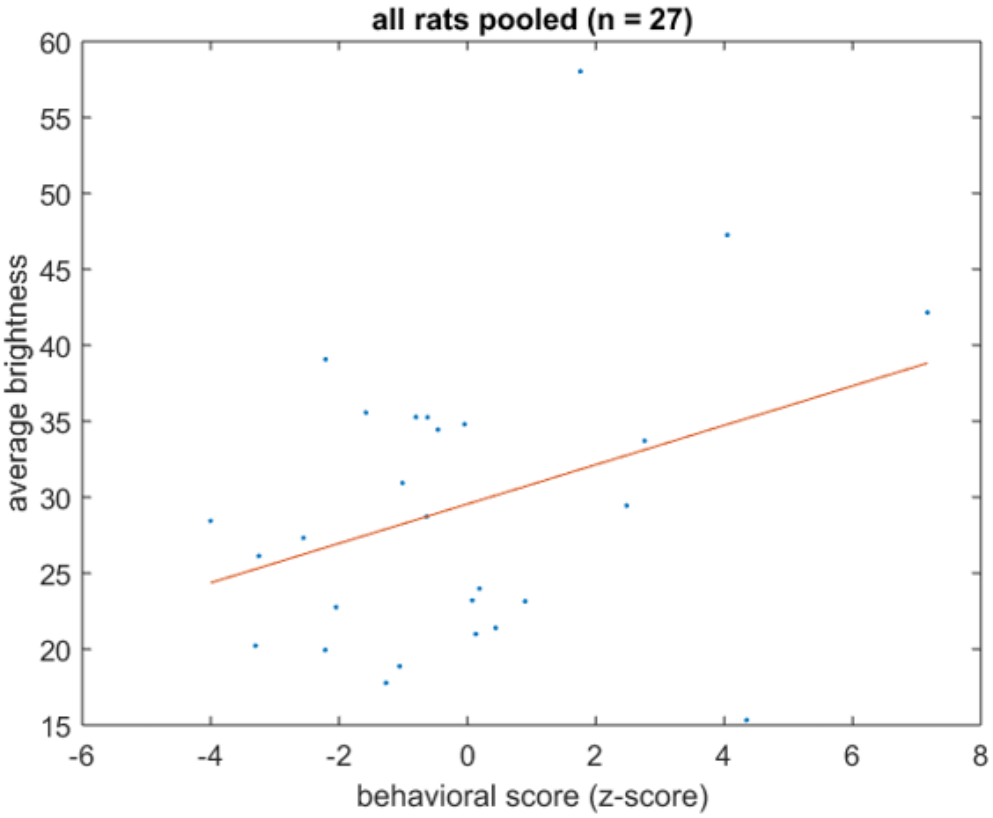

Figure 8: BDNF expression and behavioral exploration are not significantly correlated. An exploration index (z-score) was obtained for each rat by averaging z-scores obtained for each measure for each rat (x-axis). Average pixel brightness in the NC was obtained, for each rat, by averaging across hemispheres, sections and ROIs (y-axis). A correlation coefficient was calculated between the two variables (correlation coefficient, $r = 0.338$; $p > 0.05$; $n = 27$).

