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Protein Kinase M Zeta-Mediated LTP Maintenance in the Non-Human Primate
Hippocampus: A role for Stress and Serotonergic Signaling in Affective Processing

by

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Date

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Signature

December 21, 2016

Date

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Signature of Second Reader

Project Summary:

Early Life Stress (ELS) is associated with an increased vulnerability to developing mood disorder later in life (35). While many of the structural and neurochemical consequences of ELS have been studied, it is not well understood how these changes may translate to the deficits in cognitive and affective function associated with mood disorders. The constitutively active Protein Kinase C kinase isoform Protein Kinase M zeta (PKMzeta) has a critical role in the molecular mechanisms of long-term potentiation (LTP) maintenance (1, 2), and memory storage. Recent preclinical studies indicate PKMzeta expression at the synaptic membrane directly mediates cognitive processing in the hippocampus (33, 34). Building on our previous findings of serotonergic dysfunction and hippocampal hypotrophism in the highly clinically relevant Variable Foraging Demand (VFD) Non-Human Primate (NHP) model of ELS, the current study aims to further characterize the ELS phenotype with respect to this key marker of hippocampal LTP. Furthermore, we have recently presented evidence of impaired neurogenesis in the VFD dentate gyrus compared to non-VFD reared animals subjected to chronic adult stress (93) suggesting that ELS and concurrent adult stress affect hippocampal processes differentially. In the current report, we utilize our extensive NHP tissue bank, which includes hippocampal samples from animals subjected to ELS and chronic adult stress, respectively, in order to explore these differential effects of ELS vs. concurrent stress on hippocampal LTP, and the implications of those effects on cognitive aspects of stress-related depressive symptoms in NHPs.

This is the first report of PKMzeta detection and analysis in the NHP brain – an important model for translational psychiatric research. We hypothesize that ELS

rearing is associated with reduced PKMzeta expression, and that this effect may be linked to cognitive deficits often observed in animal and clinical studies of ELS. Semi-quantitative evaluation of PKMzeta signal intensity via neuroimmunostaining across defined subregions in the NHP hippocampus will allow us to assess the effects of differential rearing conditions and stress paradigms on key components of hippocampal LTP maintenance as a consequence of ELS versus concurrent adult stress. We further hypothesize, based on recent mechanistic reports, that PKMzeta expression in the hippocampus is interconnected with both serotonergic signaling and Brain-derived Neurotrophic Factor (BDNF) expression – two critically important processes involved in hippocampal function (94). Importantly, our NHP cohort includes bonnet macaques harboring both the “long” and “short” alleles of the serotonin transporter gene polymorphism. The “short” STG allele produces a functional serotonin transporter with reduced capacity leading to reduced serotonin signaling, allowing us to observe how impaired serotonergic signaling may interact with rearing conditions to affect expression of these markers.

Finally, we hypothesize that reduced PKMzeta in ELS subjects may manifest in behavioral alterations in adulthood. Previous studies in rodents and primates have definitively shown that PKMzeta expression in the hippocampus has a direct effect on cognition and behavior (33, 34). Thus, by correlating hippocampal PKMzeta signal intensities with previously obtained behavioral data from this same cohort, we aim to delineate the neurobiological phenotype of altered hippocampal processing in the NHP model of ELS – further linking the distribution and activity of the molecular substrate of L-LTP to its behavioral and physiological manifestations.

While many studies have utilized PKMzeta localization as a tool for identifying maintenance of spatial memory in the rodent dorsal hippocampus (20), few have

examined the distribution of PKMzeta in the non-human primate brain or attempted to investigate the role of PKMzeta in the affective processing circuits of the hippocampus (25). Nonhuman primates provide a powerful model in which to use postmortem analyses to identify the mechanisms by which early stress and genetic makeup interact to produce long-term changes in brain development, stress reactivity, and risk for psychiatric disorders. Improved understanding of these processes will likely lead to the development of novel strategies for the prevention and treatment of neuropsychiatric disorders.

Specific Aims:

1. Assess differential patterns of PKMzeta expression in the NHP hippocampus by quantifying average PKMzeta signal intensity in specific subregions to detect alterations in VFD animals compared to control. We are particularly interested in exploring the role of serotonergic signaling, a key monoamine implicated in depression, in mediating PKMzeta expression. We will examine PKMzeta signal intensity in differentially reared subjects harboring the “Long” versus “Short” polymorphic alleles of the Serotonin Transporter Gene (STG) promoter.
2. Determine how PKMzeta expression in VFD subjects compares to hippocampal PKMzeta expression in concurrent adult stress subjects, in order to finely define specific phenotypes of early life stress versus adult stress.
3. Quantify the BDNF concentration in key sub-groups of our extensive NHP cohort in order to determine if there is a relationship between PKMzeta and BDNF expression, potentially indicating a role for neurotrophic deficits in the cognitive symptoms of stress-induced affective dysregulation. Results from this analysis may indicate if the

neurobiological alterations we have previously observed in VFD models of ELS are associated with cognitive processes.

4. Correlate previously collected data, including maladaptive behavioral response to intruder stressors, from this same cohort of NHPs to the quantification of PKMzeta concentration distribution across the hippocampus with the goal of determining the extent to which the consistent VFD maladaptive behavioral phenotype is linked to LTP maintenance in the hippocampus.

Background:

Protein Kinase M Zeta and the Molecular Mechanisms of LTP Maintenance

Molecular Mechanism of PKMzeta in LTP maintenance

One of the most exciting recent discoveries in the field of molecular neuroscience is the identification of constitutively active atypical PKC kinase isoform PKMzeta as a critical factor in the molecular mechanism of late-phase long-term potentiation (LTP) maintenance (1,2). LTP is an activity-dependent and persistent strengthening of synaptic transmission, and one of the most likely candidate processes underlying long-term memory (3). This potentiation is achieved through a complex set of intracellular molecular signaling cascades that ultimately converges on the synaptic machinery responsible for increasing the number of excitatory α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors (AMPA) in the post-synaptic density of the neuronal cell membrane (4). LTP can be separated into two distinct phases: the initial induction phase triggers potentiation, and involves the rapid synthesis and activation of calcium-dependent proteins to quickly enact the structural and proteomic changes required for modulating synaptic plasticity (5,6). Importantly,

many of the molecules that were discovered to be important to forming long-term memory, such as neurotransmitters and their receptors, Ca²⁺-dependent second messenger kinases, and downstream transcription factors and growth effectors were found to be critical for LTP induction, but not required for the second and longer-lasting maintenance phase (76). Inhibiting any of these LTP induction factors does not affect storage of previously consolidated memory (6, 76). Instead, the process of LTP induction is thought to coincide with memory encoding, while the second and longer-lasting LTP maintenance phase sustains storage of a distributed long-term memory engram (6). Recently, it was discovered by Dr. Todd Sacktor's group at SUNY Downstate that the persistently active protein kinase C isoform PKMzeta is both necessary and sufficient to maintain LTP and memory storage (8,19). This atypical PKC isoform has several unique characteristics that specify its central function in sustaining synaptic potentiation. PKMzeta is exclusively expressed in brain tissue and is specifically enriched in the forebrain, particularly in the hippocampus and neocortex (9). Furthermore, the PKMzeta isoform is transcribed from an internal promoter at the PKC locus, and thus lacks the autoinhibitory domain of typical PKC molecules, allowing it to autonomously and continuously phosphorylate its target substrates (10). Importantly, this unique persistent catalytic activity is the basis for PKM z's role in long-term LTP maintenance.

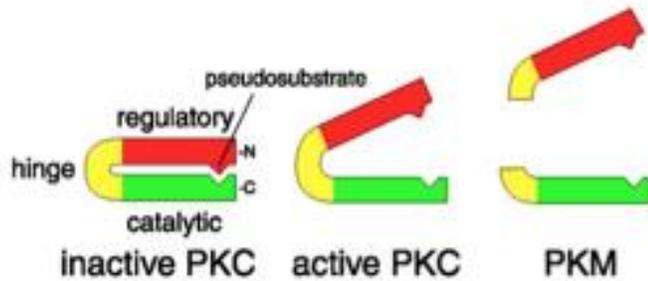


Figure 1: Shows a graphic model of the inactive and active conformations of the typical PKC kinase and the truncated PKM. PKC consists of a C-terminal catalytic domain (green) tethered by a hinge (yellow) to an N-terminal regulatory domain (red), which contains an autoinhibitory pseudosubstrate sequence. PKC is maintained in an inactive state by the interaction between the pseudosubstrate and the catalytic site. PKC is activated by second messengers, which produce a conformational change that releases the autoinhibition. PKM is the independent catalytic domain of PKC, synthesized from an internal promoter at the PKC gene locus. Because PKM lacks a regulatory domain, it is constitutively active. [Taken from Ref. 73]

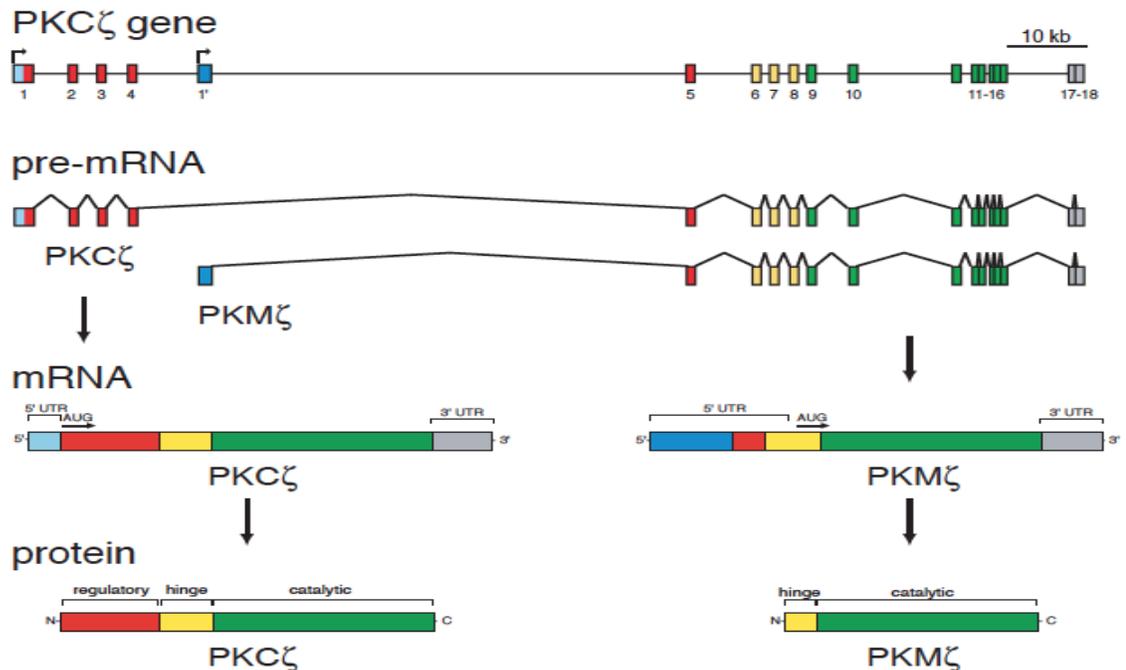


Figure 2: PKM ζ mRNA formation from an internal promoter within the PKC ζ gene : The intron-exon structure of the human PKC ζ gene shows two exon clusters separated by a large intron: exons 1-4, encoding the PKC ζ 5'UTR (in light blue) and regulatory domain (in red), and exons 5-18, encoding the remaining regulatory domain, hinge (yellow), catalytic domain (green), and 3' UTR (grey). The unique 5' PKM ζ mRNA sequence is in a single exon (exon 1', dark blue) within the large intron. PKC ζ mRNA transcription begins at exon 1. PKM ζ transcription is initiated from exon 1' and alternative splicing to exon

