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Brain-derived neurotrophic factor (BDNF) signaling mechanisms that regulate learning and memory in rats

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Abstract: Brain-derived neurotrophic factor (BDNF) is a widespread neurotrophin implicated in cellular processes underlying memory, socialization, and emotion; however, little is known about how genetic modification of BDNF interacts with BDNF modulators (e.g., hormones) to regulate BDNF-TrkB signaling. BDNF binds to the TrkB receptor and triggers signaling cascades underlying neuroplasticity, learning, and memory. Inhibitory phosphorylation of one downstream target of the BDNF cascade - GSK3β – is also linked to memory improvements. The Val66Met allele – a single nucleotide polymorphism (SNP) in the *Bdnf* gene - produces impairments in regulated BDNF release from the cell, as well as deficits in spatial memory related to the hippocampus and increases in anxiety-like behavior associated with the striatum in rodent models. The Met allele causes deficits in regulated BDNF release by impairing the trafficking of this neurotrophin to the correct vesicles; however, prior literature has shown increased BDNF and TrkB levels in the hippocampi of Met/Met rodents, indicating a possible compensatory mechanism for said deficits. The goal of this investigation was to explore if the Met allele, when coupled with estrogen, a known promoter of BDNF signaling, suppresses or increases downstream phosphorylation of GSK3β in the striatum. Female middle-aged Val/Val and Met/Met rats were ovariectomized, treated with either vehicle (sesame oil) or 17β-estradiol (EB), and subjected to an object recognition paradigm, which assesses striatum-sensitive response memory. Semiquantitative western blotting was performed on striatal samples from these rats to detect phospho-GSK3β vs. total GSK3β protein signal (i.e., to measure inhibition of GSK3β). GSK3β phosphorylation trended towards being greater in the striata of Met/Met rats than those of the Val/Val rats. However, EB treatments did

not alter GSK3β phosphorylation in either Val/Val rats or Met/Met rats. The apparent trend of Met-related increase in GSK3β phosphorylation in the striatum supports the hypothesis that the Met allele may boost BDNF and TrkB levels in compensation for impairments in BDNF secretion. Furthermore, the lack of difference in GSK3β phosphorylation between EB- and vehicle-treated Met/Met rats and between EB- and vehicle-treated Val/Val rats suggests that both genotypes are insensitive to the BDNF-enhancing effects of estrogens, possibly due to increases in regulated BDNF release in response to learning during the dOR task, resulting in saturated BDNF release that could not be enhanced by estradiol.

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Table of Contents

List of Illustrative Materials

Introduction

Rationale. In this project, I explored how a CRISPR-generated known variant of the gene encoding the neurotrophin BDNF – namely, the Met substitution - can interact with or alter the neuromodulatory effects of estradiol to shape signaling cascades downstream of BDNF and the receptor TrkB in the striatum. Ovariectomized Met carrier rats were compared to those of the predominant Val genotype after being treated with either estradiol or a vehicle control to examine the effects of the steroid on the activation status of GSK3β, a downstream enzyme in the BDNF-TrkB pathway.

BDNF is a desirable target for study in this investigation, as it is a highly prevalent neurotrophin that underlies the neuroplastic changes involved in memory formation and maintenance, including long-term potentiation and long-term depression (Bramham & Messaoudi, 2005; Miranda et al., 2019). Augmented expression of BDNF in the hippocampus has been connected to improvements in hippocampus-sensitive learning and memory (Tyler et al., 2002; Yamada et al., 2002; Yamada & Nabeshima, 2003). Furthermore, BDNF has been implicated in social and emotional functions, as BDNF deficits in the striatum lead to social impairments, as well as higher anxiety- and depression-related phenotypes in rodent models, rescued only by BDNF treatment (Blechkarz-Klin et al., 2018; Marais et al., 2009).

BDNF binds to the TrkB receptor, which activates PI3K, ILK, and/or p70 S6K, all of which phosphorylate and inhibit GSK3β downstream (Beurel et al., 2015; Fig. 1A). GSK3β is an important target for exploration in this pathway, as its activation state shapes synaptic and neural processes underlying cognition. For instance, experimental hyperactivity or overexpression of this enzyme in animal models and cell cultures has