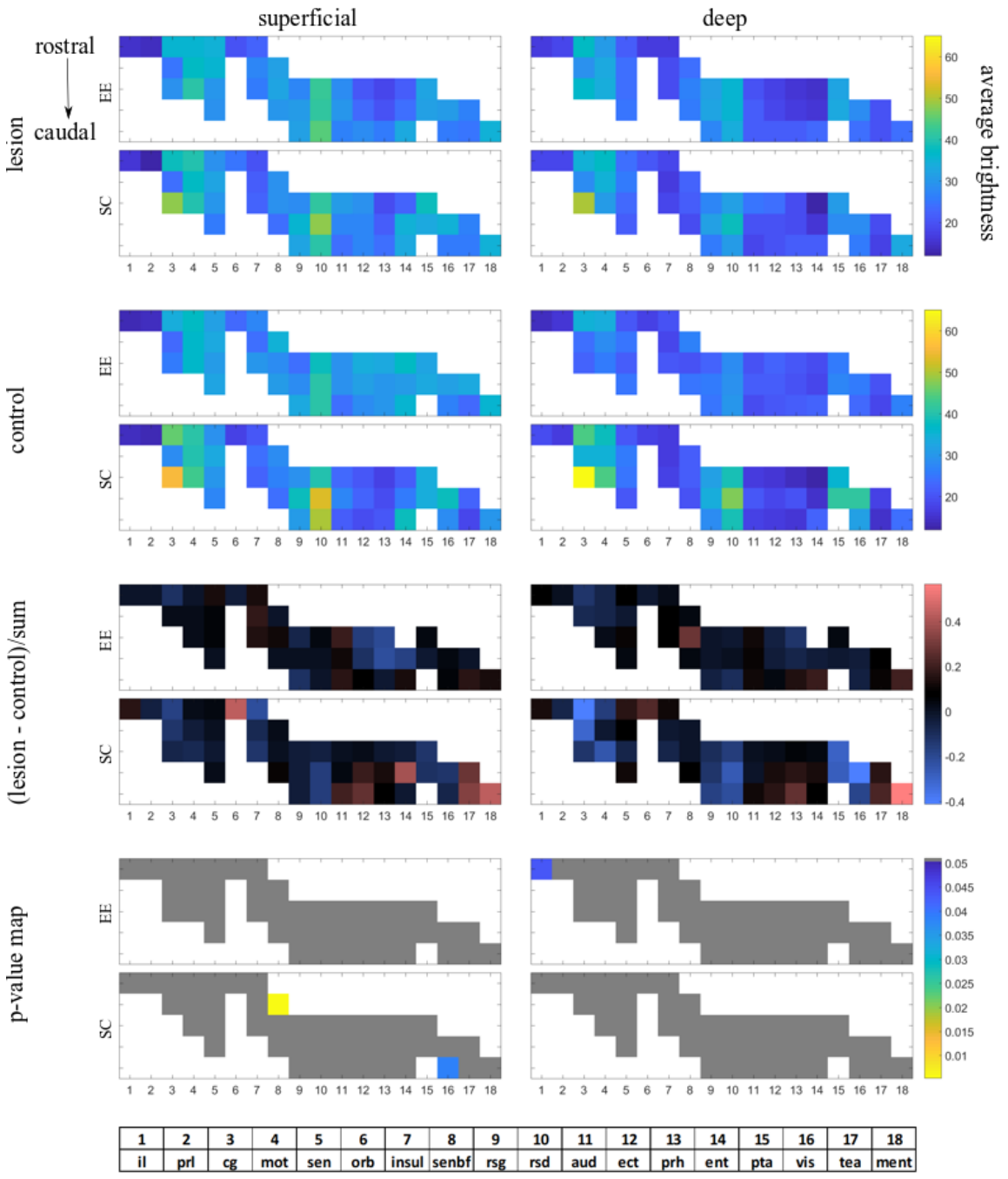

Figure 9: Heatmaps representing BDNF expression across entire NC in unilateral lesion conditions. The top two sets of heatmaps represents average brightness, the third set are normalized difference maps and the fourth set are uncorrected p-value maps. Heatmaps (top two sets) were created by averaging across sections and across animals within each condition. The heatmaps are organized into superficial (left column), deep (right column), enriched (upper panel) and social (lower panel) groups, labelled EE and SC respectively. Each panel consists of tiles, each tile representing a single ROI in the NC. The vertical axis represents tiles from rostral (top) to caudal (bottom); and the horizontal axis represents 18 ROIs measured across the NC (bottom table). The Difference maps shows the control hemisphere matrix subtracted from the lesion hemisphere matrix. All panels in the control and lesion groups have the same colour scale and all panels in the difference group have the same color scale. Enriched n = 4; social n = 4.

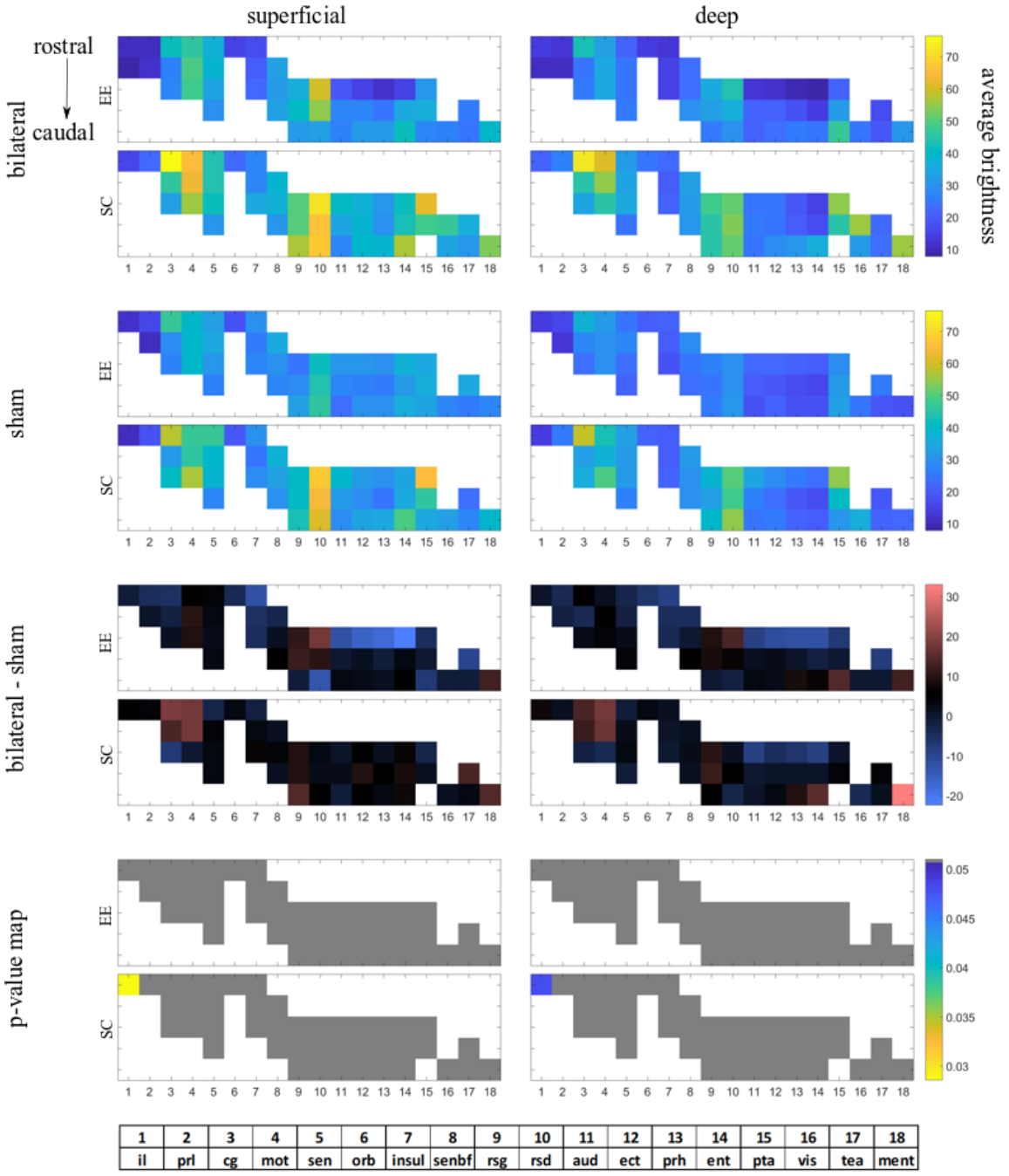

Figure 10: Heatmaps representing BDNF expression across entire NC in bilateral and sham lesion conditions. The top two sets of heatmaps represents average brightness, the third set are difference maps and the fourth set are uncorrected p-value maps. Heatmaps (top two sets) were created by averaging across sections and across animals within each condition. The heatmaps are organized into superficial (left column), deep (right column), enriched (upper panel) and social (lower panel) groups, labelled EE and SC respectively. Each panel consists of tiles, each tile representing a single ROI in the NC. The vertical axis represents tiles from rostral (top) to caudal (bottom); and the horizontal axis represents 18 ROIs measured across the NC (bottom table). The Difference maps shows the control hemisphere matrix subtracted from the lesion hemisphere matrix. All panels in the control and lesion groups have the same color scale and all panels in the difference group have the same color scale. Bilateral enriched n = 5; bilateral social n = 6; sham enriched n = 4 and; sham social n = 4.