5 generates PKM ζ mRNA. [Taken from Ref. 73]

At the synapse, the strength of signal transmission between two neurons is tightly regulated to preserve relevant connections and degrade irrelevant ones. At resting state, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) cation channels are a main regulator of synaptic membrane depolarization thresholds and are constantly removed from the membrane and targeted for degradation by the ubiquitin-proteasome pathways that work to recycle proteins in the dendritic compartment (9). The constitutive PKMzeta kinase appears to sustain LTP maintenance after induction through regulating AMPA Glutamate receptor 2 (GluR2)-subunit dependent ionotropic receptor trafficking at the post-synaptic density (10) and preventing AMPAR endocytosis and degradation, in order to generate non-decaying LTP (11). The mechanistic details of this process are just now beginning to be understood: PKMzeta interacts directly with activity-dependent factors that traffic excitatory AMPA-glutamate receptors to the membrane during LTP induction (12). The kinase maintains LTP by acting on the N-ethylmaleimide-sensitive factor (NSF) - Protein Interacting with C Kinase – 1 (PICK1) AMPA receptor trafficking pathway to increase intracellular trafficking of the GluR2 subunit-containing AMPA receptor to the postsynaptic density following LTP induction, thereby potentiating the postsynaptic intracellular response to future excitatory neurotransmitter signaling at a given synapse (9,13).

In the past few years, mounting evidence has demonstrated the importance of PKMzeta in LTP maintenance and long-term memory storage. Many of these studies have utilized the ζ -pseudosubstrate inhibitory peptide (ZIP), a specific inhibitor of PKMzeta, to disrupt PKMzeta activity. In a wide range of preclinical

investigations, microinjection of ZIP into specific brain regions abolishes both spatial memory and fear conditioned memories for up to three months post-training, far longer than the average half-life of the PKMzeta protein (14,15). In order to preserve synaptic potentiation for these long periods, PKMzeta must be continuously produced and upregulated locally to constantly work against this recycling process in neurons actively participating in long-term memory, (16) thus making it an excellent potential marker of long-term information storage and preferred processing pathways in memory circuitry.

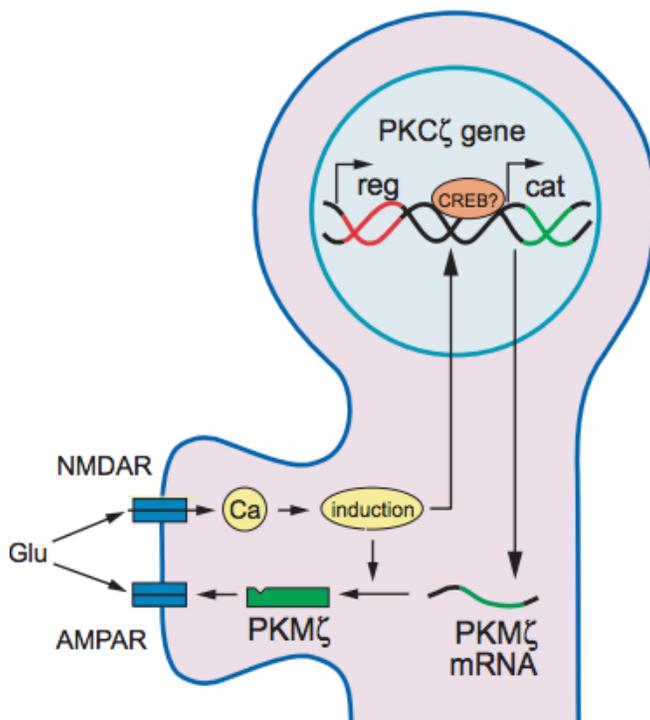


Figure 3: Model of LTP maintenance by PKM ζ synthesis. Activation by the excitatory neurotransmitter glutamate (Glu) at the NMDA receptor (NMDAR) during a tetanus triggers the induction of PKM ζ formation. PKM ζ phosphorylation potentiates α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPA) mediated synaptic transmission in LTP maintenance. Formation of PKM ζ during LTP occurs through increased de novo protein synthesis from the PKM ζ mRNA, produced by a dedicated internal promoter within the PKC ζ gene. Potential activity-dependent regulatory mechanisms for PKM ζ formation include enhanced transcription by CREB and increased local translation. [Taken from Ref. 73]

PKMzeta as a Marker of Neuronal Activation and Potential Indicator of Memory Engram

PKMzeta is preferentially expressed in the hippocampus as a downstream target of many major signaling cascades in response to neuronal activation (1). Since the identification of PKMzeta as the major synaptic molecule responsible for LTP maintenance, two contrary studies have suggested that PKMzeta null mice are still capable of maintaining long-term memory (17), implying that the ZIP inhibitor of PKMzeta used to validate its critical role in long-term memory may act nonspecifically to disrupt proteins other than PKMzeta (18). Until recently, these reports cast doubt on the central role of PKMzeta in LTP maintenance. However the latest investigation into this process has now supplied definitive evidence that in these PKMzeta null mice, alternative atypical PKMs are able to compensate for PKMzeta function, thus reinstating PKMzeta as the endogenous mechanism for LTP maintenance. Tsokas et. al. (19) circumvented the use of potentially problematic off-target effects of ZIP by synthesizing an antisense RNA to efficiently degrade and Knock-Down (KD) both PKMzeta and its closely related family members, Protein Kinase M Delta and Iota (PKMdelta and PKMiota). The authors show that in PKMzeta null mice, long-term memory does indeed remain intact (17,18). However, when the alternate atypical PKMs are also depleted in these same PKMzeta null mice, long-term memory is inhibited. Furthermore, in wild-type (WT) mice, depletion of alternate PKMzeta had no effect on long-term memory. Taken together, these data confirm that PKMzeta is required to maintain LTP and long-term memory, but that in the absence of PKMzeta, alternate mechanisms are recruited to perform LTP maintenance (19).

At the circuit level, PKMzeta's role in LTP maintenance has been confirmed in a wide range of brain regions known to be involved in various types of memory. In the hippocampus, PKMzeta mediates place memory, spatial learning, and object recognition (14, 20, 21). Injection of ZIP into the amygdala disrupts hippocampal-dependent fear memory in rats, (22) and fear potentiated startle (23). In the rodent reward centers of the brain, the Ventral Tegmental Area (VTA) and the Nucleus Accumbens (NAc), ZIP infusion alters cocaine sensitization and reduces memory for cocaine preference (24). In addition to using PKMzeta inactivation and overexpression to characterize PKMzeta function, many of these studies have demonstrated that intracellular PKMzeta protein expression positively correlates with specific memory retention (assessed behaviorally) and strengthened neuronal connectivity (assessed electrophysiologically) in mice models (20, 21, 22).

A more recent study found that while fear learning paradigms induced PKMzeta expression in the hippocampi of young control rats, this increase in PKMzeta was significantly reduced in aged rats. The PKMzeta reduction in aged rats was associated with cognitive impairment on several behavioral assessments, and was linked to increased cytosine methylation of the PKMzeta internal promoter loci (33). Furthermore, PKMzeta expression appears to be critically related to cognitive performance – both overexpression of PKMzeta and enriched environment improved cognitive performance in aged rats to a similar degree (33). However, in aged rats harboring a dominant negative version of PKMzeta, the loss of PKMzeta function prevented any improvement in performance after enriched environment (33), demonstrating the direct connection between PKMzeta expression levels and cognitive performance.

Overall, while many studies have utilized PKMzeta localization as a tool for identifying maintenance of spatial memory in the rodent dorsal hippocampus (20), few have examined the distribution of PKMzeta in the non-human primate brain or attempted to investigate the role of PKMzeta in the affective processing circuits of the ventral hippocampus (25).

Protein Kinase M Zeta and Affective Processing in the Hippocampus

Stress and Affective Processing in the Hippocampus

The hippocampus is a key structure in the limbic system with an anterior segment (homologous to the ventral region in rodents) that mediates affective response to contextual changes in environment, and a posterior segment (homologous to dorsal region in rodents) that modulates spatial navigation and memory (26). Extremely sensitive to stress, hippocampal function is closely associated with affective disorder, with extensive afferent and efferent connections to limbic structures that regulate mood and cognition (71), such as the amygdala and ventromedial prefrontal cortex (vmPFC) (95). In both clinical and animal models of depression, researchers have found decreased hippocampal volume, reduced hippocampal activity, hypothalamic-pituitary axis (HPA) hyperactivity (indicative of hippocampal dysfunction) and reduced neurogenesis in the dentate gyrus (27-30). In addition, significant subsets of patients with major depressive disorder present with cognitive deficits, which can be recapitulated by inducing hippocampal lesions in nonhuman primates (31, 32).

Given the large number of studies validating the role of the hippocampus in these processes, we aim to examine if PKMzeta expression can be used as a marker for

LTP and information storage integrity in these pathways. Two recently published reports support the relevance of PKMzeta in both cognitive and affective processing in the hippocampus. The first, by Hara et. al. (33) is particularly relevant for its use of the rhesus monkey as a model organism to examine the connection between PKMzeta subcellular localization and performance on cognitive and memory tasks. This study looked at perforant spines in the dentate gyrus of aged monkeys and found decreased density of synaptic GluA2 spines co-expressing PKMzeta compared to young monkeys – a deficit that correlated with impaired recognition memory (33). They found that the total densities of PKMzeta in dentate gyrus dendritic spines significantly correlated with both acquisition and performance on a delayed nonmatching-to-sample memory (DNMS) task, where monkeys with higher PKMzeta densities learned the task more rapidly and more accurately (33).

