Changes in NR2B-NMDA Receptor Expression Relate to Individual Differences in Adaptability to Food Restriction-Stress

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Abstract — The eating disorder Anorexia Nervosa has an extremely high mortality rate of 5.9% (Arcelus et al. 2011) and a poorly understood neurobiology. Research indicates the animal model Activity Based Anorexia (ABA) may provide insight to the neural correlates of its behavioral and physical markers, hyperactivity and weight loss. Previously, female ABA rodents were shown to have increased levels of the NR2B subunit of NMDA receptor (NMDAR), an excitatory glutamate receptor, in stratum radiatum of dorsal hippocampal CA1. Increased NR2B-NMDAR levels may increase excitability in hippocampus, an anxiety-regulating brain region, possibly increasing anxiety –like behavior (Chen et al. 2017) such as hyperactivity (Wable et al. 2015). Here we tested whether adolescent female mice exposed to a second food restriction (ABA2) also demonstrate the behavioral and physical markers of ABA. NR2B-NMDARs in ABA2 and control mice were immunolabeled for electron microscopy and quantified at excitatory synapses of CA1 at various pre-, post-, and peri-synaptic regions. Electron microscopy was chosen to selectively observe NR2B-immunoreactivity at distinct pre- and post-synaptic compartments because NMDARs can operate differently depending on how closely they are anchored to the synaptic junction immediately apposed to presynaptic release site (Hardingham and Bading 2010). We found that ABA2 animals had more NR2B-labeling in presynaptic terminals and postsynaptic cytoplasm than controls. Increased NR2B-labeling at postsynaptic density correlated with increased running activity during food access and weight loss, suggesting that NR2B-NMDARs at postsynaptic density may mediate vulnerability to ABA2. Increased levels of NR2B-labeling at extra-synaptic membrane of dendritic spines correlated with less running during ABA2, suggesting NR2B at this site may mediate ABA resilience. The results indicate localized NR2B expression correlates with adaptability to second exposure to food restriction and support a neurobiological basis for ABA.

I. INTRODUCTION

Anorexia Nervosa (AN) is a psychiatric eating disorder that can be characterized by restriction of food intake, extreme weight loss, distorted body image and excessive exercise (National Eating Disorders Association 2016). Exercise-based hyperactivity, which is evident in 40-80% of AN patients is not only unhealthy, but also compulsive and compensatory for calorie intake (Holland et al. 2014), thereby maintaining the disorder (Holtkamp et al. 2004). Etiology ranges from socio-cultural factors, such as the media's construction of the ideal body type, to trauma, personality traits and other mental health conditions (Bemis 1978, Schmidt et al. 1997); for instance,

symptoms of anxiety and restricted food intake may actively contribute to hyperactivity and worsen symptoms of AN (Holtkamp et al. 2004). Familial and twin genetic studies have also revealed susceptible chromosomal loci and candidate genes involved in AN pathophysiology, providing evidence for both a biological and experiential bases to the disorder (Pinheiro et al. 2009).

The disorder typically manifests amongst the adolescent female population and has the highest mortality rate of any psychiatric disease (Arcelus et al. 2011). Treatment commonly consists of psychotherapy or counseling but can be challenging and with poor outcomes, since about 41% relapse within a year of recovery (Carter et al. 2012). Medications such as antidepressants and antipsychotics may be prescribed but improve symptoms by targeting pathways involved with depression and bipolar disorder rather than AN itself (American Psychiatric Association). Understanding the neurobiological causes of AN and how they may contribute to AN pathogenesis and progression is therefore critical for developing an alternate route for effective and targeted therapy for AN patients.

The neural correlates of Anorexia Nervosa may be explored using activity-based anorexia (ABA), the animal model for the disease (Chowdhury et al. 2015). Built upon the physiological premise that experiencing hunger induces foraging behavior, the model manipulates rodent's food restriction and access to a running wheel to induce reduced (voluntary) food intake, hyperactivity, and extreme weight loss (Chen et al. 2017). For rodents that demonstrate ABA, implementing food restriction increases voluntary wheel running such that the rodent chooses to run even during times of food availability and a hungered state (Chen et al. 2017). What is intriguing about the model is that rodents experience behavioral symptoms such as hyperactivity in the absence of exposure to the socio-cultural factors often ascribed to human etiology of the AN, suggesting that a neurobiological change may be sufficient for exacerbating the behavioral symptoms in rodents that are analogous to those of AN.

Invoking ABA in rodents thus provides a route to explore vulnerability to AN. Age, weight, and sex, for instance, are factors established to affect the extent of wheel running and weight loss in ABA. Animals who are older, heavier, and male tend to demonstrate less hyperactivity and weight loss than younger, lighter or female counterparts- trends that parallel manifestation of AN in patients (Boakes and Dwyer 1997, Pare et al. 1978), who are typically adolescent females. Experimental evidence reveals distinct neural changes that occur in conjunction with ABA to explain these trends.