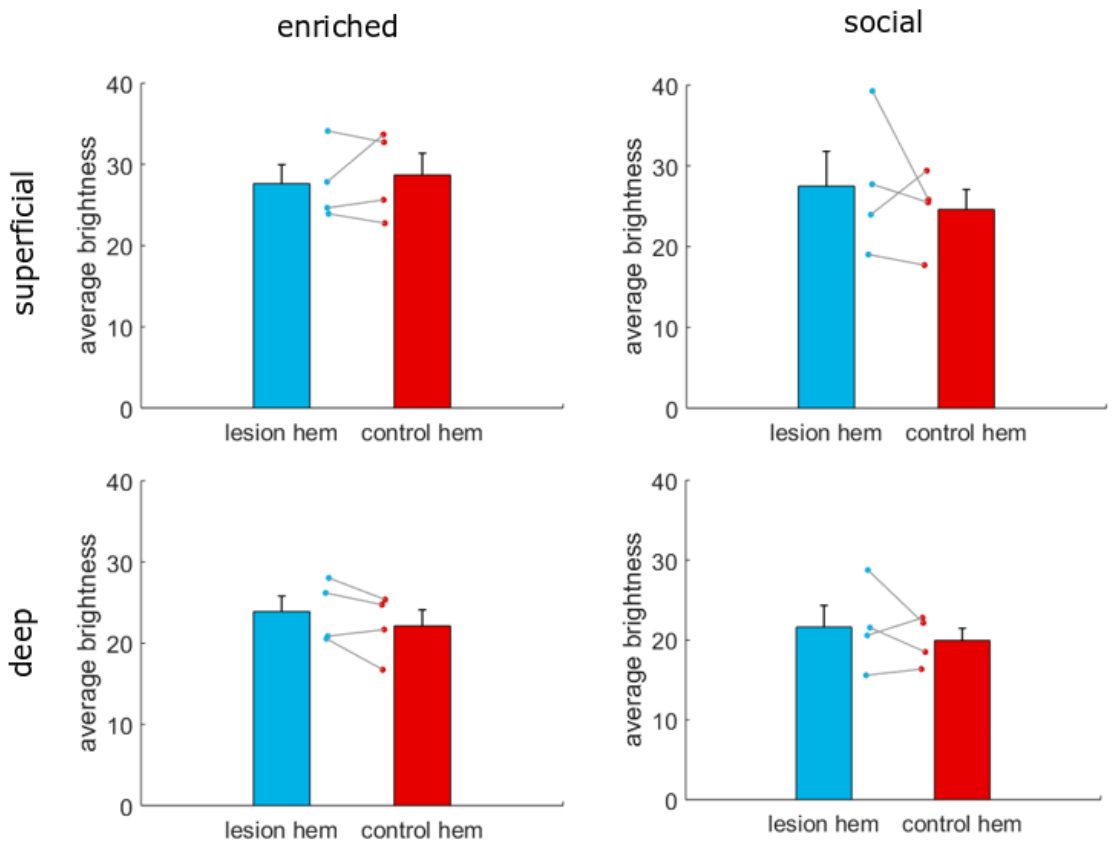

Figure 11: Unilateral lesions of the hippocampus had no effect on BDNF expression in NC. Neocortical BDNF expression in both lesion and control hemispheres was compared. ROIs, sections and rats were averaged across. No detectable difference was found between lesion and control hemispheres in any condition (paired-sample repeated measures t-test; $p > 0.05$; error bars represent +SEM; $n = 4$).

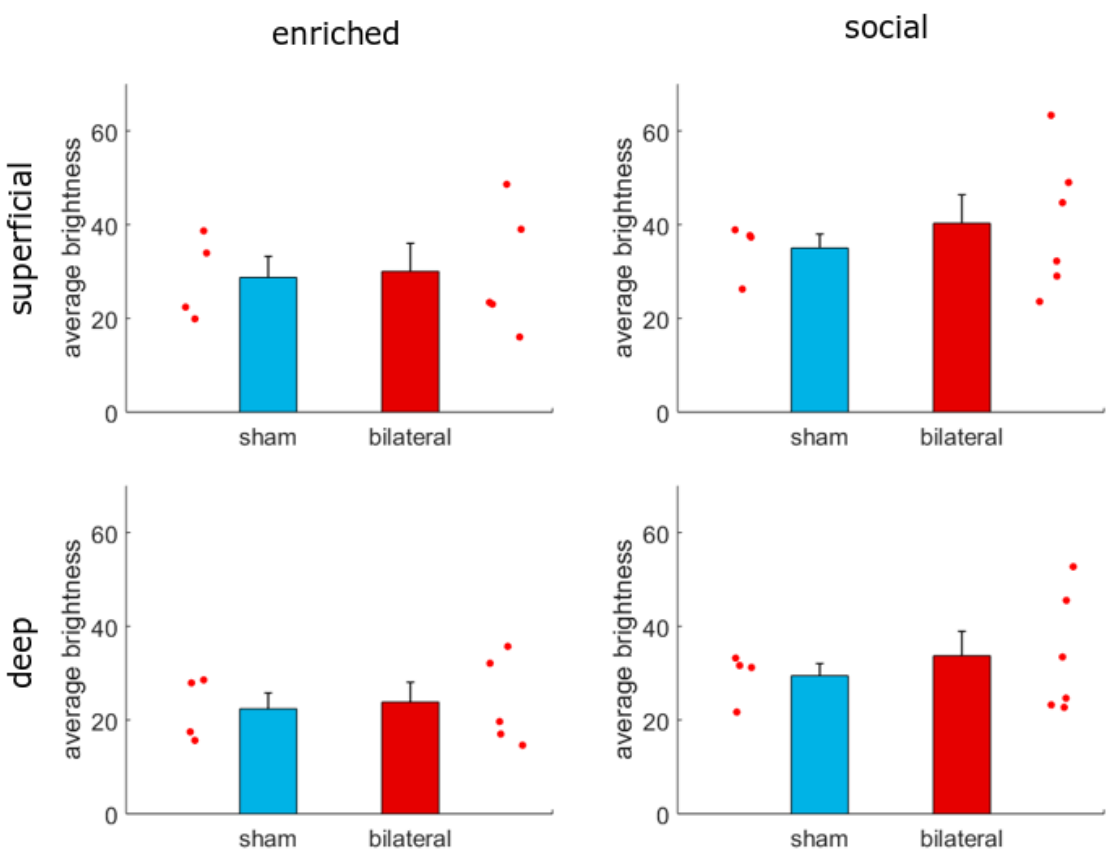

Figure 12: Bilateral lesions of the HPC had no significant effect on BDNF expression in the NC when compared with sham lesioned brains. Sham (blue) and bilateral (red) lesion conditions were compared in superficial (top row) and deep (bottom row) cortex as well as in enriched (left column) and social (right column) housing. ROIs, sections and rats were averaged across within each group. No significant effect of lesion was found (two-sample t-tests; $p > 0.05$; error bars represent +SEM; sham enriched n = 4; bilateral enriched n = 5; sham social n = 4; bilateral social n = 6).