The second study utilized a rodent model of PTSD called re-stressed SPS to show that hippocampal PKMzeta can mediate anxiety and depressive behaviors related to this disorder (34). By modifying the classical SPS paradigm for modeling PTSD in rats to include a re-exposure to the original stressful context, the authors were able to more fully recapitulate PTSD symptomology in their subjects, resulting in impaired extinction of contextual fear, enhanced glucocorticoid negative feedback, and increased anxiety-like behavior (34). In re-stressed SPS rodent subjects, the authors observed a 3x increase in PKMzeta protein and PKMzeta mRNA transcripts in the hippocampus compared to control animals correlating with enhanced depressive or anxiety-like behaviors (34). Next, they found that inhibiting PKMzeta by administering ZIP into the hippocampus alleviated these depressive and anxiety-like responses in re-stressed SPS animals while not significantly affecting these

same measures in control animals (34), suggesting that PKMzeta-dependent LTP mediates these affective responses in the hippocampus.

Given these findings, we intend to utilize PKMzeta as a marker for LTP maintenance in the non-human primate hippocampus in order to characterize its role in cognition and affective processing. Our unique and extensive non-human primate brain tissue bank contains whole brain slices from subjects with a wide range of experimental and developmental conditions, most notably Early Life Stress (ELS) rearing paradigms. A series of studies has provided a mounting body of evidence that ELS may confer susceptibility to psychiatric disorder later in life (35). Given its importance in the pathophysiology of affective disorder, we aim to determine the effects of ELS on PKMzeta expression in the hippocampus.

A Putative Role for Protein Kinase M Zeta in the Pathophysiology of Affective Disorders

Early Life Stress is a Risk Factor for Affective Disorder

Early life stress (ELS) exerts a profound influence on behavior and physiology, putting victims at risk for depression, anxiety disorders and substance abuse (35-38). Indeed, ELS not only increases the risk for developing these disorders later in life, but may also increase the likelihood of comorbidity and resistance to clinical treatment (39-42). In order to examine the effects of early life adversity, our laboratory has developed and characterized the Variable Foraging Demand (VFD) model of ELS in bonnet macaques (43). In the VFD model, mother-infant dyads are presented with unpredictable variations in the ease of food accessibility. Using foraging carts, food is either readily accessible to the mother (low foraging demand)

or more difficult to obtain (high foraging demand). This unpredictability leads to maternal neglect of the infant, generating a translational model of human anxiety and mood disorder in the offspring (44-48). These subjects have psychological symptoms similar to those in patients with anxiety and mood disorders: blunted affect, diminished capacity for affiliative engagement, and social subordination (71). Using this paradigm, we have found significant neurobiological alterations in the central nervous system and peripheral tissues post-ELS conditioning that may underlie its capacity as a major risk factor for affective disorders into adulthood. These stress-related abnormalities in VFD versus non-VFD animals include: altered monoamine neurotransmission (43,45,49), impaired hippocampal neurogenesis (50), maladaptive behavioral response to acute stressors (51, 52), abnormal regional activation via MRI imaging (53), and neuroanatomical changes including reduced hippocampal size (54). Thus, by correlating hippocampal PKMzeta neuroimmunological signal intensities with previously obtained behavioral data from this same cohort, we can delineate the neurobiological phenotype of altered hippocampal processing in the NHP model of ELS -- effectively linking the distribution and activity of the molecular substrate of L-LTP to its behavioral and physiological manifestations.

Methods:

Non-Human Primates

Males:

Variable Foraging Demand Rearing Procedure: Mother-infant dyads were group-housed in pens of 5–7 dyads each, and stabilized for at least 4 weeks prior to VFD

onset (74). After infants reached at least 2 months of age, dyads were subjected to a standard VFD procedure that involved 8 alternating 2-week blocks in which maternal food was readily obtained (LFD) or more difficult to access (high foraging demand; HFD). During HFD conditions, the mothers had to find food by digging through clean wood-chip in a foraging cart. Food can be accessed by mothers through apertures in the sides of the foraging cart. In the control non-VFD condition, the mothers' food access was continuous. Adequate food was always available under both conditions, and there were no differences in weight between VFD and non-VFD mothers or infants. However, the unpredictability of foraging conditions putatively prevented VFD mothers from adequately attending to their infants. The early-life stress paradigm putatively occurs through the disruption of normative patterns of maternal rearing and infant attachment (45). After infancy, no experimental manipulations occur that may confound the VFD-rearing effects.

Females:

Adult female bonnet macaques were matched based on age, weight, social rank, and timing of menstruation, and randomized to a Control pen (n=6) or a Stress pen (n=6).

Separation Stress: Using the chronic stress paradigm developed in rhesus macaques, we exposed subjects in the Stress pen to social isolation for two days followed by social reunion on the remaining 5 days, repeated for a total duration of 15 weeks. The monkeys in the Control pen remained in social housing for those 15-weeks.

Fluoxetine: During this period, half the subjects in each pen (Control-Drug and Stress-Drug groups) were treated with the selective serotonin reuptake inhibitor

(SSRI), fluoxetine. In order to minimize the stress of administration, we used Prozac-weekly preparation (Eli Lilly Corp.), at a dose of 13.5 mg/kg infused via nasogastric tube (NGT) under sedation (ketamine 5 mg/kg and xylazine 1 mg/kg), once per week for 15-weeks. This dose was equivalent to a daily dose of 2 mg/kg of the drug. The remaining half (Control-Placebo and Stress-Placebo) received the same treatment with saline placebo via NGT.

Sacrifice: All groups were injected with the thymidine analog bromodeoxyuridine (BrdU) (100 mg/kg/day, I.V.) under ketamine/xylazine sedation for 5 days during week-7 of intervention (Stress/Control+Drug/Placebo) and then sacrificed by transcardiac perfusion with normal saline (500 ml/kg) followed by 4% paraformaldehyde (500 mg/kg) under deep anesthesia with pentobarbital (15 mg/kg, I.V.) on week-16 of interventions.

Behavioral Measures of Emotional Reactivity

Each animal was individually exposed briefly to an intruder, a fear-stimulus which is a variation of a previously detailed masked intruder paradigm (46).

Males were singly housed in holding cages and the “intruder” entered into the pen and stood about six feet in front of the cage making direct eye contact with the monkey. Emotional responsivity was rated by two experimenters blind to Rearing status using a 3-point scoring scale. To receive a score of one for intruder distress, subjects exhibited “confrontational” behaviors including: fang-baring, growling, direct eye contact, pilo-erection, ear flexing, cage shaking, and mouth gaping. The least distressed response received a score of “three” which was characterized by an animal that was minimally confrontational, averting eye contact, submissive and

displaying lip smacking, receding to the back of the cage and exhibiting “timidity” in response to the intruder. A score of two describes a subject with intermediate or alternating levels of both confrontational and timid behaviors. One hundred percent inter-rater reliability was observed for the intruder behavioral scoring system.

Genotyping:

Genotypes were determined by PCR amplification followed by size fractionation on a 2% agarose gel (75). Primers used were CAG CAC CTA ACC CCC TAA TGT CCC TG and GAT TCT GGT GCC ACC TAG ACG CCAG. Each 10 µl reaction contained 20 ng DNA, 1 µM of each primer, 1 M betaine, 10 µM dNTPs, and 0.1 unit KlenTaq polymerase, in manufacturer's PC2 buffer. Cycling parameters were 95°C for 5 min followed by 30 cycles at 95°/72° for 30 and 60 s respectively, using an MJR thermal cycler.

Neurohistology:

PKMzeta and BDNF Fluorescent: Left brain sections cut to 40µm thickness were rinsed three times (5min) in PBS, pH 7.4, transferred to .2% PBST to permeabilize. Sections were then incubated for 10 min with 0.1% glycine in PBS to quench free aldehydes, and rinsed with PBS for 10 min. The sections were then incubated in blocking buffer (5% normal goat serum in PBS) for 1 h and then overnight in primary antibody (either PKMzeta or BDNF @ 1 : 200) in blocking buffer (a–c), or blocking buffer alone as control (d). The sections were then rinsed in PBS, four times for 20 min each and incubated with goat anti-rabbit Alexa Fluor 488 (1 : 200) in blocking buffer overnight on a rotator at room temperature in the dark. The sections were rinsed three times in PBS, washed in distilled water and mounted with DAPI

Vectashield Mounting Medium on glass slides with Prolong Gold (Molecular Probes, Eugene, OR, USA). Negative control was hippocampal tissue without primary antibody.

PKMzeta Peroxidase: Sections of the left hippocampus was removed from cyroprotectant, heat-induced epitope retrieval boiling in citrate buffer ph 6.0, washed in .2% PBST, peroxidase activity quenched with 2% H2O2, blocked in Normal Serum, incubated in Primary Antibody @ 1:500 (Rabbit Anti-PKMzeta, Provided by Dr. Todd Sacktor, SUNY Downstate) for 72hrs, washed in .2% PBST, Incubated in Secondary antibody @ 1:200 (Biotinylated Goat-Anti Rabbit, Vector Labs), Incubated in avidin-biotin solution (ABC elite, Vector Labs), chromagen developed using DAB (vector labs), mounted on subbed slides, counterstained with Neutral Red (vector labs), dehydrated, cleared, and coverslipped with DPX mountant (Sigma). Negative control was hippocampal tissue without primary antibody.

Quantification of Average Signal Intensity:

Hippocampal slides were imaged with using an Olympus BS 52 research microscope fitted with the Olympus DP72 camera at 10x. In order to achieve appropriate images for quantification, images had to be processed before cell-counting program could be applied to optimize resolution, artifacts, and uneven fluorescence. These parameters were optimized for each group of control and experimental animals to account for differences in tissue quality. Images were analyzed using ImageJ to quantify PKMzeta or BDNF-labeled cells. Briefly, background noise was subtracted using averaged ROI measures from blank slide,

image was converted to grayscale, and each subregion of the hippocampus was traced out using rhesus monkey atlas (no bonnet atlas exists).

Mean PKMzeta Signal Intensity by hippocampal subregion: Adapted from Dr. Sacktor's methods, ROIs of each subregion were traced out, and mean signal intensity was calculated using ImageJ analysis software.