The hippocampus is a highly plastic region that has not yet reached maturity during adolescence in rodents; for instance, the dentate gyrus (DG), which is part of the hippocampal formation dorsal to the ventral CA1 region,

is still undergoing neurogenesis, migration and maturation during puberty and adolescence (Stagni et al. 2015). The ongoing development of the hippocampus during this time makes it a particularly vulnerable target for experience-dependent plasticity in response to environmental stressors. Coincidentally, adolescent female rats that underwent acute induction of the ABA paradigm demonstrated suppressed non-neuronal cell proliferation in the DG and surrounding areas (Barbarich-Marsteller et al. 2013). While the direct consequence of this abnormality has yet to be discovered, it is well known that normal development of the hippocampus is important for learning and memory formation. However, the hippocampal formation is especially relevant for ABA experiments because it regulates stress and anxiety (Shen et al. 2007), a condition symptomatic or comorbid with AN (Kaye et al. 2004). Maladaptive alterations to the hippocampus can therefore be detrimental to stress and anxiety management, contributing to the behavioral symptoms of ABA.

In response to stress, there are elevated levels of glutamate, the excitatory neurotransmitter, in the hippocampus (Kazi and Oomenn 2014). When glutamate binds NMDA receptors on the post-synaptic membrane, and if the membrane potential is sufficiently depolarized, there is an influx of calcium into the cell that activates intracellular signaling cascades that may activate receptor trafficking pathways and exocytotic events. Higher concentrations of glutamate mediate increased excitability in the hippocampus, which strongly correlates with stress-induced anxiety (Shen et al. 2007). For animals that are vulnerable to ABA, there is a significantly higher concentration of NMDARs within regions of dorsal hippocampus (Chen et al. 2017), in addition to an altered dendritic arborization of CA1 pyramidal cells in dorsal hippocampus (Chowdhury et al. 2014).

The experience-dependent changes in CA1 spines in response to ABA can therefore be used to study vulnerability to ABA. The dendrites of CA1 branch together within stratum radiatum (SR), receiving inputs from CA3 Schaffer collaterals and entorhinal cortex (EC), and sending outputs to the EC layer V and subiculum (Olypher, Lytton and Prinz 2012). For adolescent female mice that have undergone one round of ABA (ABA1), there is a marked increase in levels of the NR2B subunit of the NMDA receptor in CA1, thereby mediating increased excitability and anxiogenesis within hippocampus (Chen et al. 2017). Functional NMDARs are comprised of four subunits: two subunits of NR1 and two subunits of NR2A and/or NR2B. This experiment implemented a second exposure to food restriction to the wheel-acclimated mice after seven days of recovery from ABA1. Although both NR2A and NR2B are NMDA subunits of interest, only the relationship between NR2B levels and ABA2 behaviors was considered here.

Rodents who are exposed to food restriction once demonstrate the behavioral and physical markers of ABA. Here we test whether or not adult female mice with a second exposure to food restriction also demonstrate the behavioral and physical markers of ABA and have a redistribution of NR2B-NMDA at hippocampal CA1. Using immunocytochemistry and post-embed gold (PEG) labeling for electron microscopy, NR2B-NMDA receptors were visualized at axo-spinous synapses within SR of dorsal CA1 of ABA2 animals and age-matched controls. Electron microscopy was chosen for visualization of the PEG-labeled NR2Bs because it allows visualization of both pre- synaptic and post-synaptic regions of the CA1 junction and differentiation between axo- spinous excitatory synapses verses axo-dendritic shaft inhibitory synapses. Because the location of the NR2B containing NMDAR dictates its function (Hardingham and Bading 2010, Petralia et al. 2010), it was important to quantify PEG-particles across various synaptic regions; the subcellular synaptic compartments analyzed included at the postsynaptic density, near the postsynaptic density, postsynaptic cytoplasm, post-extrasynaptic membrane, at/near presynaptic membrane, presynaptic cytoplasm, pre- extrasynaptic membrane and the synaptic cleft.

The hypothesis was that neural circuitry modifications in the hippocampus do not get restored after ABA1, and there are therefore increased levels of NR2B-NMDA in CA1 pyramidal cells during and after ABA2. Higher levels of NR2B-NMDA can mediate increased excitability in the hippocampus, leading to increased anxiety-like behavior and increased hyperactivity, mediating vulnerability to ABA. We found that ABA2 animals demonstrated significantly higher levels of NR2B-immunoreactivity within the postsynaptic cytoplasm, presynaptic cytoplasm and pre-extrasynaptic membrane than age- matched controls. ABA2 animals also demonstrated individual differences in NR2B levels that correlated to adaptability to ABA2, such as extent of wheel running activity and weight loss. This provides evidence that a second exposure to food restriction also correlates with the behavioral and physical markers of ABA.

II. METHODS

Animals

Mice used for this experiment were handled according to the Institutional Animal Care and Use committee of New York University (Animal Welfare Assurance No. A3117- 01). There were a total of 15 mice used in the experiment, 8 in the ABA condition and 7 age-matched controls. The control animals did not experience any food restriction. All mice were female, bred and housed within the NYU animal facility, and with a C57BL/6J strain background (see Chowdhury et al. 2013, Chen et al. 2016).