resulted in various pathologies, including tau hyperphosphorylation, progression of neurofibrillary tangles (NFTs), neuronal apoptosis, synaptic loss, LTP deficits, neurogenesis deficits, and cell death (Bhat et al., 2004; Bhat et al., 2000; de Barreda et al., 2010; Hanger et al., 1998; Hooper et al., 2007; Liu et al., 2017; Kimura et al., 2007; Kimura et al., 2010; Pardo et al., 2016), as well as deficits in memory associated with the prefrontal cortex and hippocampus (Albeely et al., 2022; Barker & Warburton, 2011). These abnormalities are historically rescued when GSK3β is experimentally phosphorylated by treatment with inhibitors (Beurel et al., 2015; Choi et al., 2011; Chuang et al., 2011; Franklin et al., 2014; King et al., 2014; King & Jope, 2013; Leeds et al., 2014; Scheuing et al., 2014). The phosphorylation state of GSK3β is therefore not only a downstream indicator of the release and binding of BDNF to TrkB, but it also underlies a myriad molecular and cognitive processes. The activation state of GSK3β thus serves as crucial target for further exploration into BDNF-TrkB signaling and its implications in the neuroplastic underpinnings of cognitive function.

The implication of BDNF signaling – and ultimately, the phosphorylation state of GSK3β – in cognition, socialization, and emotion necessitates my exploration into how modifications of BDNF – including those at the genetic level – can impair these functions. One such genetic alteration – the Met allele – involves the substitution of a methionine in the place of a valine at codon 66 in humans and 68 in rats due to a single nucleotide substitution in the *Bdnf* gene. This allele, which alters trafficking of BDNF (Chen et al., 2005; Szarowicz et al., 2022), is highly prevalent among humans (Mercado et al., 2021; Petryshen et al., 2009). The presence of the Met allele has been linked to performance deficits in verbal, working, and spatial memory tasks (Dempster et al.,

2005; Hansell et al., 2007; Goldberg et al., 2008; Miranda et al., 2019), as well as alterations in experience-dependent plasticity changes (Kleim et al., 2006) and increased stress susceptibility (Yu et al., 2012). The high prevalence and significant implications of the Met allele in molecular and cognitive functions thus necessitates further investigation into 1) how these deficits are mediated at the biochemical level in the BDNF-TrkB signaling pathway by monitoring the activation state of GSK3β, and 2) if said deficits can be experimentally mitigated or even reversed.

Sex steroids like estrogens are well-established enhancers that shape neural physiology and behavior via BDNF signaling mechanisms. Evidence suggests that estrogens transactivate the TrkB receptor (Arpino et al., 2008; Barletta et al., 2004; Cikla et al., 2016; Huang & McNamara, 2010; Spencer et al., 2008; Spender-Segal et al., 2011; Wang et al., 2016) and boost mature and pro-BDNF expression by increasing histone H3 acetylation at the *Bdnf* gene promoters pII and pIV (Kiss et al., 2012; Luine & Frankfurt, 2013; Pan et al., 2010). These steroids can also modulate various types of learning and memory in rats in a dose-, task-, and age-dependent manner (Korol & Kolo, 2002; Pisani et al., 2012; Wang et al., 2008; Zurkovsky et al., 2006; Zurkovsky et al., 2007; Zurkovsky et al., 2011).

This evidence therefore raises the question of whether estrogens can rescue dampened regulated BDNF release in Met carriers, thereby providing a potential therapeutic avenue for treatment of Met-related memory and neuroplasticity dysfunctions, or if Met carriers are simply insensitive to the BDNF-boosting impacts of estrogens due to said deficits in regulated BDNF release. Therefore, the premise of my project is to test for BDNF-TrkB signaling – in the form of downstream GSK3β

phosphorylation – in the striata of Met vs. Val homozygote rats given either a control treatment or estradiol.

BDNF and its downstream targets. The neurotrophin brain-derived neurotrophic factor (BDNF) is abundantly expressed and broadly distributed throughout the mammalian central nervous system (Aid et al., 2006; Barde et al., 1987; Schinder & Poo, 2000; Yamada & Nabeshima, 2003). The mature BDNF protein (mBDNF) is released on both a constitutive and activity-dependent basis (Balkowiec & Katz, 2002; Goodman et al., 1996; Guo et al., 2018). Regulated, activity-dependent secretion of BDNF results from high frequency neuronal stimulation and excitation (Guo et al., 2014; Goodman et al., 1996). In contrast, constitutive secretion occurs regardless of neuronal activity, generating a more gradual increase in released BDNF (Guo et al., 2018). This increase is thought to drive neuronal processes like dendritic spine growth and long-term potentiation (LTP) (Guo et al., 2018). Both constitutive and regulated BDNF release mechanisms occur in cells (neurons and glia) in the hippocampus, cortex, amygdala, and striatum (Brigadski & Leßmann, 2020), as well as in endothelial and muscular cells (Cefis et al., 2020).