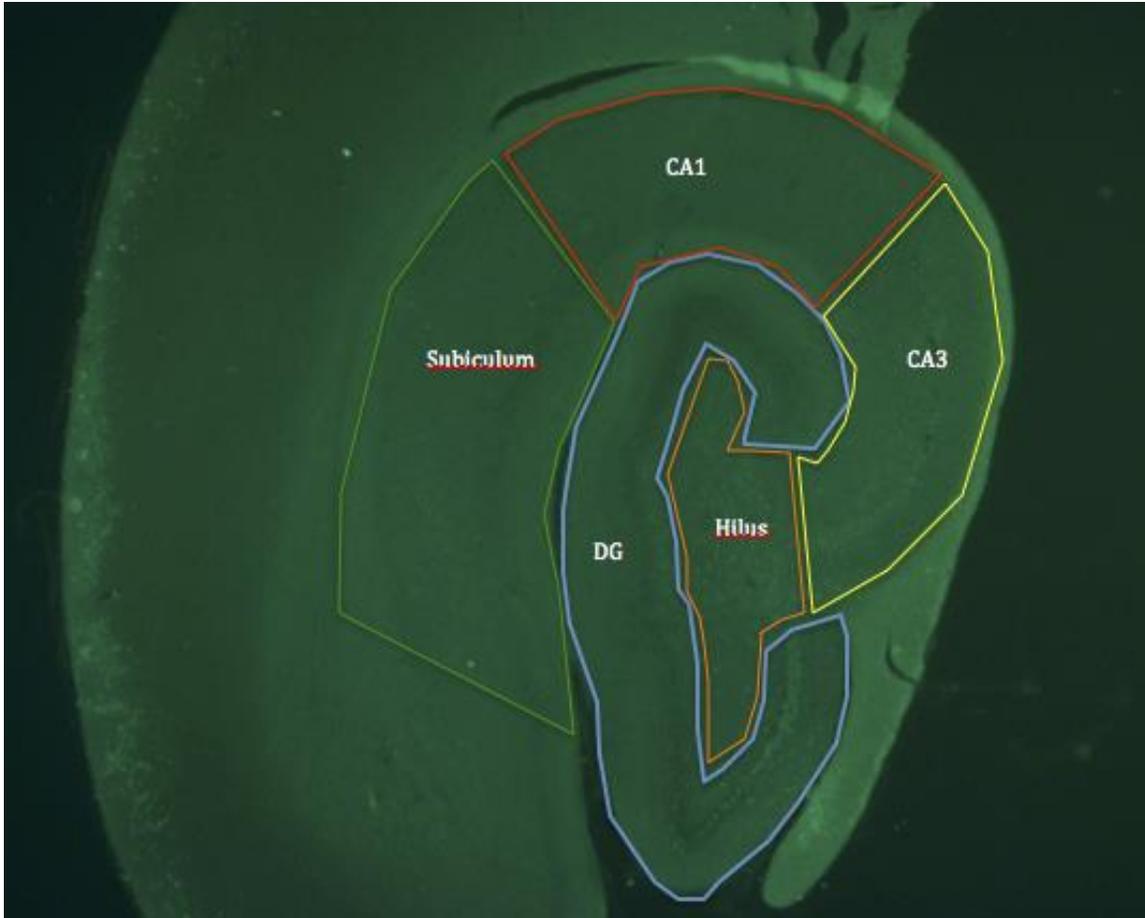


Figure 4: Example of ROIs traced for each hippocampal subregion. Image shows *Control Animal (Non-VFD, Long allele) PKMzeta IF test, showing localization of PKMzeta in green in hippocampus. Magnification 4x. Abbreviations: Dentate Gyrus (DG), Cornu Ammonis 3 (CA3), Cornu Ammonis 1 (CA1)*

ImageJ Method Validation: Protocols were validated using independent rater counts. Two independent raters, blind to experimental condition of animal,

conducted all measurements of total PKMzeta and BDNF counts manually. The total number of unambiguously labeled PKMzeta+ or BDNF+ cells in each sub-region was counted. For both counts, either ImageJ-generated or manual, the total number of counted cells was divided by the volume of the subregion (area of each region measured by ImageJ in $\mu\text{m} \times 40 \mu\text{m}$ thickness before post-mounting shrinkage of 50%–70%) (see Figure 5). Rates of labeled cells were expressed as a density per mm^3 of the given subregion. Images taken using an Olympus BS 52 research microscope fitted with the Olympus DP72 camera.

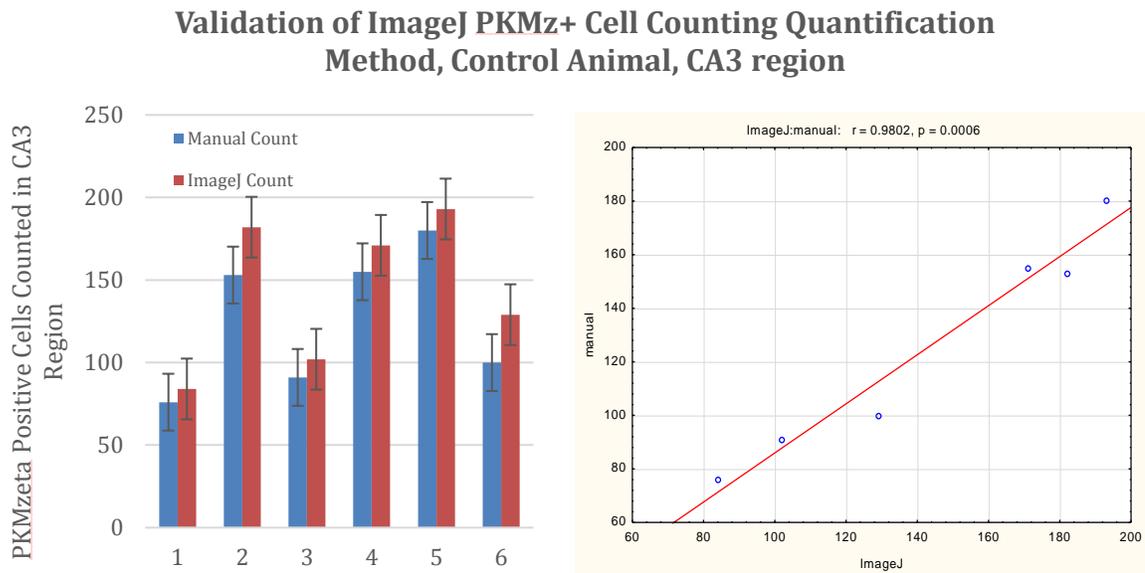


Figure 5: Validation of ImageJ PKMzeta+ Cell Counting Method Right graph shows comparison of manual count vs. ImageJ count over 6 separate specimens. Left graph shows correlation between two counting methods. ($p = 0.0006$)

Statistical Analysis:

Males: PKM ζ Intensity Signal in Subregions of the Nonhuman Hippocampus: A General Linear Model (GLM) was performed in the males using group [VFD short

(n=4), VFD long (n=2), and LFD long (N=4)] as the categorical variable, based on the subjects' serotonin transporter gene (STG) polymorphism status. No subjects were available for the "LFD -short group", which prohibited use of a factorial ANOVA. Subregions of the hippocampus were used as repeated measures. Post-hoc effects were examined using Newman-Keuls testing.

Females: A GLM was employed in normally reared females using concurrent stress versus no stress as the categorical variable and hilar PKMZETA mean signal intensity in the hilar subregion of the hippocampus as the dependent variable. Age [years: mean (SD) = 9.13 (3.20)] and weight [kg: mean (SD) = 8.58 (3.03)] were confounds and not used as covariates.

Relationship of Hippocampal Subregional PKMzeta to Behavioral Response to a Human Intruder: For males possessing PKMzeta mean signal intensity data, acute behavioral response to a human intruder, prior to sacrifice, was grouped into confrontational (N=3) or timid (n=6). Using a GLM, the categorical variable was defined by nature of acute behavioral response, confrontational versus timid, whereas subregions of the hippocampus were used as repeated measures.

Interaction of Concurrent Stress and Serotonin Transporter Gene Polymorphism:

Effect of SSRIs A GLM was performed using two categorical variables – Condition: No Stress + placebo (N=4), Stress + placebo (n = 7) and stress + fluoxetine (N=4) – whereas the second categorical variable was serotonin transporter gene polymorphism [short (N = 7) versus long (N = 8)] and using a factorial ANOVA, their interaction. The repeated measures were PKMzeta mean signal intensity for the hilar and CA3 hippocampal subregions.

Results:

Early Life Stress Interacts with Short Allele of Serotonin Transporter Gene to produce PKMzeta deficit across all Hippocampal Subregions in Male NHPs

Our first specific aim was to detect and quantify average PKMzeta signal intensity in the NHP hippocampus across differentially reared subjects. Using a subset of our male NHP cohort (N=10), we first utilized IHC to detect and quantify the average signal intensity of PKMzeta protein in postmortem NHP fixed hippocampal tissues. Using the methods described above, each hippocampal subregion was traced out according to the rhesus macaque brain atlas (Figure 4). We next compared the average PKMzeta signal intensity in six hippocampal subregions across three groups: Low Foraging Demand (LFD) + Long Allele Serotonin Transporter Gene (STG), VFD + Long STG, and VFD + Short STG. There was an overall group effect: (Group Effect $F(2, 7)=30.36$ $p=.0.00035$)—there was a marked reduction in PKMZETA signal mean intensity in the VFD-short allele group versus the other two groups, whereas the VFD-long allele and LFD-long allele were not statistically distinguishable. For every hippocampal subregion the identical pattern of differences was evident on post-hoc testing (indicated by the asterisks in the figure

below). Mean age of subjects (N=9; one value missing) = [mean (SD) = 8.88 (1.23) years] whereas mean weight of subjects [mean (SD) = 9.11 (2.83) kg]. No PKMzeta variable was significantly related to weight or age and therefore these were not used as covariates. Thus, there was a highly significant STG genotype x rearing effect – average PKMzeta signal intensity was severely reduced in all six hippocampal subregions in the VFD + Short STG group (Figure 6).