The ABA1 and ABA2 paradigm is as described in Chowdhury et al. (2013), except we used 2 hours of food restriction from 7pm-9pm, during the first two hours of the dark cycle. The ABA paradigm begins with a baseline period starting at P36, when animals are acclimated to the running wheel in individual cages and have normal access to food (See Fig. 1A). Starting at P41, the animals are subject to ABA. This involves continuous access to the running wheel in conjunction with food restriction. Animals in ABA only have access to food during the hours of 7-9pm, during the first two hours of the dark cycle (See Fig. 1B). The animals' dark cycle begins at 7pm and terminates at 7am, during which time the lights are kept off. At the end of each food access period, animals were moved to a new cage and the amount of food consumed and the wheel count recorded. Wheel counts were

recorded at these intervals: 1am-7am (during the dark cycle), 7am-1pm, 1pm-7pm (FAA), 7pm-9pm (food access) and 9pm-1am. This allowed for comparison of wheel activity during distinct periods of the day and a summation of wheel counts to calculate total activity during ABA. After completion of ABA1, animals were given a recovery period until P51 in order to restore a healthy body weight (See Fig. 1A). From P51-P55, animals underwent a second baseline period with continuous running wheel access to reacclimate to the wheel. ABA2 began on P55 through P59 and followed the same protocol as for ABA1. The paradigm was culminated with euthanasia by perfusion on P60. Wheel activity and weight were monitored throughout this time.
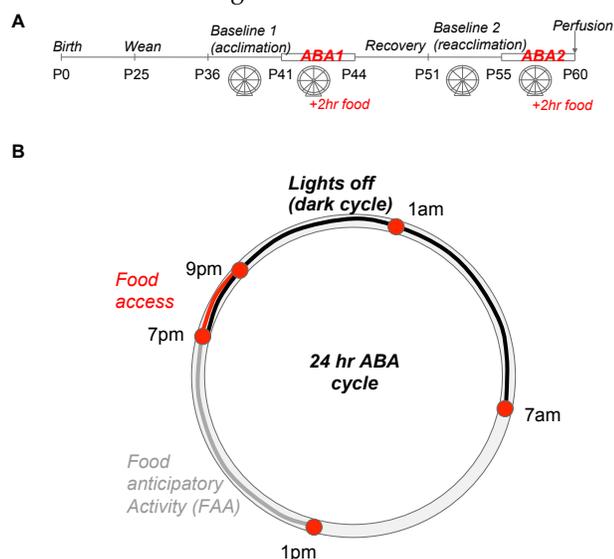
Figure 1: Schematics of the ABA paradigm. (A) Timeline outlining sequence of events for ABA2 mice from birth (P0) to perfusion (P60). During ABA1 and ABA2, mice had continuous access to a running wheel but limited access to food. (B) Typical day of ABA: mice received food only from 7pm-9pm, during the first two hours of the dark cycle that continues until 7am. Wheel running is monitored throughout ABA; food anticipatory activity is seen and measured from 1pm to 7pm.

Brain Tissue Preparation

ABA animals and age-matched controls were euthanized on P60 (after the second ABA), using a urethane based anesthesia (i.p. 34%, 0.15mL/20g), at a time between 12pm and 4pm (Chen et al. 2016). Animals were then transcardially perfused using a buffer containing 4% paraformaldehyde (PFA). Fixation with glutaraldehyde was not conducted until post-embed tissue processing for the purpose of antigen retention (Chen et al., 2016). After extraction, brains were post-fixed in a PFA fixative for 9h, and sectioned on a coronal plane using a vibratome (40μm). Tissue sections were stored at 4°C in 0.01M phosphate buffer, 0.9% saline (buffer pH = 7.5) and 0.05% sodium azide (PBS-azide). Sections containing dorsal hippocampus were then selected for post-embed processing.

Antibodies

Primary antibodies used in the experiment are previously described (Aoki et al. 2009a, b, and Chen et al. 2017). The antibody used against NR2B (cat. #06-600, Millipore (previously available from Upstate Biotechnology)) was developed in rabbits and targets the last 20 amino acids of the rodent NR2B C-terminus. Antibody specificity was confirmed with Western blots on rat brain microsome, which revealed a single band at ~180kD corresponding to the molecular weight of NR2B (Chen et al. 2017) and no indication of cross-reactivity with NR1 or NR2A subunits of the NMDAR (Rinaldi et al. 2007). The ABA2 and CON tissue was also labeled with anti-NR2A primary antibody for a portion of the experiment that will be considered in the near future. The secondary antibody used to detect the primary NR2B antibody was anti-rabbit IgG, produced in donkey, conjugated to 10nm colloidal gold particles (Cat. #25705, Electron Microscopic Sciences).