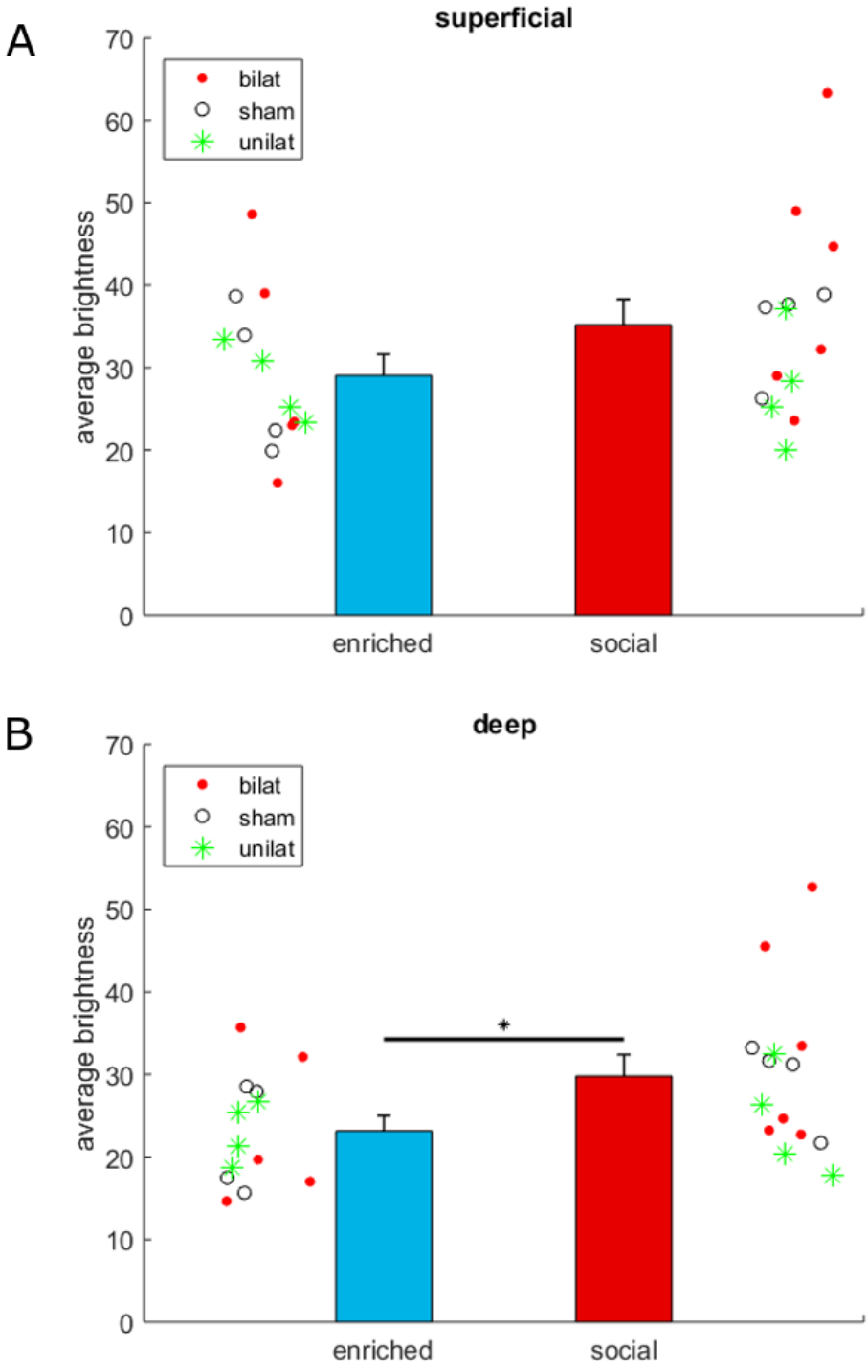

Figure 13: EE significantly reduced BDNF expression in the deep layers of NC regardless of lesion condition. All lesion conditions were pooled together to examine the overall effect of environmental housing condition in superficial (A) and deep (B) cortex. Deep layers of NC showed significantly reduced BDNF expression in enriched rats (two-sample t-test; $p \leq 0.05$; enriched n = 13; social n = 14; error bars represent +SEM). A similar trend was observed in superficial layers of NC as well (two-sample t-test; $p = 0.115$; enriched n = 13; social n = 14; error bars represent +SEM)