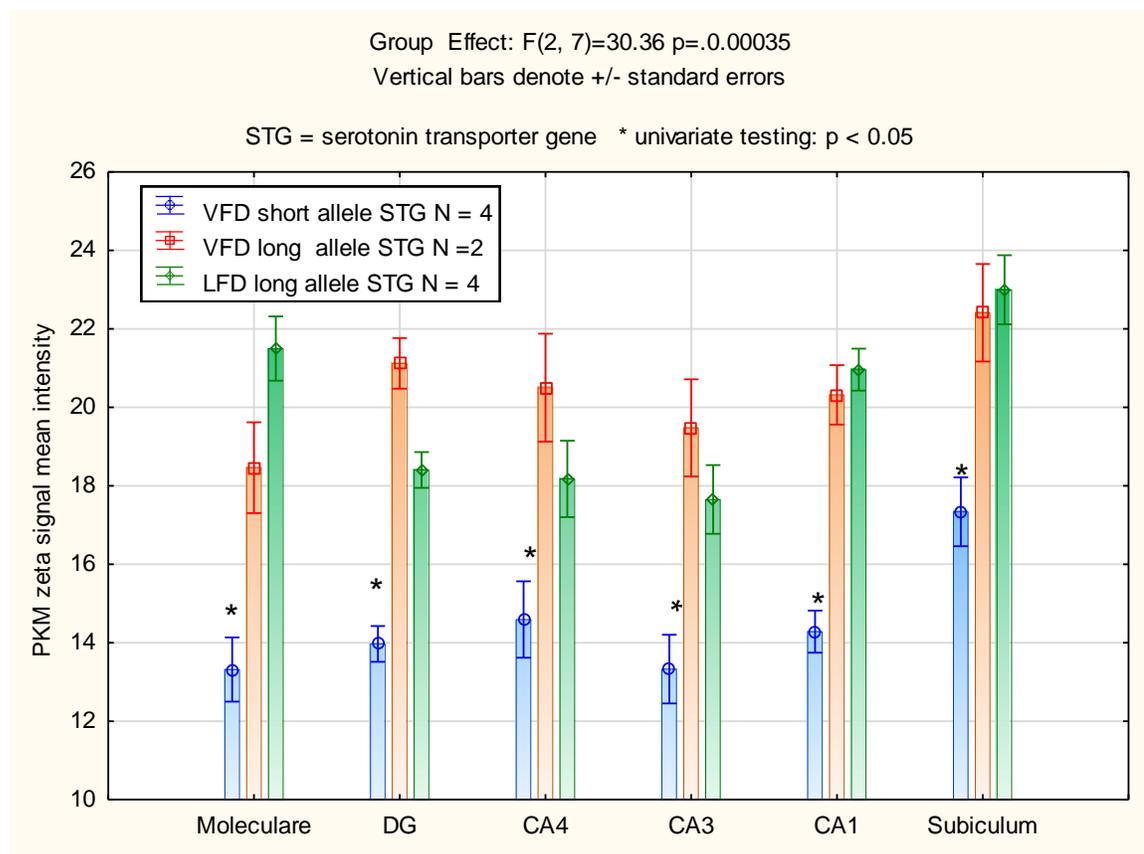


Figure 6: A combination of ELS + Short STG leads to reduced average PKMzeta signal intensity across all six subregions of the hippocampus measured.

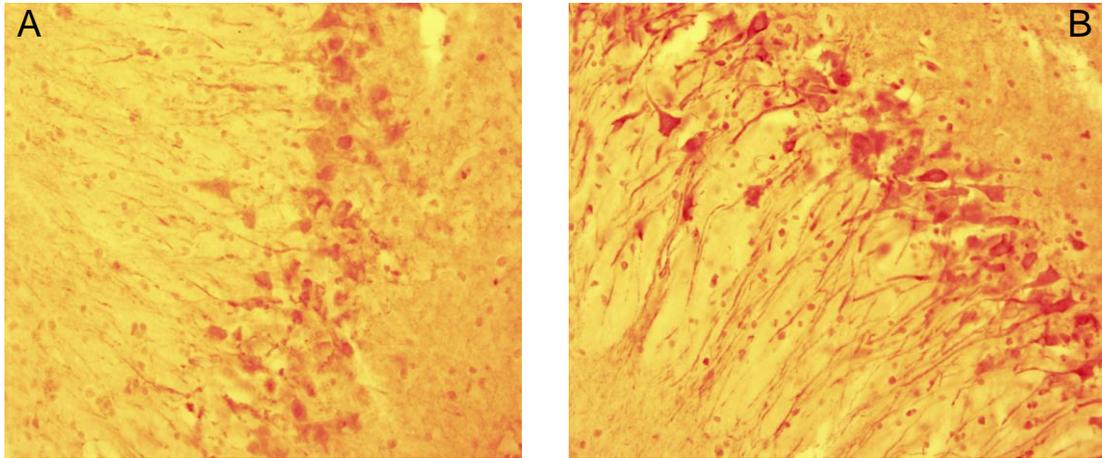


Figure 7: Reduced PKMzeta expression in VFD Short STG (A) Shows VFD Short STG Allele subject PKMzeta peroxidase test for intracellular localization, showing PKMzeta in brown in CA3 region. Magnification 40x. (B) Shows Control Animal (Non-VFD Long allele)

These results demonstrate that serotonergic signaling mediates a severe deficit in hippocampal PKMzeta expression in ELS subjects. Notably, neither ELS rearing nor Short STG allele alone was enough to affect PKMzeta expression. However, ELS in combination with the Short STG allele leads to a PKMzeta deficit. This is partially consistent with our main hypothesis, that ELS would negatively affect PKMzeta. However, this effect appears to be mediated through serotonergic alterations. Interestingly, in a previous study (77) we have reported that VFD animals experience abnormal serotonin neurotransmission to forebrain regions from midbrain serotonergic nuclei. Given these results, we posit that while this effect is not sufficient to reduce PKMzeta in adulthood on its own, additional perturbations to serotonin signaling due to impaired serotonin transporter function can cause significant attenuation in PKMzeta expression. These results then lead us to ask if reduced PKMzeta expression in VFD + Short STG animals was associated with changes in behavioral phenotypes.

Decreased PKMzeta Signal Intensity in VFD + Short STG subjects is Correlated with Behavioral Timidity

We next set to investigate if there is a relationship between hippocampal PKMzeta expression observed in the VFD + Short STG and our previously obtained behavioral measures in these subjects. We hypothesized that reduced PKMzeta would translate to changes in behavior that may stem from reduced LTP maintenance, and in turn, reduced capacity for hippocampal processing. When we examined average PKMzeta signal intensity across all 6 subregions of the hippocampus and compared these values to our behavioral measures of emotional reactivity, we found a significant correlation between reduced PKMzeta expression and behavioral timidity – a maladaptive, anxiety-like behavior in male bonnet macaques (Figure 8). An overall group effect was observed [$F(1,7) = 6.29, p = 0.04$] indicating that the confrontational nature of response was associated with greater PKMzeta signal overall in comparison to the timid group. In addition, a repeated measures by group effect was observed [$F(5,35) = 3.02, p = 0.022$]. Using post-hoc t-tests, significant group effects were noted for the zona moleculare, dentate gyrus and CA3 subregions of the hippocampus (see * in the figure below) whereas differences tapered off toward the subiculum. Group effects remained significant when covarying for weight whereas repeated measures x group effects remained significant when covaried for age. Timidity is described as a subordinate behavior, with the subject displaying low emotional reactivity to direct confrontation with an intruder. This is in contrast to the more typical male bonnet macaque response – a demonstration of confrontation and distress at an intruder. Furthermore, subjects

that displayed a confrontational response to intruder had significantly higher PKMzeta expression across all hippocampal subregions. Together, these results suggest that PKMzeta levels in the hippocampus are linked to affective processing – lower PKMzeta is associated with anxiety-like behaviors.

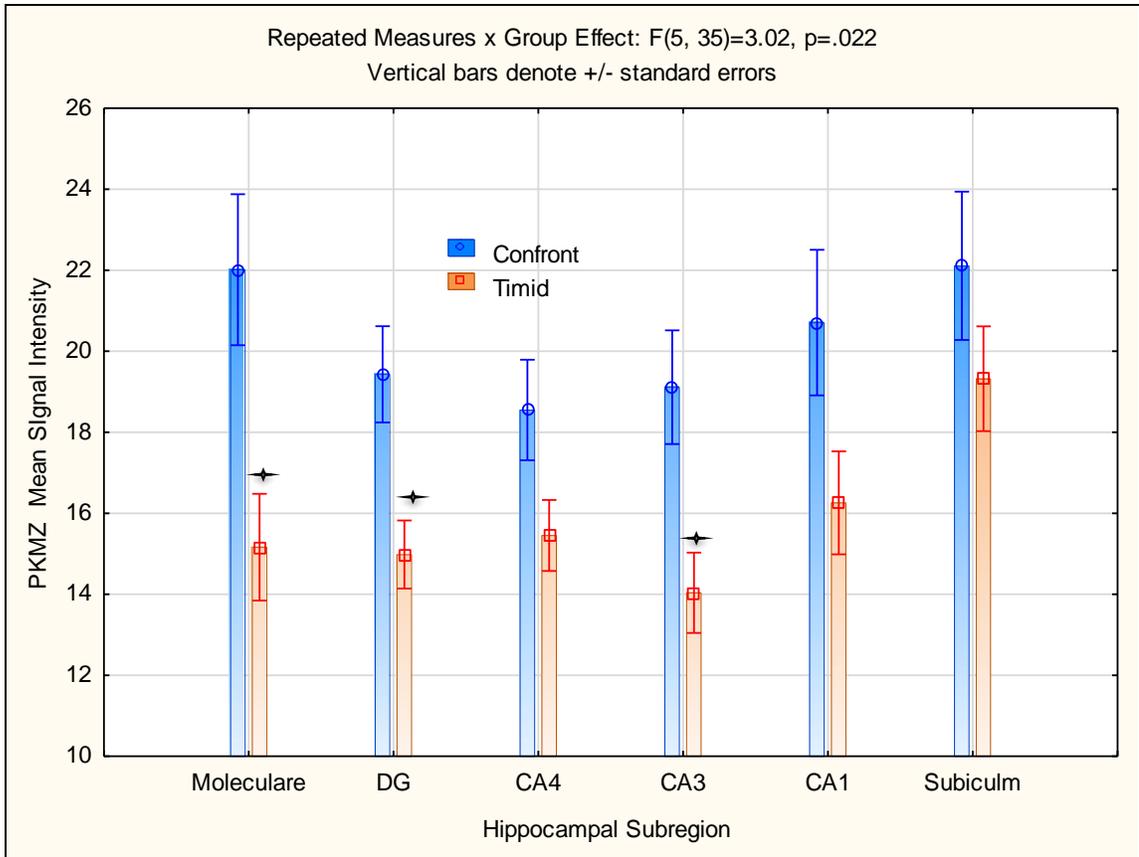


Figure 8: Reduced PKMzeta expression is associated with anxiety-like behaviors in response to intruder confrontation across all hippocampal subregions in NHP males

Chronic Adult Separation Stress Leads to Reductions in Average PKMzeta Signal Intensity in the Hilus Region of the Female NHP Hippocampus

In order to compare the relative effects of ELS versus chronic adult stress, we next investigated the effects of adult stress (N=4) on average PKMzeta signal intensity in

our female cohort of NHPs compared to non-stressed control animals (N=7). Interestingly, we found that concurrent, chronic adult stress severely reduces average PKMzeta signal intensity in the NHP hilus region of the hippocampus ($p = .018$) (Figure 9) in female subjects. Due to time constraints, we were not able to quantify all six subregions of the female NHP hippocampus. However, in our analysis with the male cohort, all six subregions followed the same correlative pattern, with no regional effect differences. For this reason, we used the hilus and CA3 regions in the female NHP subjects as representative ROI. The concurrent stress condition was associated with a significant reduction in hilar subregion of PKMZETA signal mean intensity (see stats in Figure 9). Overall, while ELS alone did not significantly affect PKMzeta expression later in life (Figure 6), concurrent stress does affect PKMzeta expression.