Immunocytochemistry

Procedures for targeting NR2B using immunocytochemistry are as previously described in Aoki et al. 2000, 2003, 2009a, 2009b, Fujisawa and Aoki 2003). Processing for immunolabeling was done on two vibratome sections (randomly selected) per animal that were post fixed with 2% glutaraldehyde in PBS for 10 mins. These sections were then processed using the osmium-free procedure for electron microscopy and embedded in plastic. The plastic-embedded blocks were then ultrathin sectioned, producing five EM grids per section. Two of the grids were used for NR2B labelling, two for NR2A, and one as a no primary antibody control. Post-embed immunogold labelling was conducted as described previously (Aoki et al. 2009b). The grids were incubated with anti-NR2B (10ug/ml, Millipore) overnight and anti-rabbit IgG secondary antibody (1:100, EMS) for 1 hour at room temperature.
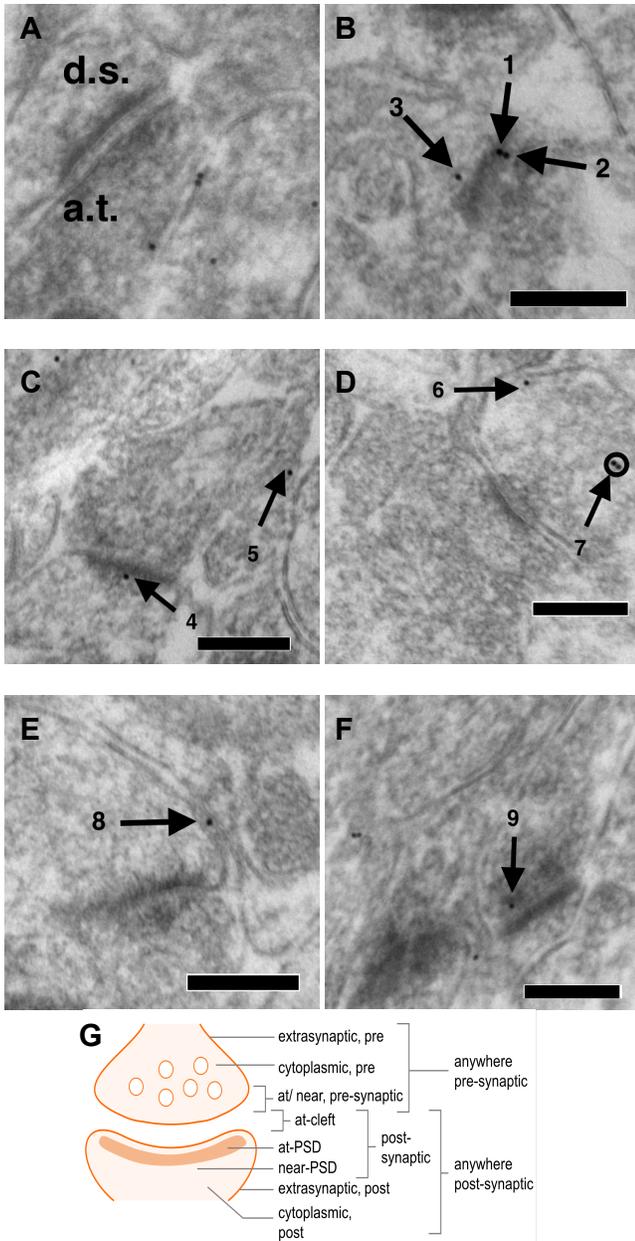
Electron Microscopy

Visualization and imaging of immuno-labeled sections was conducted using a JEOL 1200XL transmission electron microscope and AMT XR80 camera system and software (Boston, MA). Typically, one grid, randomly picked from pairs of immunolabelled grids, was selected per animal for imaging. The grids contained anatomical markers including CA3, dentate gyrus and hippocampal fissure that were used to locate the CA1 region. A second grid was only used in cases when the tissue was torn over the region of interest, making it challenging to capture good images for quantitative analysis. All images captured were within SR of CA1 in dorsal hippocampus, and the synapses accounted for were asymmetric axo-spinous, identified using a thick post-synaptic density opposite to a presynaptic terminal containing vesicles. The identity of the animals, either ABA2 or control, was not revealed until after image capturing and PEG quantification to prevent bias from seeking higher or lower levels of NR2B-immunoreactivity during image capturing. Images were analyzed for PEG-particle quantification using ImageJ (NIH).

PEG-Particle Analysis and Statistics

Because each NR2B-immunoreactivity was analyzed by quantifying PEG particles at distinct subcellular compartments (See Fig. 2A, B) - pre-extrasynaptic, pre-cytoplasmic, at/near presynaptic membrane, cleft, at/near postsynaptic density, post-extrasynaptic, post-cytoplasmic- at axo-spinous synapses in SR of dorsal CA1. PEG particles that were clustered in groups of 2 or more particles that were less than 20nm apart were quantified as a single NR2B label. PEG particle analysis was conducted in two ways: using *animals* as independent units and using *synapses* as independent units.

Figure 2: Electron micrograph images from ABA tissue to demonstrate categorization of immuno-labeled NR2B subunits at distinct axo-somatic synaptic compartments. Numerically annotated images contains a bar to scale to 200nm. (A) A presynaptic axon terminal is indicated by "a.t." and

postsynaptic dendritic spine by "d.s.". NR2B-immunoreactivity is observed through PEG immunolabelling, as indicating by numbers 1-9, categorized accordingly: (B) 1 at postsynaptic density, 2 in cleft, 3 in postsynaptic cytoplasm, (C) 4 near postsynaptic density, 5 and (D) 6 at pre-extrasynaptic (pre-ES) membrane, 7 at presynaptic cytoplasm- the cluster of two PEGs at 7 was counted as a single labeled NR2B subunit, since the PEGs were < 20nm apart- (E) 8 at post-extrasynaptic (post-ES) membrane, (F) 9 at presynaptic membrane. (G) Schematic to show how PEGs were categorized at distinct locations within excitatory synapses.