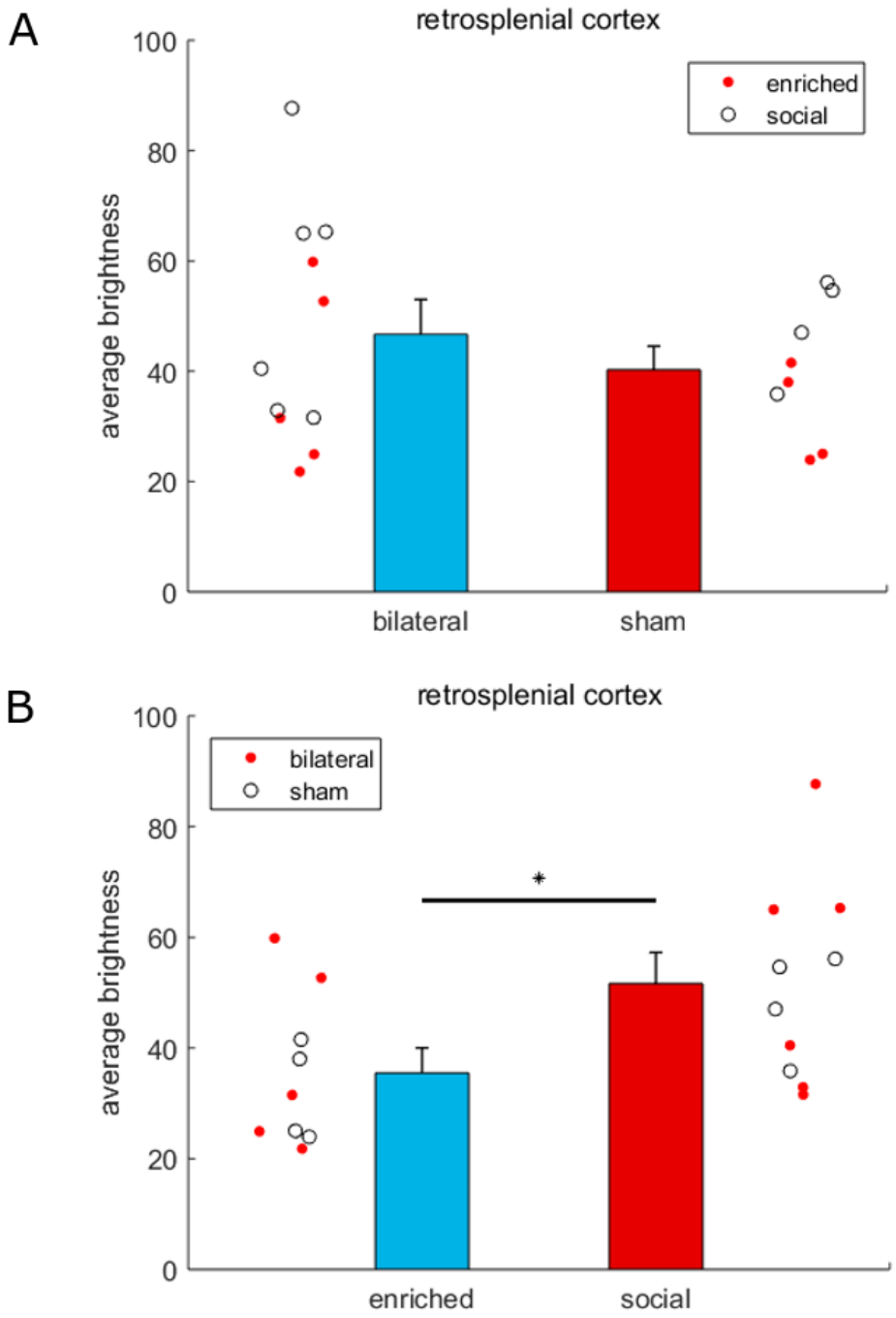

Figure 14: EE significantly reduced BDNF expression in RSC regardless of lesion condition. Panel A compares bilateral lesioned brains with sham lesioned brains. Retrosplenial cortex subregions were averaged together and averaged across sections with environmental conditions pooled. There was no overall effect of lesion (two-sample t-test; $p > 0.05$; error bars represent +SEM; bilateral n = 11; sham n = 8). Panel B compares overall housing conditions with lesion condition pooled. RSC of enriched rats exhibited a significant decrease in BDNF expression relative to social rats (two-sample t-test in RSC; $p < 0.05$ error bars represent +SEM enriched n = 9; social n = 10).

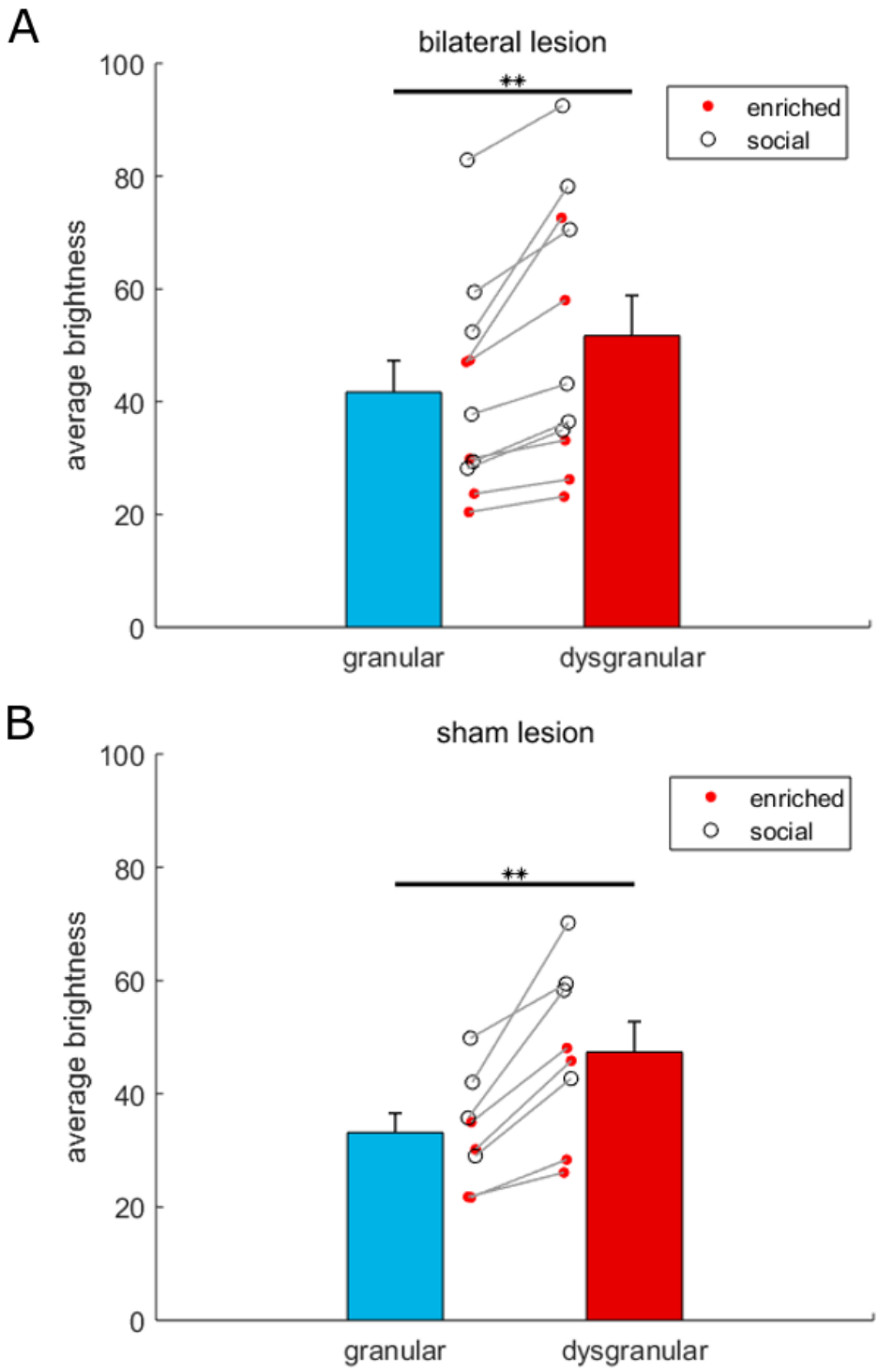

Figure 15: Granular RSC had significantly reduced BDNF expression relative to dysgranular RSC regardless of lesion condition. Granular and dysgranular subregions of RSC were compared in bilateral (A) and sham (B) lesion conditions, averaged across sections. Granular RSC showed significantly reduced BDNF expression relative to dysgranular regardless of lesion (paired-sample repeated measures t-test; $p < 0.01$; error bars are +SEM; bilateral n = 11; sham n = 8).