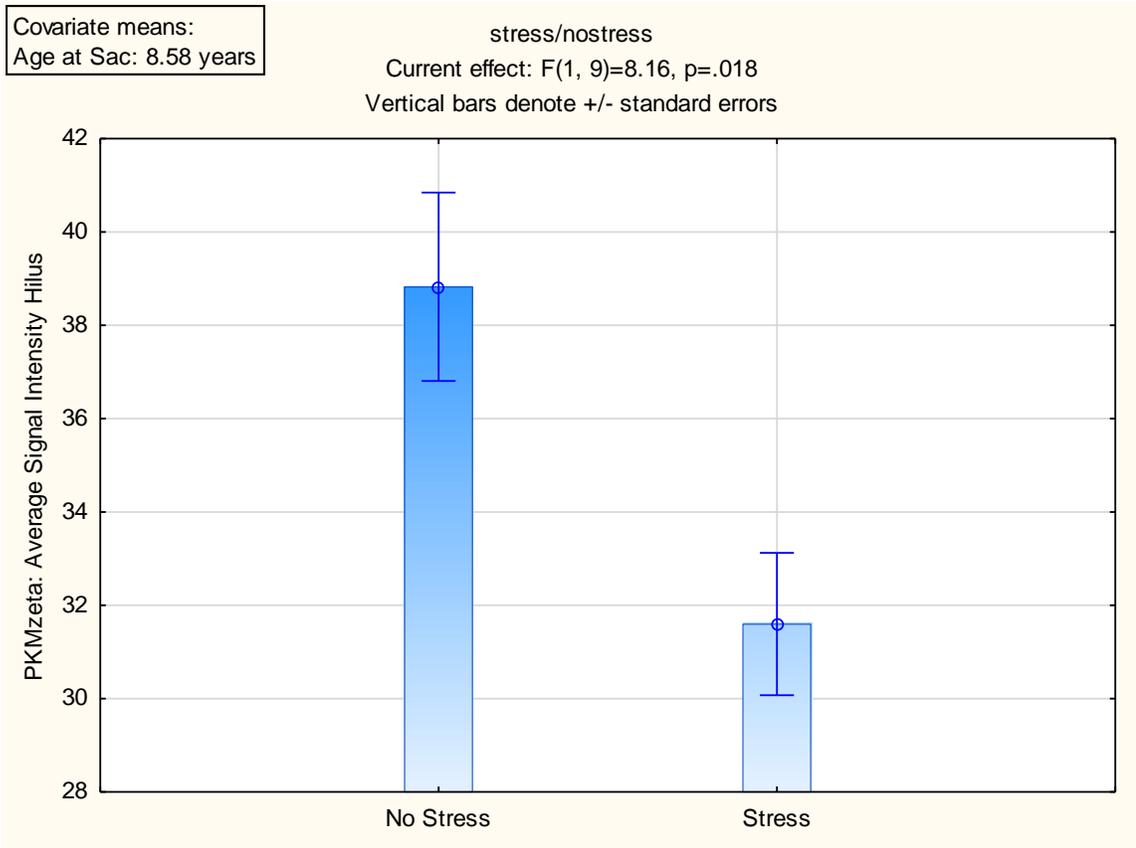


Figure 9: Concurrent Adult Stress reduces PKMzeta Average Signal Intensity in Female NHP Hilus
 Control animals did not undergo any stress paradigms and were normally housed. Stress animals were subjected to the standard separation stress paradigm, described in Methods.

The Short STG allele leads to decreased PKMzeta expression in the Hilus + CA3 Subregions of Female Stressed NHP Hippocampus compared to Long STG Allele Subjects

Having established that concurrent stress decreases PKMzeta expression in the hilus region of Long allele STG subjects, we next asked if STG genotype modulates PKMzeta expression in the female in a similar fashion as it did in the male cohort. Thus, we looked at PKMzeta signal intensity in the hilus and CA3 regions of female subjects that underwent concurrent adult stress and examined the effects of the Short STG allele vs. the Long STG allele. We found a highly significant correlation

between the Short STG allele and decreased PKMzeta signal in the hilus and CA3 regions of the hippocampus, indicating that in animals experience chronic adult stress, the Short STG allele mediates a further reduction in PKMzeta expression.

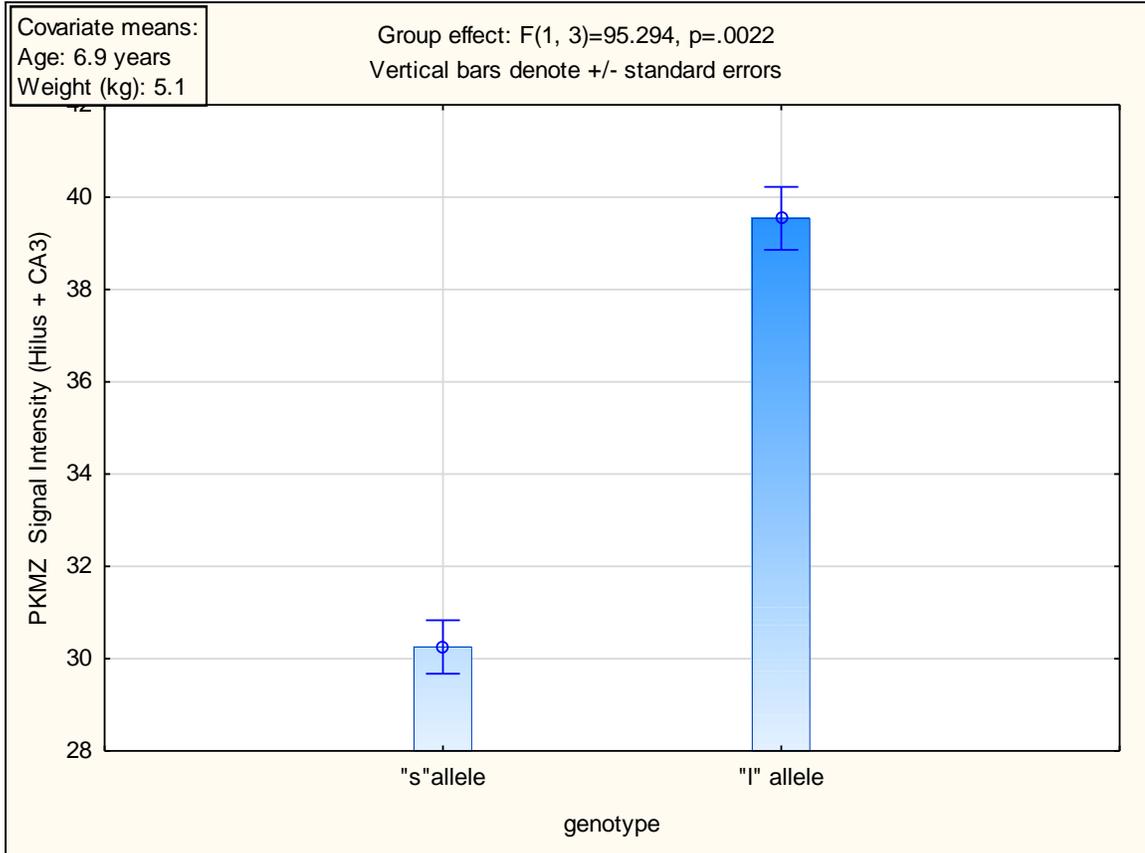


Figure 10: STG genotype Mediates Average PKMzeta Signal Intensity in Concurrently Stressed Adult Female NHP. Figure shows the PKMzeta signal intensity means for the Hilus + CA3 region, for both Short STG allele subjects and Long STG allele subjects.

Increasing Hippocampal Serotonin via Fluoxetine Increases PKMzeta

Expression in Hilus of Chronically Concurrently Stressed Short STG NHP

Female Hippocampus Compared to Placebo Stressed Short STG Subjects

Next we observed how PKMzeta expression would be affected by administration of the Selective Serotonin Reuptake Inhibitor (SSRI) Fluoxetine, a pharmacological

agent that blocks the serotonin transporter, increasing the amount of extracellular serotonin available and potentiating the signal strength of serotonergic transmission. Based on our findings, STG genotype plays a role in modulating PKMzeta expression after both ELS (Figure 6) and adult concurrent stress (Figure 10). We thus hypothesized that increasing the amount of serotonin available in the hippocampus via fluoxetine would increase the amount of PKMzeta signal detected. We obtained the average PKMzeta signal intensity of the Hillus + CA3 regions in NHP females and compared these measures across six groups: No Stress + Placebo (Short and Long STG allele); Concurrent Adult Stress + Placebo (Short and Long STG allele); and Concurrent Adult Stress + Fluoxetine (Short and Long STG allele). SSRI treatment, for the short allele, has no discernable effect whereas for the long allele, SSRIs appear to reduce PKMZETA signaling into the range of values observed with the short allele, and is significantly less (see asterisk) than the other two long allele groups – No Stress + Placebo and Stress + Placebo. The fluoxetine effects are counterintuitive in comparison to human data in which SSRIs appear to perform optimally in depressed subjects with the long allele, and not to reduce the expected value (1). The results are shown in Figure 11; on the left side of the graph, the measures for the Short Allele STG animals were consistent with our hypothesis. Administration of the SSRI fluoxetine increased PKMzeta expression to Control levels in the Stress + Short Allele STG group. Thus, increasing serotonin also increased PKMzeta signal intensity in these two hippocampal subregions in stressed, Short STG Allele subjects. Interestingly, we found a contradictory effect in the Long STG allele group. Although concurrent adult stress did reduce PKMzeta expression compared to control animals (as previously shown), subjects that received fluoxetine had severely reduced levels of PKMzeta. This was an

unexpected result, and suggests that fluoxetine has differential effects on PKMzeta expression in Short versus Long STG allele stressed subjects.