Using synapses as independent units compares group differences in NR2B- immunoreactivity between the ABA2 group and the control group using Mann-Whitney U tests (See Fig. 3). Approximately 200 synapses were analyzed per animal, and sorted into groups of 10 (per-10-synapses) to assess for the level of PEG particles and proportion of NR2B-immunoreactivity present across the subcellular compartments per tissue. The sums of the PEGs per 10 and per animal (200 synapses) or sums of synapses immunolabeled per 10 and per animal were then pooled together. NR2B-immunoreactivity at each subcellular synaptic site in ABA2 and control animal tissue was compared using Mann-Whitney U tests for nonparametric data because the data was not normally distributed.
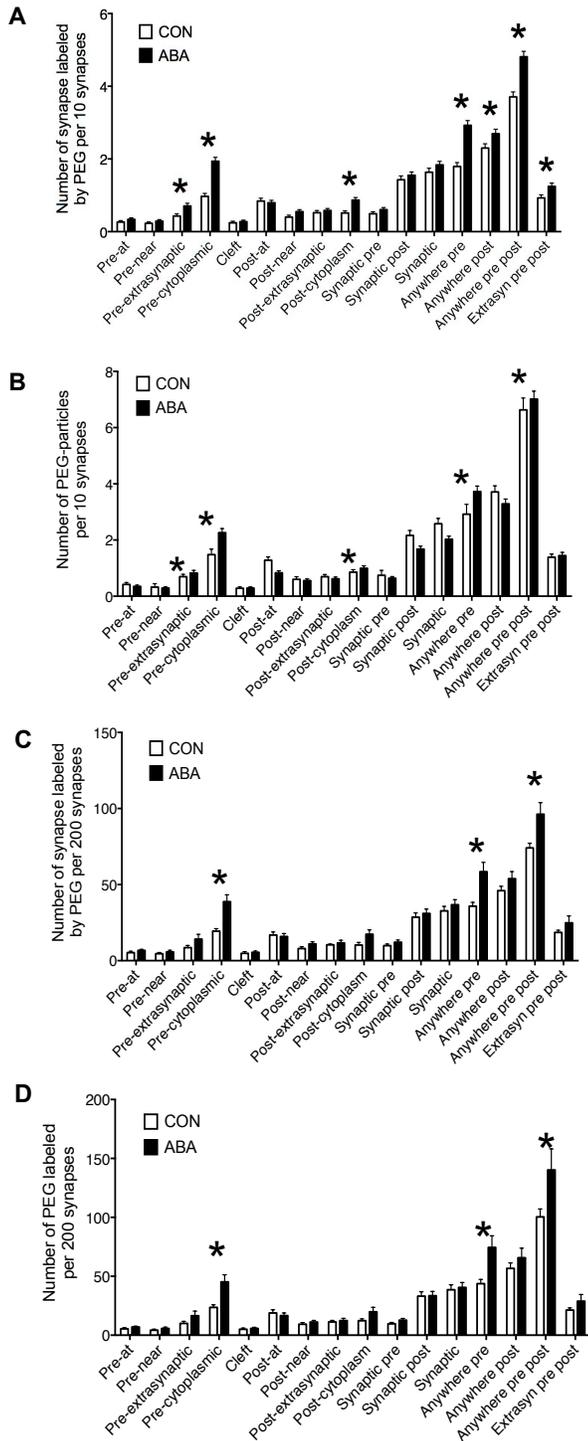
Using each animal as an independent unit compares how individual differences in NR2B- immunoreactivity correlate with wheel running activity and change in body weight (See Figs. 4, 5). The per-10-synapses sorting was repeated 20 times to obtain a sum of the PEGs or immunolabeled synapses per 200 synapses. The sum of the PEGs per animal (or 200 synapses) or sum of synapses immunolabeled was then used to determine significant individual differences in ABA2 tissue immunolabeling and behavioral symptoms of ABA. NR2B-immunoreactivity was related to wheel running activity and weight loss by conducting a Pearson's correlation analysis on Prism (La Jolla, Calif., versions 7.0). The p value used to determine statistical significance was p< 0.05.

III. RESULTS

ABA2 evokes a distribution of NR2B in SR of hippocampal CA1 that is different from controls and ABA1 animals

Animals that experienced a second ABA (ABA2) demonstrated a distribution of NR2B-PEGs that was different from age-matched control animals. NR2B detection by PEG immuno-labeling revealed that ABA2 animals had a greater proportion of synapses with PEG-labeled NR2B in the pre-synaptic cytoplasm (Mann-Whitney U (MWU)= 6515, P= <.0001, Fig. 3A) and postsynaptic cytoplasm (MWU= 8749, P= 0.003, Fig. 3A) relative to age-matched controls. ABA2 animals also had higher levels of NR2B- immunoreactivity, as quantified by the number of PEG particles, within the postsynaptic cytoplasm (MWU= 9091,P=0.0023, Fig. 3B), presynaptic cytoplasm (MWU= 6900, P= <0.0001, Fig. 3B), and pre-extrasynaptic membrane (MWU= 9716, P= 0.0255, Fig. 3B). While ABA2 and control animals did not demonstrate a significant difference in NR2B- immunoreactivity at other subcellular presynaptic sites, there was still an overall

increase in NR2B-PEGs within presynaptic terminals (MWU = 6821, P= <0.0001, Fig. 3B) compared to controls.

comparisons in levels of NR2B-PEGs using synapses as independent units. (C) Group comparisons in proportion of synapses labeled with NR2B using animals as independent units. (D) Group comparisons in levels of NR2B-PEGs using animals as independent units.