DISCUSSION

NTs are dynamic modulators of neuroplasticity (Poo, 2001; Schinder & Poo, 2000; Xu et al., 2006) and the HPC is thought to contribute to neuroplasticity in the NC (Sutherland et al., 2010). We hypothesized, therefore, that BDNF protein expression in the NC would approximate the magnitude of neuroplastic change in the NC; and the removal of hippocampal input would result in downregulation of BDNF. We found no detectable difference in BDNF expression between lesion and control conditions three months after the removal of the HPC unilaterally (Figure 11) or bilaterally (Figure 12). NT-3 showed similar results in enriched rats (Appendix; Figure 17). This study was largely based on findings from Sutherland et al. to gain a better understanding of the contribution the HPC plays in neocortical plasticity. Our study also addresses two major problem with their paper; first, the hippocampal lesions were all right unilateral lesions, and; second, they restricted their analysis to pyramidal cells in parietal cortex. We addressed these problems by having both left and right unilateral hippocampal lesions as well as bilateral lesions of the hippocampus. In addition, our analysis covered the entire NC with both superficial and deep aspects. A major sacrifice for the additional analysis, however, are small sample sizes.

A significant lesion to the HPC should, at least in theory, negatively affect neuroplasticity in the NC. To find no effect on BDNF or NT-3 expression after almost total lesions, therefore, is unlikely and it is more probable, rather, that we simply did not detect it. There are a couple possible explanations for the lack of lesion effect observed in this study; of which, will be discussed here: First, our study was underpowered due to small sample sizes and relatively large variance in the bilateral lesioned animals (Appendix; Figure 18). Therefore, even if effects of lesions are present, they would be undetectable.

Second, hippocampal lesions may have had a significant impact on NT expression early after the lesions and subsequently returned to a baseline expression prior to sacrifice.

The time-course of experience-dependent BDNF protein expression is an important consideration and occurs relatively rapidly with observable increases at 14 days and reaching significance at 28 days in the HPC of voluntary exercising rats (Adlard, Perreau, Engesser-Cesar, & Cotman, 2004). Our study, enriched rats approximately three times longer, moreover, it could be that BDNF upregulation was significant at some time point within the first 30 days and subsequently dropped to levels below that of controls at the time of sacrifice. Duration of enrichment is therefore an important consideration because, for example, 30 days of EE has greater effects on cortical thickness than 80 days of EE (Diamond, 2001); however, there were regional differences in these effects. Male rats enriched for 30 days exhibit significant changes in the thickness of somatosensory cortex, whereas 80 days shows no effect in this region. In addition, the occipital cortex showed significant changes in cortical thickness at both 30 and 80 days of EE (Diamond, 2001). These findings suggest that the time course for changes in the brain vary considerably and choosing an appropriate point for sacrifice may be difficult.

Enriching the environments of rodents upregulates BDNF expression in multiple brain regions (Gomez-Pinilla, Ying, Agoncillo, & Frostig, 2011; Ickes et al., 2000; Pham et al., 1999; Pham et al., 2002; Van Praag et al., 2000). Our study, contrary to much of the literature, found a downregulation of BDNF in the NC following enrichment, reaching significance in deep layers, independent of lesion condition. Downregulation of BDNF is usually associated with psycho/neuro-pathologies, which include but are probably not limited to: post-traumatic stress disorder (Green, Corsi-Travali, & Neumeister, 2013),

suicide risk (Dwivedi, 2010), Parkinson's disease (Mercado, Collier, Sortwell, & Steece-Collier, 2017), Huntington's disease (Zuccato & Cattaneo, 2007), Alzheimer's disease (Allen, Watson, & Dawbarn, 2011; Travaglia, Pietropaolo, La Mendola, Nicoletti, & Rizzarelli, 2012) and depression (Autry & Monteggia, 2012). Certain types of stress such as chronic isolation stress (Murínová, Hlaváčová, Chmelová, & Riečanský, 2017) also downregulate BDNF expression.

It is possible that the observed down-regulation of BDNF in this study was a result of stress from field trips. These field trips often entailed freely exploring an open field, a maze, and a linear track which were all approximately 1m off the floor which may have been stress-inducing rather than enriching leading to a downregulation of BDNF in the two weeks before sacrifice.

Granular RSC (grRSC) also showed a significant downregulation of BDNF relative to dysgranular RSC (dysRSC) in animals with both sham and bilateral lesions of the HPC (paired-sample repeated measures t-test; $p < 0.01$; bilateral (n=11), sham (n=8); Figure 15). Neural representations in RSC are sparse (Mao, Kandler, McNaughton, & Bonin, 2017), BDNF is activity dependent, thus, speculatively, it is possible that dysRSC and grRSC have differential levels of sparsity in their neural representations, leading to differential expression levels of BDNF.

Another important consideration is the potentially short half-life of the BDNF mature protein which has been speculated to be less than 10 minutes (Wurzelmann, Romeika, & Sun, 2017), however, NGF, a close relative of BDNF, has a half-life of around 2.3 hours (Tria, Fusco, Vantini, & Mariot, 1994). Thus, the half-life of BDNF is essentially unknown but remains an important consideration for this study. As mentioned, prior to behavior, rats

were placed in small transport containers to deprive the rats of sensory input to allow pre-expressed IEGs to dissipate (for a separate experiment). Which may in effect allow any pre-expressed BDNF to dissipate as well; resulting in decreased BDNF expression. Although, it is important to note that all rats received this experience and it could be assumed their BDNF levels would drop by the same amount if this in fact occurs.

Monoamines

It is important to mention the monoamine neurotransmitters (which include serotonin and dopamine), how they are affected by EE and how they interact with BDNF. EE has been shown to have antidepressant effects and increase serotonin in the PFC (Brenes, Rodríguez, & Fornaguera, 2008). The antidepressant effects of EE and exercise, however, may not be due to serotonin (Rogers et al., 2017). Serotonin and BDNF belong to what seems like two completely different signaling systems (Martinowich & Lu, 2007); yet BDNF promotes the survival and differentiation of serotonergic neurons (Martinowich & Lu, 2007) and serotonin has been shown to regulate BDNF expression during stress (Jiang et al., 2016).

Dopamine, an important neurotransmitter in the brains reward system, is also affected by EE. EE downregulates the dopamine transporter in rat medial PFC (Zhu, Apparsundaram, Bardo, & Dwoskin, 2005) and has been shown to decrease the rewarding effects of heroin (Rawas, Thiriet, Lardeux, Jaber, & Solinas, 2009). BDNF and dopamine also interact; BDNF promotes the survival of dopaminergic neurons (Hyman et al., 1991) and controls the dopamine D_3 receptor expression (Guillin et al., 2001).

An attenuation in overall behavioral exploration in social rats with a right lesion was observed in our study. Behavioral effects in rats with unilateral hippocampal inactivation

have been reported in older rats before (Poe et al., 2000). Poe et al found impairments in older male rats with left hippocampal inactivation, opposite to what we found. Since males likely use different strategies for exploring their environments than females, it is possible they rely more heavily on different hemispheres to accomplish navigation; and this may account for the results.