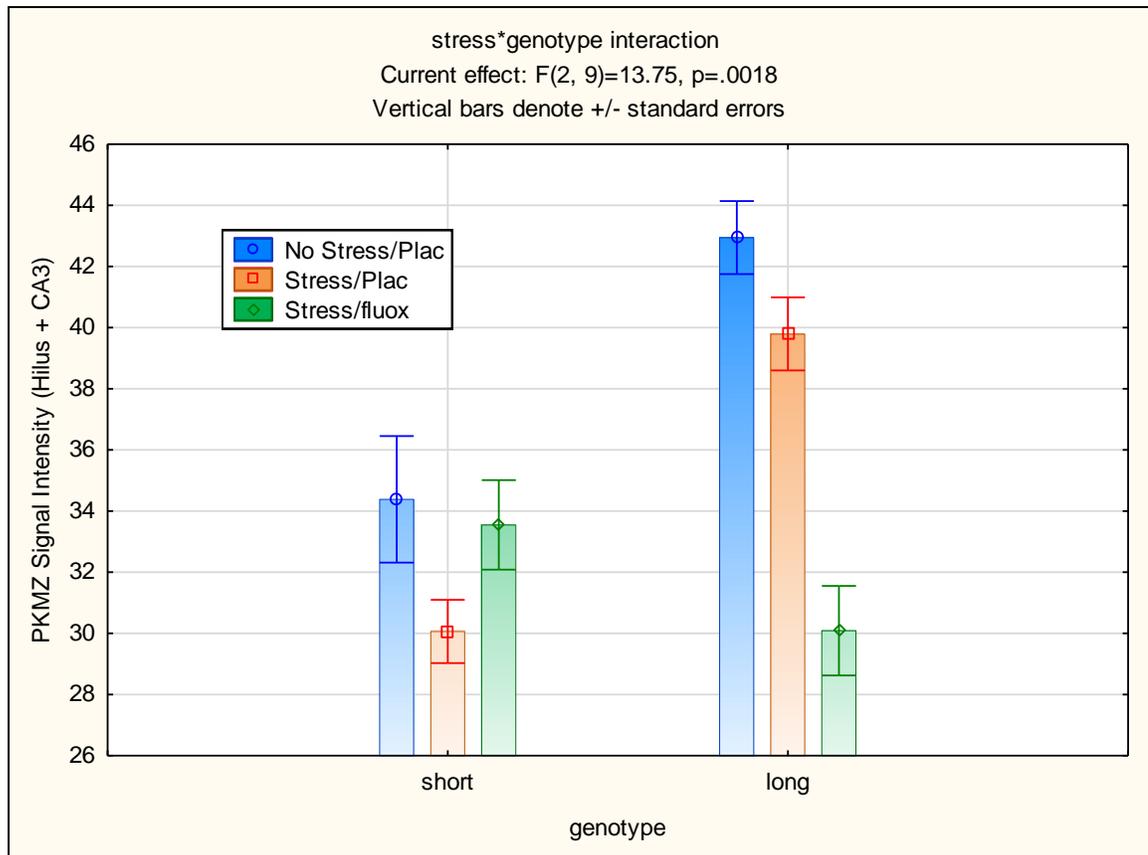


Figure 11: Fluoxetine increases PKMzeta expression in Hilus + CA3 compared to Placebo in Concurrent adult stress + Short STG allele female NHPs, but NOT in Long STG allele subjects.

Increasing Hippocampal Serotonin via Fluoxetine Increases BDNF Expression in Hilus of Concurrently NHP Female Hippocampus Compared to Placebo Stressed Subjects

Finally, we used IHC to detect and quantify BDNF average signal intensity in the female NHP hippocampus in order to determine if BDNF expression is linked to serotonergic signaling in these same subjects. In our previous study, we outlined a putative model for serotonergic dysfunction in the VFD NHP, in which attenuated

serotonin neurotransmission from the dorsal raphe nucleus to forebrain limbic regions resulted in compromised neurotrophic activity (77). This model is drawn from preclinical reports (59,60) that the main trophic factor responsible for maintaining neuronal growth and integrity, BDNF, is intimately connected with serotonin signaling. Thus, we hypothesized that increasing hippocampal serotonin via SSRI fluoxetine administration would result in increased BDNF expression. In Figure 12, we show that BDNF expression is significantly increased in the Hilus of concurrently stressed female NHPs with fluoxetine, compared to subjects given a placebo. Thus, when serotonin is putatively increased, BDNF is also increased. This data supports our hypothesis that low serotonin may lead to low BDNF levels, leading to reduced neurotrophic activity and LTP capacity through BDNF's mediation of PKMzeta.

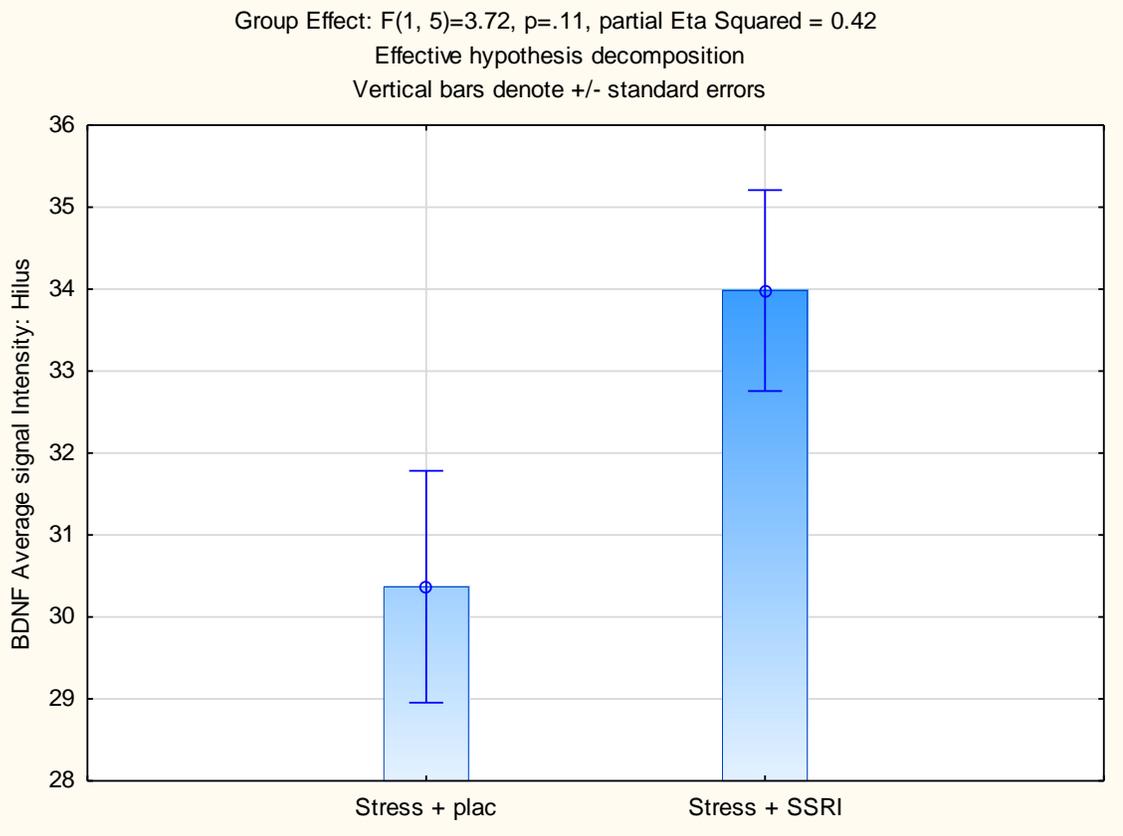


Figure 12: Regardless of STG genotype, SSRI Fluoxetine increases BDNF expression in concurrently stressed female NHP hilus, compared to subjects receiving placebo.

Conclusions

1. In Male NHPs, ELS + Short STG allele is correlated with PKMzeta expression deficit across hippocampus, a deficit associated with anxiety-like behaviors
2. In Normally-Reared Female NHPs, Adult Concurrent Stress Reduces PKMzeta Expression in the Hippocampus, an Effect Exacerbated by Short STG allele and Relieved by SSRI administration
3. Hippocampal PKMzeta Expression is Associated with Serotonergic Function in Both Male and Female NHP Cohorts, Revealing a Connection to Both ELS and Concurrent Adult Stress

4. SSRI treatment is Related to Increased BDNF expression in the Female NHP Hippocampus

Discussion

In Male NHPs, ELS + Short STG allele is correlated with PKMzeta expression deficit across hippocampus, a deficit associated with anxiety-like behaviors

In this study, we first present data indicating that ELS in combination with the Short STG allele negatively regulates PKMzeta expression in the male NHP hippocampus. Our initial hypothesis predicted that ELS would lead to decreased PKMzeta in the adult subjects – however we found that ELS alone was not sufficient to produce a PKMzeta deficit into adulthood, when PKMzeta expression was measured across six hippocampal subregions via IHC. Interestingly, in VFD subjects harboring the short STG allele, we found significant decreases in PKMzeta expression compared to VFD subjects with the Long STG allele or Control animals. These data indicate that PKMzeta expression in ELS animals may be mediated directly by serotonergic signaling. These findings extend our previous work showing gene by environment (short allele by ELS) effects in nonhuman primates exposed to VFD-rearing for reduced corpus callosum cross sectional area (77) and CSF CRF concentration elevations (78). Additionally, these reports are consistent with rhesus macaque work from other groups showing a similar short allele by ELS interactions (i.e., peer rearing) on HPA reactivity in response to stress (79-81). Several studies investigating the influence of risk factors for depression and other stress-related psychiatric disorders have found a role for the short allele of the serotonin

transporter linked polymorphic region (5-HTTLPR) in increased vulnerability to these diseases (82). These studies suggest that the short STG allele may be linked to increased sensitivity to stress, starting early in life (83). It has found that the short allele STG genotype is linked to increased anxiety-related behaviors beginning in development, (84), and continuing into adolescence and early adulthood, providing a valid developmental model for understanding how the 5-HTTLPR short allele might modulate stress reactivity.

In the current study, we find that the short STG genotype mediates reduced PKMzeta expression in the hippocampus when present in animals reared in a VFD ELS paradigm, suggesting that this allele may also be contributing to LTP maintenance and cognitive processes in these subjects. Supporting this hypothesis, we found that reduced PKMzeta expression in these same subjects was associated with timidity and anxiety-like responses to an intruder stressor task. This timid, maladaptive response to intruder stress has been found to be indicative of an increased susceptibility to various forms of affective disorder (85). Indeed, based on a wide body of work, a conceptual framework has been developed that suggests that differences in behavioral strategies to deal with this same intruder paradigm may have implications in terms of vulnerability to stress-related pathologies (85). Here, we report that STG genotype interacts with ELS exposure to reduce PKMzeta, which directly predicts the patterns and types of behavioral responses that NHP subjects exhibit to an acute stressor. This finding supports the hypothesis that PKMzeta expression in the hippocampus may be used as a marker of affective processing, and may represent a potential target for therapeutic applications in the treatment of psychiatric disorders related to stress.