When NR2B-PEG levels were compared using each animal as an independent unit, a significant difference in immunoreactivity relative to controls was only seen in the presynaptic cytoplasm, since ABA2 animals had a greater proportion of terminals labeled (MWU= 0, P= 0.003, Fig. 3C) and more abundant NR2B-immunoreactivity (MWU = 1, P= 0.0006, Fig. 3D) within this region. Although NR2B labeling anywhere in the presynaptic spine was significantly greater (MWU= 5, P= 0.0059, Fig. 3D) between ABA2 animals and controls, the animals did not exhibit a significant differences in NR2B distribution anywhere in the postsynaptic spine.

These results additionally indicate that NR2B distribution for ABA2 animals was different from ABA1 animals'. Previous findings conducted by Chen et al. 2017 that considered how the ABA induction affects NR2B distribution in SR of hippocampal CA1 found significantly greater NR2B-immunoreactivity at both the presynaptic axon terminals and postsynaptic spines, relative to control animals' (See Chen et al. 2017 fig 3B). The new findings suggest that experiencing ABA2 results in a loss of the upregulated postsynaptic NR2B present during ABA1, since there is no consistent significant difference between ABA2 and control animals within postsynaptic regions (Fig. 3C, 3D).
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Increased NR2B-immunoreactivity at the postsynaptic density correlates with individual differences in running activity during food access and increased weight loss after ABA2

Running during food access is an indication of hyperactivity and self-starvation in ABA because the animal is choosing to exercise rather than eat its food. ABA2 animals demonstrated individual differences in running activity during food access. For animals that ran more during the feeding hours of the last three days of ABA2, there was a higher number of NR2B-PEGs at the postsynaptic density (PSD) (R= 0.902, p = 0.005, Fig. 4A). Interestingly, there was no significant correlation between NR2B-immunoreactivity at the postsynaptic density and running activity within the reacclimation period, which occurred prior to ABA2 (data not shown).

This may suggest the role of NR2B at the PSD involved not in general locomotion, but instead when there is a conflict between an innate urge to forage (physical activity) versus feed. If upregulated NR2B at the PSD has a role in exacerbating physical activity during ABA2, specifically during times of food availability, animals with more NR2B at the PSD should also demonstrate increased weight loss after ABA2 due to their voluntary food restriction. The ABA2 animals' weight loss (%) at perfusion was used to indicate the weight lost after ABA2 relative to the animals' weight before beginning ABA2. Indeed, individual differences in ABA2 animals' weight loss (%) at perfusion correlated positively and significantly with NR2B-PEGs at the PSD (R= -0.89, p= 0.018, Fig. 4B). No other behavioral data during ABA2

Figure 3: The distribution of NR2B in SR of hippocampal CA1 is different in ABA2 tissue compared to control tissue. Mann-Whitney U test for nonparametric data was conducted to determine significant differences in NR2B-immunoreactivity. Significant differences are marked with an asterisk (*). (A) Group comparisons in proportion of synapses labeled with NR2B using synapses as independent units. (B) Group

correlated significantly with NR2B-PEGs at the PSD (data not shown), suggesting NR2B at the PSD may independently increase susceptibility to ABA2 by increasing anxiety, which promotes physical activity during food access, thereby increasing weight loss.
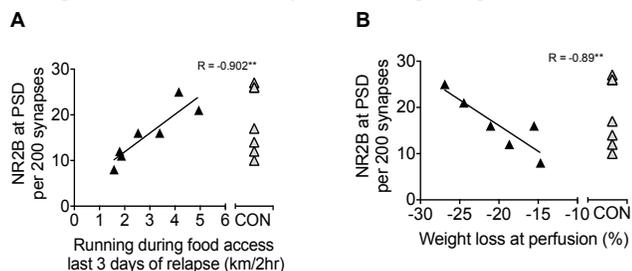
Figure 4: Pearson correlation results for NR2B-immunoreactivity at the postsynaptic density (PSD) and individual differences in ABA vulnerability. (A) Running during food access, measured from 7pm- 9pm (the hours of food availability), during the last three days of ABA2 correlated significantly and positively with NR2B at the PSD. (B) Weight loss at perfusion, relative to the animals weight before ABA2, was greater in animals that had increased NR2B at the PSD, indicating increased vulnerability to ABA with increased NR2B at the PSD. Black triangles indicate data from ABA2 tissue; grey triangles indicate data from age-matched controls (CON) tissue. Pearson correlation R values are marked with two asterisks (**) to indicate p<0.01.