Future research

NTs are a complicated group of growth factors and, in the case of BDNF, take multiple forms. For example, pro-BDNF is the immature protein which is cleaved to form the mature BDNF protein measured in this study. Others have reported differential and often opposing functions for pro-BDNF and BDNF (Gibon, Barker, & Séguéla, 2016). BDNF also can be measured by its mRNA which adds an extra dimension to studies measuring effects on BDNF. In addition, the high-affinity tyrosine kinase receptor for BDNF (TrkB) can also be measured, adding to the complexity. For example, our study found a decrease in BDNF in the NC after EE; however, one could ask if this downregulation of BDNF is accompanied by an upregulation of the TrkB receptor. An interesting study may examine both BDNF as well as its receptors in rats living in enriched and impoverished conditions.

Since unilateral hippocampal lesions yield far superior statistical power it would be interesting to perform a similar study to the present one, except only lesion the HPC unilaterally but sever all commissural fibers as well, which would disallow interhemispheric communication; and measure BDNF as well as changes in axons, dendrites and dendritic spines.

Conclusion

The results of this study are interesting, however, largely inconclusive. How the HPC contributes to neocortical plasticity and therefore memory remain unclear. NTs remain a likely candidate for hippocampal-driven plasticity in the NC which, further studies will elucidate how the hippocampal-cortical relationship stores and retrieves memories; and how these memories are transformed into semantic knowledge, which is intelligence.

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APPENDIX

Behavioral Observations

Logistical barriers prevented much of the rodent behavior, throughout the experiment, from being quantified (i.e. home-cage behavior, field trip behavior or behavior in transport containers). However, anecdotal observations occurred regularly throughout the experiment (i.e. during cage cleaning, field trips and handling). Thus, it is important and relevant to include these observations which may lead to additional, future research questions to explore.

Post-lesion

As others have reported (Teitelbaum & Milner, 1963), hyper-excitability and increased activity levels following hippocampal lesions were observed upon fully waking from hippocampal lesion surgery; which seemed to diminish greatly over the few days following surgery.

Assigned Housing

Rats housed in the enrichment condos were more physically active than socially housed rats. Enriched rats would often engage in activities such as: scaling the wire mesh walls to get to upper levels of the condo (as opposed to using the provided ramps), stashing food in different locations of the environment and ripping up paper towel into small pieces to use in nest building. Rats housed in the social-only environments, which were presumably more impoverished, were mostly observed sleeping in a corner of the environment together and occasionally observed engaging in exploratory rearing.

Transport Container

Once rats were fully habituated to the transport containers, they appeared to sleep or at least enter quiescence shortly after being placed in these containers and remained in this state until being transported for behavior.

Experimental Caveats

Throughout this study there were some important caveats that should be mentioned. First, during the environmental exposure for cohort 1 there was construction occurring simultaneously in and around the facility and others in the building have verbally reported puzzling results during this time. Second, a malfunctioning vibratome resulted in some variable tissue thickness issues during sectioning for cohort 1. Third, immunohistochemical labelling of NTs was somewhat inconsistent and likely added some variability in the data. Fourth, the lamp brightness on the Nanozoomer Digital Pathology microscope system decreased as a function of hours of use until it needed replacing thus tissues imaged at the beginning of the life of the lamp are brighter than tissues imaged at the end of the lamp life.

Table 1: Number and distribution of animals in each experimental group analyzed for BDNF. Total number of animals analyzed in each environmental condition (enriched or social) and in each HPC lesion condition (bilateral, sham, left and right). Values are the sum of cohort 1 and cohort 2 rats in the same lesion and environmental conditions.

HPC lesion condition	Enriched	Social
Bilateral Lesion	5	6
Sham Lesion	4	4
Left Lesion	2	2
Right Lesion	2	2

Table 2: Number and distribution of animals in each experimental group analyzed for NT-3. Total number of animals analyzed in each condition. Values are the sum of cohort 1 and cohort 2 rats in the same lesion and environmental conditions. One bilateral lesion had poor NT-3 immuno labeling and thus was excluded from the study. One unilateral left lesion was processed to see if there is any effect of unilateral lesion on NT-3 expression in NC.

HPC lesion condition	Enriched
Bilateral Lesion	4
Sham Lesion	7
Left Lesion	1
Right Lesion	-

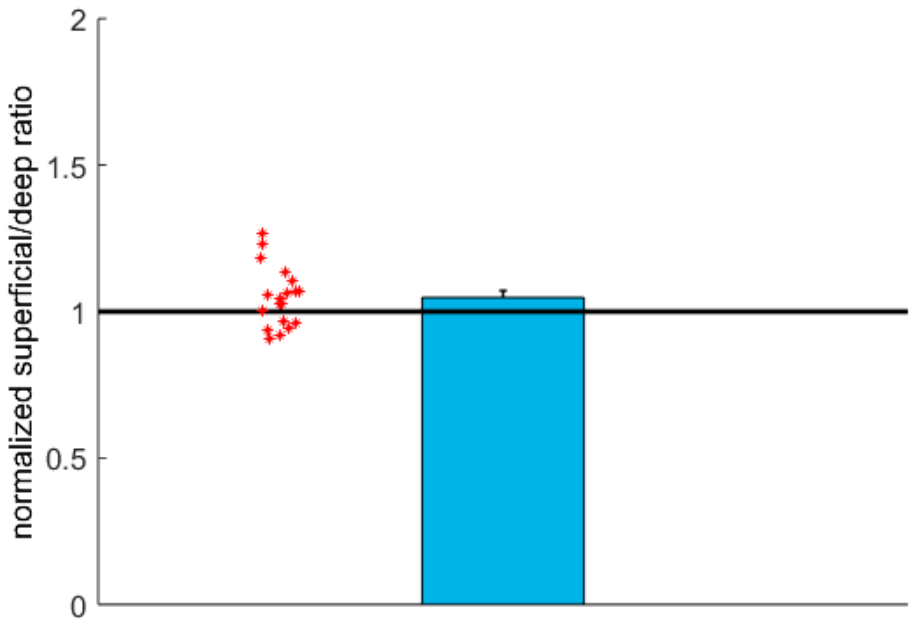

Figure 16: A strong trend of higher BDNF expression in superficial cortex remains after normalizing expression to cell density. Each animal's brightness was divided by their cell density to obtain a normalized brightness measure, this was performed for superficial and deep cortex. Once all values were obtained, superficial data was pooled, deep data was pooled, and the overall superficial/deep ratio was obtained. After using this method of normalization, the superficial/deep difference is not significant however, a strong trend of higher BDNF expression in superficial layers remains (one-sample t-test; $p = 0.055$; $n = 19$).