In Normally-reared Female NHPs, Adult Concurrent Stress Reduces PKMzeta Expression in the Hippocampus, an Effect Exacerbated by Short STG allele and Relieved by SSRI Administration

Another intriguing result from this study was the finding that adult concurrent stress reduces hippocampal PKMzeta expression in normally-reared female NHPs. This finding is consistent with our previous work on this same female NHP cohort, showing a variety of stress-related neurobiological alterations (50). Notably, we have published data showing that adult concurrent stress leads to depression-like anhedonia behaviors in these animals (50) – a finding that supports our current finding of PKMzeta being linked to behavior (in male NHPs) and its role as a marker of affective processing. In that paper, exposure of monkeys (Stress-Placebo group, n=3) to repeated social separation stress led to gradual increases in behavioral scores for anhedonia (a behavioral composite of collapsed postures, inactivity, and blank stares) and decreases in scores for hierarchy (total subordinate behaviors subtracted from total dominant behaviors) (50). The bonnet macaques exposed to repeated social separation stress displayed increases in anhedonia that involved a cluster of symptoms typically seen in depressive monkeys (86), including macaques (87). Critically, this behavioral profile possesses significant face validity as an analog of clinical anhedonia, a core symptom of major depression (88). The increases in the anhedonia scores were accompanied by decreases in hierarchy scores. Social subordination is a hallmark of both chronic anxiety and depression in monkeys (86). This behavior of the NHPs in the setting of repeated social separation stress parallels clinical depression, as interpersonal loss is the predominant trigger of depression in humans (88) and chronic stress is a major epidemiological risk factor for major depression and chronic anxiety disorders (88).

Building on this finding, we next turned to compare the effects of the STG allele in concurrent adult stress subjects. Based on our previous findings in the male NHP cohort, we predicted that the short STG allele would combine with concurrent adult stress to further decrease PKMzeta expression in the hippocampus of stressed females.

One of the most important findings in this report is the contribution of STG transporter genotype in mediating PKMzeta expression in both ELS (male) and normally-reared animals subjected to concurrent adult separation stress (female). As discussed above, ELS + short STG allele reduced PKMzeta expression, and was correlated with anxiety-like behaviors. In our female NHP cohort, we found that while concurrent adult stress alone affected PKMzeta expression in the hippocampus compared to control animals. Investigating further, we looked at PKMzeta expression in Long vs. Short STG allele animals in this cohort of concurrently stressed subjects, we found that, as in the males, Short STG allele exacerbated the negative effects of stress on PKMzeta hippocampal expression, even further reducing PKMzeta expression in both subregions examined (hilus + CA3) below levels found in Stress + Long STG allele subjects.

In several previous studies, we have reported that administration of the SSRI fluoxetine improves several depression-like symptoms in this cohort of bonnet macaque females (50), including measures of neurogenesis and anhedonia-related behaviors (50). Thus, we utilized this group of SSRI-treated animals in order to compare PKMzeta expression in these animals versus stressed, placebo-treated animals. We reasoned that due to our previous findings that serotonin transporter function is clearly tied to PKMzeta expression in both ELS and normally-reared concurrent adult stress conditions, increasing serotonin via SSRI treatment would

increase PKMzeta expression compared to the placebo group. Indeed, we found that in short STG allele females that were subjected to concurrent adult stress, treatment with SSRIs increased PKMzeta expression over the levels seen in the placebo-only stressed group. It is important to note that we did not see the same effect in stressed animals harboring the Long STG allele – that is, Stress + Long STG allele + Placebo subjects had higher PKMzeta expression than Stress + Long STG allele + SSRI. This was an unexpected result, and not consistent with our hypothesis. However, in a recent study (unpublished results) we have seen several counterintuitive results in this same cohort of female NHPs between the placebo and SSRI groups, indicating that fluoxetine affects different groups in unpredictable ways. Much further analysis and study is needed in order to fully characterize the effects of SSRIs in differential STG genotype subjects. Nevertheless, there was a significant effect in the Short STG allele group with PKMzeta expression comparing Placebo vs. SSRI conditions. Given these results, we have begun to develop a putative model for the connection between serotonergic signaling and PKMzeta expression in the NHP hippocampus in stress conditions.

Hippocampal PKMzeta Expression is Associated with Serotonergic Function in Both Male and Female NHP Cohorts, Revealing a Connection to Both ELS and Concurrent Adult Stress

Previously, in a NHP model of ELS, our laboratory has found evidence of an intriguing mechanistic connection between serotonergic neurotransmission to the hippocampus and PKMzeta-mediated LTP, putatively bridged by the highly important neurotrophin brain-derived growth factor (BDNF). This relationship between the

serotonin system, PKMzeta, and BDNF may explain many of the neurobiological symptoms observed post-ELS, and elucidate the pathophysiology of affective disorder. One of the major consequences of VFD rearing we have focused on in our research is dysfunctional serotonin neurotransmission. The serotonergic system is crucially important to the pathophysiology and the treatment of mood disorders, and its disruption in ELS may underlie the increased risk of depression and anxiety (55, 45). Accordingly, one genetic risk factor that contributes to increased vulnerability to depression is the SLC6A4 serotonin transporter gene, which is critical to the regulation of serotonin function throughout the brain. The “s” allele encodes an attenuated promoter segment, and is associated with reduced transcription and functional capacity of the serotonin transporter (89). In bonnet macaques, this endogenous single nucleotide polymorphism is conserved, allowing us to study the effects of serotonin neurotransmission in our ELS animal model. In our cohort of VFD and non-VFD subjects, we have reported a gene x environment effect for both CSF CRF concentration, and amygdala volume (77,78). Thus, the short allele of the 5-HTTLPR is linked with an increased risk for developing symptoms associated affective disorder after ELS, pointing to the important role of serotonergic signaling in modulating these processes. Additionally, we have found elevated cisternal, periraphe CSF 5-HIAA in three separate cohorts of VFD animals compared to normally reared controls, which we hypothesized reflects a form of serotonin neurotransmission dysfunction specific to ELS (54). ELS persistently activates stress-mediated circuitry, compromising these networks’ responses to future stress (1,56). A particularly relevant example of such circuitry is the glutamatergic afferents emanating from Layer V of the medial prefrontal cortex to stimulate serotonin release in the midbrain raphe nuclei (57). Stress-induced excess serotonin release may

trigger an endogenous negative-feedback mechanism mediated by serotonin 5-HT1A autoreceptors in the raphe itself (57,58) reducing serotonin projections to critical forebrain areas such as the hippocampus. In support of this model, we have previously found that high VFD cisternal 5-HIAA measures correlated with reduced right hippocampal volume, indicating downstream neurotrophic effects (77). The mechanisms responsible for these effects, however, are not currently understood. In the current report, we suggest that stress, both during early life and in adulthood, negatively affects serotonin signaling, leading to downstream consequences, particularly with regard to neurotrophic activity.

SSRI treatment is Related to Increased BDNF Expression in the Female NHP Hippocampus

The current study aimed to determine if PKMzeta, and by extension, LTP maintenance, has a role in mediating the neurobiological symptoms of ELS-induced affective disorder. Preclinical studies of the effects of stress suggest a minimum of four mechanisms by which hippocampal atrophy may result from stress: dendritic atrophy, synaptic remodeling (synaptic overproduction and pruning), increased neuronal death, and decreased rates of neurogenesis (90-92), all processes that are intimately tied to BDNF and serotonergic signaling (59,60). Indeed, hippocampal volume is closely reliant on the presence of neurotrophic factors such as brain-derived growth factor (BDNF) to maintain neuronal growth and survival (59). Importantly, this key growth factor has a synergistic relationship with the serotonin system, regulating serotonergic neuronal survival even while serotonin-activated second messengers modulate BDNF production (59-62). In fact, the hippocampus is particularly vulnerable to changes in BDNF level; missense polymorphisms in the

BDNF gene are directly correlated with reduced hippocampal volume (63). These data imply that reduced serotonin neurotransmission at the hippocampus is directly linked to reduced volume through BDNF production (60).

BDNF has a Central Role in Memory and Regulates PKMzeta During LTP

As described above, our previous research has suggested a connection between serotonergic dysfunction precipitated by ELS and reduced neurotrophic activity in the hippocampus, which is mediated by BDNF. BDNF has a well-documented role in mediating LTP in the hippocampus (65) and other regions involved in memory, such as the amygdala (64). Importantly, BDNF has been found to be required not only for acquisition but also for persistent storage of long-term fear memory (66). In fact, BDNF can rescue LTP even when protein synthesis is inhibited, demonstrating that the factor is both necessary and sufficient for expression of synaptic potentiation (67). A recent study by Sacktor et al. (68) reported a mechanistic explanation for this finding – in acute mouse brain slices, they showed that inhibition of PKMzeta reversed this BDNF-dependent form of LTP. Molecular analyses revealed that BDNF can maintain levels of PKMzeta at the synapse through directly associating with it, and preventing its degradation, allowing for continued trafficking of AMPARs to the post-synaptic density even when PKMzeta cannot be synthesized (68). Additional studies have confirmed this relationship between BDNF and PKMzeta, demonstrating that BDNF enhances PKMzeta expression, controls PKMzeta nascent synthesis via translation machinery mTORC1, and also enhances PKMzeta phosphorylation and activation (69).

In support of our model of BDNF bridging the connection between serotonin and PKMzeta, here we present evidence that BDNF levels are directly connected with

serotonergic levels- increasing extracellular serotonin and serotonergic signaling via SSRI administration increased BDNF expression in the hippocampus of NHP female subjects for both control and stress conditions.

Taken together, these data lay the foundation for a model in which ELS-related serotonergic dysfunction leads to reduced BDNF in the hippocampus, compromising neurotrophic activity and negatively affecting regulation of PKMzeta, the only known molecule to be necessary and sufficient for LTP and long-term memory storage (70). While many of the structural and neurochemical consequences of ELS have been studied, it is not well understood exactly how these changes might translate to the deficits in cognitive and affective function associated with mood disorders. By connecting our previous findings of serotonergic dysfunction in the VFD model to the neurotrophic hypothesis of depression, we conclude that PKMzeta expression may be negatively affected post ELS in subjects with compromised serotonergic signaling, and this effect may lead to reduced cognitive and affective processing.

Given these findings, we propose that studies in nonhuman primates provide an essential tool to understand the molecular mechanisms by which ELS interacts with the short allele to modify vulnerability to depression and anxiety in humans.

Limitations of the study include the relatively small number of subjects, particularly for the gene by environment component. However, we acknowledge the preliminary nature of these findings of the interaction of ELS and the serotonin transporter gene in PKMzeta expression contributing to maladaptive, anxiety-like behaviors.

Nonetheless, the current study adds to a growing body of data that suggests ELS is associated with a concert of biological changes that are evident across the lifecycle.

The VFD paradigm provides an important animal model to elucidate the cellular and

molecular mechanisms by which ELS causes clinically relevant neurobiological alterations in a translational NHP model.

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