Post-extrasynaptic NR2B-immunoreactivity in relationship to running activity during ABA2

Individual differences in running activity over the course of ABA2 (km) correlated negatively with levels of NR2B-PEGs at the post-extrasynaptic (post-ES) plasma membrane: animals that demonstrated increased hyperactivity had a smaller proportion of synapses containing NR2B-immunoreactivity at the post-ES membrane (R= -0.88, p=0.0088, Fig. 5A) and fewer NR2B-PEGs at the post-ES membrane (R= -0.87, p= 0.011, Fig. 5B). This suggests post-ES NR2B may have a role in mediating ABA resilience, since animals with greater NR2B levels in this region demonstrate less hyperactivity over the course of ABA2.
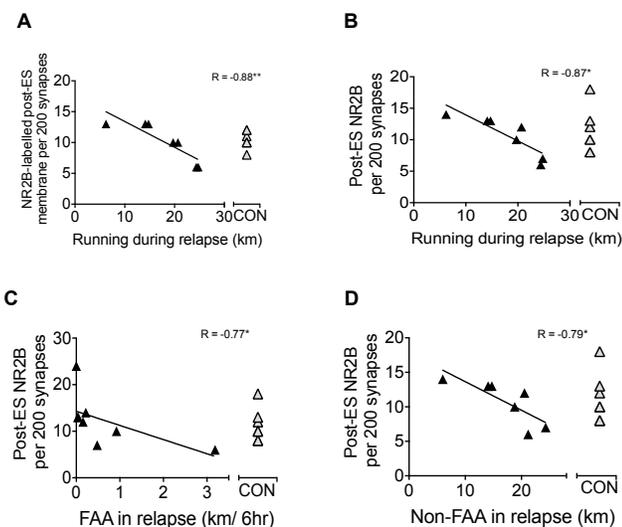
Figure 5: Pearson correlation results for NR2B-immunoreactivity at the post-extrasynaptic (post-ES) membrane and individual differences in ABA2 vulnerability. (A) A greater proportion of synapses were labeled with NR2B-PEGs at the post-ES membrane and (B) a higher level of post-ES NR2B-PEGs were present in ABA2 animals that ran less over the course of the ABA2. (C) Non- food anticipatory activity and (D) food anticipatory activity during ABA2 correlated negatively with post-ES NR2B-immunoreactivity. Black triangles indicate data from ABA2 tissue; grey triangles indicate data from age-matched controls (CON) tissue. Pearson correlation R values are marked with one asterisk to indicate 0.05>p>0.01 but two asterisks (**) to indicate p<0.01.

Although post-ES NR2B-immunoreactivity did not correlate with running activity during baseline, ABA1, or the reacclimation period (data not shown), there was a significant correlation between post-ES NR2B-PEGs and food anticipatory activity (FAA, 1pm-7pm, preceding the feeding hours) during ABA2 (R=-0.77, p=0.04, Fig 5C) as well as non-FAA activity (7pm-1pm) during ABA2 (R=-0.79, p=0.03, Fig. 5D). Food anticipation during ABA typically results in an innate increase in wheel running behavior (Mistlberger 1994). The findings suggest higher levels of post-ES NR2B levels make ABA animals less susceptible to hyperactivity during ABA2, even when anticipating food. Post-ES NR2B may therefore have a role in protecting animals form ABA2-induced hyperactivity. However, post-ES NR2B PEGs did not correlate with running activity during times of food availability, nor did it correlate with weight loss (data not shown).

IV. DISCUSSION

Functional significance of increased presynaptic NR2B-immunoreactivity in ABA2 animals compared to controls

Presynaptic receptors are known to have a role in enhancing synaptic efficacy by modulating neurotransmitter release through cascadic signaling pathways (Diguid and Smart, 2009). Presynaptic NMDARs (preNMDARs) are not classical Hebbian receptors because their activation via glutamate binding and depolarization arises presynaptically (Bidoret 2009, Dore et al. 2017) instead of postsynaptically. Yang et al. (2011) found that entorhinal cortical NR2B-NMDA presynaptic autoreceptors had a role in tonically facilitating glutamate release in chronically epileptic rats (Yang et al., 2006), suggesting that enhancement of glutamate release from presynaptic terminals can be modulated by NR2B-NMDA autoreceptors. Additionally, Dore et al. (2017) outlined the preNMDAR's roles in both evoked and spontaneous neurotransmitter release (Berretta and Jones 1996, Sjöström et al., 2013), with and without calcium+-dependency (Kunz et al., 2013). If hippocampal preNR2B-NMDARs function similarly, in that they modulate both tonic and evoked glutamate release, the concentration of glutamate would be elevated within the synaptic cleft, exciting adjacent neurons.

The level of NR2B-NMDARs within presynaptic terminals in SR of hippocampal CA1 of ABA2 animals was significantly higher than basal levels in control animals. Increased NR2B-NMDARs may thus function to enhance vesicular release of glutamate, and contribute to increased

excitability in the hippocampus that may be occuring in conjunction with ABA2. Interestingly, there were limited correlations between pre-NR2B and individual differences in ABA2 vulnerability that could be made. This suggests that while ABA tissue had significantly higher pre-NR2B-immunoreactivity compared to controls, pre-NR2B NMDAs does not have a large role in role in the two measurements we made of ABA vulnerability – wheel running and weight loss. There may be other measurements which we did not make, such as corticosteroid levels, with which pre- NR2B-immunoreactivity correlates.