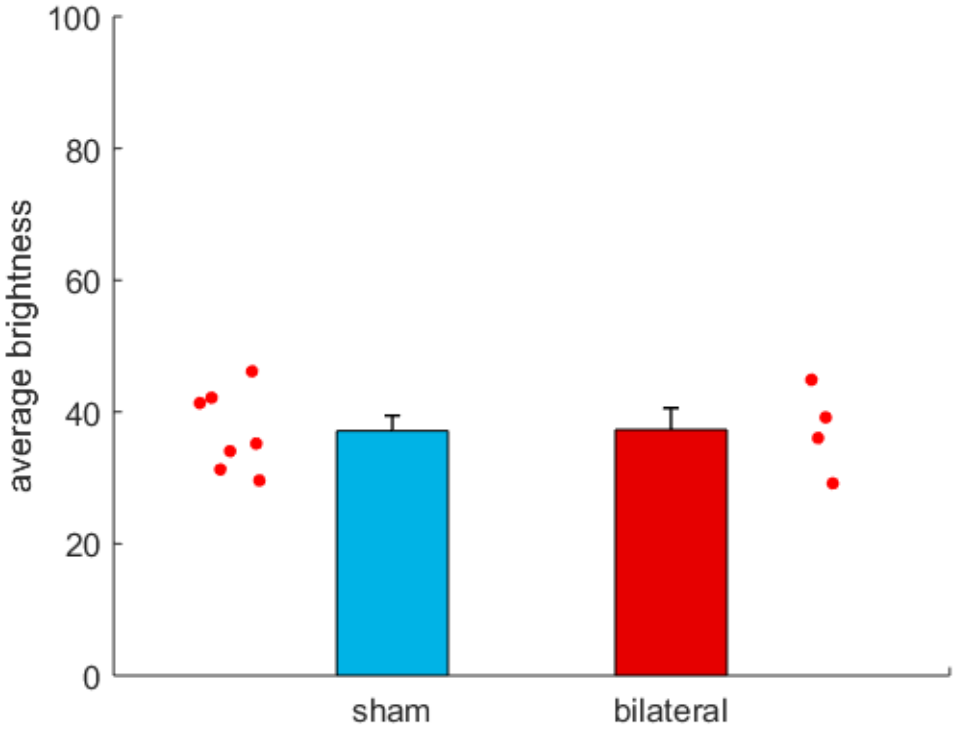

Figure 17: Lesions of the HPC had no effect on NT-3 expression in the NC of EE rats. Sham and bilateral hippocampal lesions were compared in EE rats. Hemispheres, sections, ROIs, and superficial/deep layers were averaged Average NT-3 expression comparison between sham and bilateral lesion conditions for enriched rats (sham n = 7; bilateral n = 4).

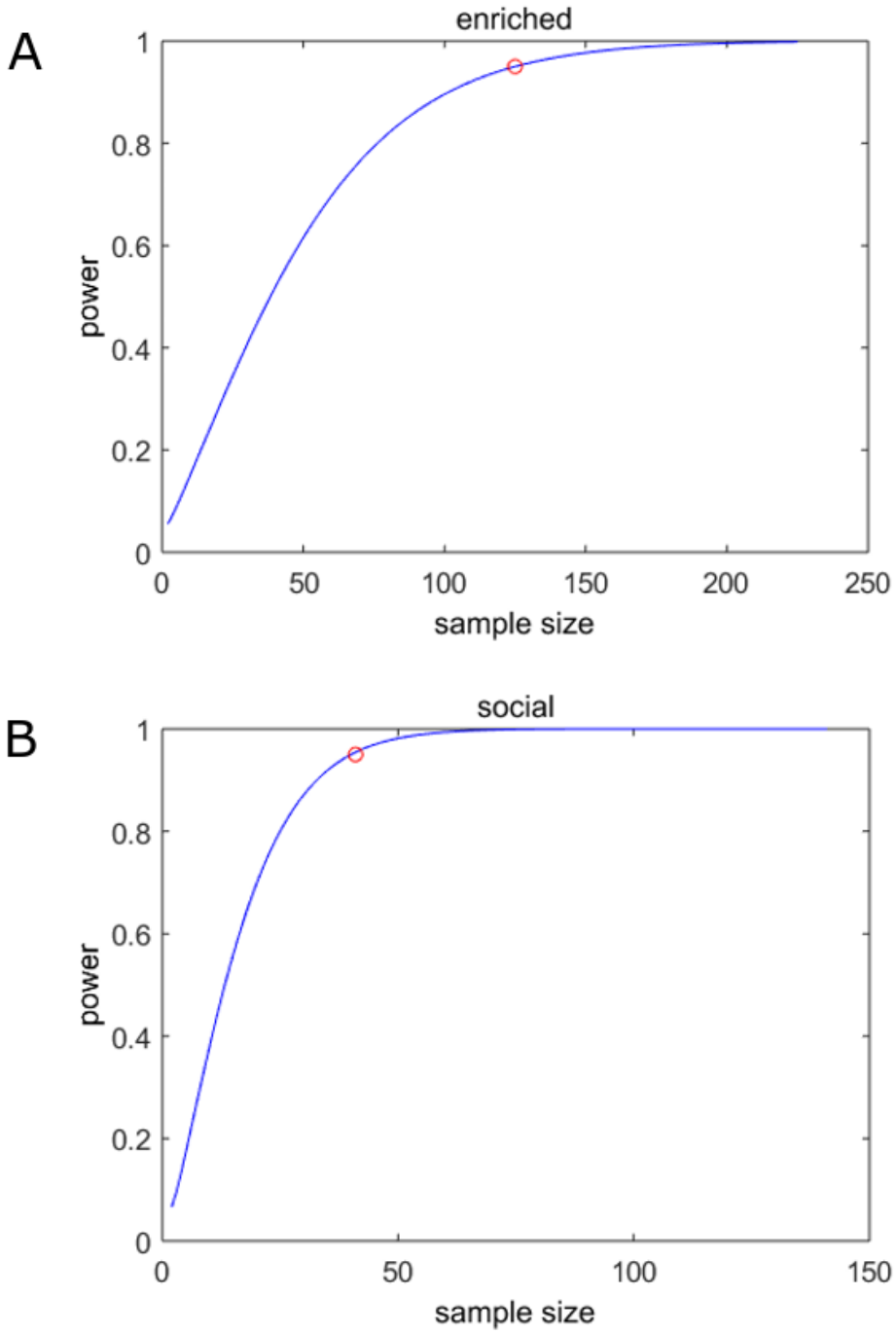

Figure 18: More subjects are required to have sufficient power. Power as a function of sample size was plotted to determine the sample size required to detect a 10% difference (if present) between sham and bilateral lesions for the enriched group (A) and social group (B) for BDNF. The red marker indicates the sample size at 0.95 power.