Functional significance of increased NR2B-immunoreactivity at the postsynaptic density correlating with increased running activity during food access

A higher concentration of NR2B-NMDA receptors receiving glutamate at the post- synaptic density correlated with increased running during food access during ABA2. Increased running during food access reduced food intake during the two hours of food availability, directly increasing weight loss, which also correlated positively with increased NR2B-immunoreactivity at the PSD. These individual differences in running during food access in ABA2 mice are a trademark behavior of ABA: the amount of energy metabolized for exercise is greater than the animal's caloric intake, ultimately leading to starvation and death. While the mechanism underlying hyperactivity despite low energy levels has yet to be determined (Kim 2012), hyperactivity may also be an indication of increased stress, and stress induced anxiety (Shen et al. 2007). In the absence of ABA, Sibold et al. (2011) found that stress significantly increased wheel-running activity in mice already acclimated to the wheel.

There is evidence to suggest hyperactivity in mice serves as an anxiolytic to relieve stress, a behavior that can be paralleled to human cases of Anorexia Nervosa, where patients who exercise excessively also demonstrate higher levels of anxiety and report exercise to be anxiolytic (Peñas-Lledó et al. 2002). While contradictory evidence suggests that hyperactivity in rodents may serve to increase anxiety instead (Garrett et al. 2012), the correlation between mice in the ABA-paradigm and increased anxiety relative to controls is consistent, since ABA mice have been shown to demonstrate increased anxiety-like behaviors in the elevated plus maze after food restriction (Wable et al. 2015). Functional NR2B-NMDARs at the post-synaptic density can increase rate of cell firing, not only increasing excitability but also increasing local concentrations of glutamate to sustain excitability. This can sustain anxiety-like behaviors in mice and contribute to the hyperactive behaviors demonstrated by ABA and ABA2 mice, which engage in high- energy-expending exercise despite having low-energy levels due to low caloric (food) intake. Future studies involving the ABA paradigm should examine the role between NR2B at the postsynaptic density and increased CA1 cell excitability in ABA or ABA2 mice that demonstrate increased anxiety and hyperactivity.

Post-extrasynaptic NR2B-NMDA as a facilitator of ABA resilience

ABA2 animals demonstrated increased NR2B-NMDA at the post-extrasynaptic membrane that correlated with decreased running during ABA2, including reducing food anticipatory activity (FAA) and non-FAA wheel running. This suggests post extrasynaptic NR2B (post-ES-NR2B) may provide resilience to ABA2. Whereas post-ES-NMDA has been shown to promote neuronal apoptosis in cultured neurons (Hardingham and Bading 2010), Chen et al. (2017) suggest that post-ES-NMDA role may have more to do with synaptic, spine and dendritic pruning as evident in adolescent animals that demonstrate voluntary exercise (Chowdhury et al 2014). Post-ES-NR2B activation may also evoke long-term depression (LTD) in hippocampal CA1 (Liu et al 2013), suggesting post-ES- NR2Bs may play a role in reducing excitability within the post-synaptic neuron. If upregulated post-ES-NR2B functions to depress synaptic efficacy via LTD and reduce CA1 excitability, this explains how post-ES-NR2B can protect ABA-vulnerable animals from exercising excessively during ABA2 and also suppress overall activity, as seen with reduced FAA in ABA2 animals that have increased post-ES NR2B.

Interestingly, the ABA2 findings for post-synaptic NR2B-immunoreactivity differ from those found after only one ABA. Chen et al. (2017) noted no significant correlation between post-extrasynaptic NR2B levels and weight loss (see Table 4 of Chen et al. 2017). This can suggest either a downregulation of NR2B from the post-ES membrane or a redistribution of NR2B from the post-ES membrane to another postsynaptic site that only occurs after the experience-dependent changes facilitated by ABA1.

Immunodetection of NR2B-NMDA is not an indication of functionality. In the absence of electro-physiological experiments on neural tissue from ABA2 animals, the ratio of functional to non-functional NR2B-NMDARs, and the true change in excitability of the hippocampus due to an increase in NR2B-NMDA, are not known. While the possibility of non-functional NR2B-NMDA present at cytoplasmic and extrasynaptic sites cannot be ruled out, there is still evidence for an upregulation, or downregulation, of NR2B at these sites. Moreover, while some differences between NR2B-immunoreactivity in ABA1 (from Chen et al. 2017) and ABA2 were noted, it is important to consider that the ABA1 animals were perfused at a younger age of P44 than ABA2 animals that were perfused at P60. This means that age is likely to have also contributed to differences in NR2B distribution between the two ABA groups, which can be determined by doing an age-matched ABA1 paradigm.

V. CONCLUSIONS

Animals that experience two episodes of food restriction in the presence of a running wheel exhibited increased NR2B-immunoreactivity in CA1 neurites that positively correlated with activity-based Anorexia behaviors such as hyperactivity (wheel running) and weight loss, both of which are analogous to behaviors symptomatic of Anorexia Nervosa. Animals that demonstrated ABA had differential levels of NR2B-

NMDA that correlated with how adaptable they were to stress evoked by food restriction. The findings of elevated NR2B-NMDARs across pre- and post-synaptic regions correlating with ABA- behaviors, after a second exposure to food restriction, indicate a route for therapy through NR2B antagonism. Future studies should examine how NR2B- selective NMDA antagonists such as ifenprodil (Chennard and Menniti 1999) administered through an implantable infusion into CA1 of dorsal hippocampus may alleviate ABA severity.

VI. AUTHOR INFORMATION

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