BDNF undergoes several steps prior to being secreted and released in its mature form. When translated from the *Bdnf* gene in the nucleus, pre-pro-BDNF first moves to the endoplasmic reticulum, where it undergoes cleavage of the pre-sequence. This leaves pro-BDNF, which consists of two domains: the pro-domain and the mature BDNF (mBDNF) domain. Pro-BDNF passes through the trans-Golgi network and undergoes cleavage to release mBDNF from the cell (Aid et al., 2006; Fig. 3A). Mature BDNF then binds to and dimerizes tyrosine kinase receptor B (TrkB) (Haniu et al., 1997; Yamada &

Nabeshima, 2003) to activate phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), or Ca^{2+}, pathways known to be involved in learning and memory and underlying neural plasticity.

The PI3K-mediated pathway results in the phosphorylation and activation of one of three downstream effectors: Protein kinase B (Akt) (Yamada et al., 1997; Yamada et al., 2001), integrin-linked kinase (ILK), and p70 ribosomal protein S6 kinase (p70 S6K) (Fig. 1B). The ERK/MAPK and Ca^{2+} pathways activate cycle-AMP response element-binding protein (CREB), a transcriptional factor that in turn regulates BDNF expression (Rosa & Fahnestock, 2015). Activation of Akt by PI3K promotes glucose use (Burkhalter et al., 2003) and angiogenesis (Shiojima & Walsh, 2002). ILK can also activate Akt via phosphorylation (Troussard et al., 1999). p70 S6K, which can also be phosphorylated by Akt (Fig. 1B), activates multiple different downstream effectors that regulate gene transcription, cell motility, synaptic strength, cell survival, and various other processes that are involved in memory acquisition and storage (Bahrami-B et al., 2014; Bekinschtein et al., 2014). Akt, ILK, and p70 S6K can all phosphorylate and inhibit glycogen-synthase-kinase-3β (GSK3β) (Fig. 1B) to promote neuronal survival and glycogen synthesis (Kim et al, 2000; Korol et al., 2013; Rosa & Fahnestock, 2015).

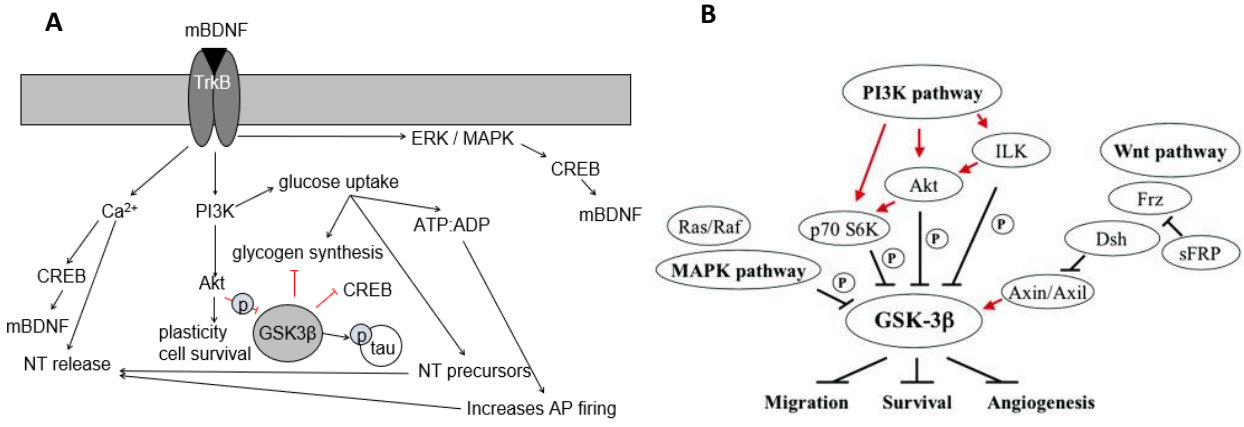


Fig 1. *GSK3β is a key downstream target in the BDNF-TrkB-PI3K-Akt signaling pathway, as well as in other pathways, governing mechanisms like plasticity, cell survival, glycogen synthesis, and tau phosphorylation*. (A) Downstream of BDNF binding to TrkB, the targets PI3K, ERK/MAPK, or Ca^{2+} can be activated. PI3K activate Akt, which phosphorylates and inhibits GSK3β. The mitogen-activated protein kinase (MAPK) and Ca^{2+} pathways activate transcriptional factor CREB to promote expression of mature BDNF. (B) P13K can also activate integrin-linked kinase (ILK) and/or p70 S6K, which can phosphorylate and inactivate GSK3β. Moreover, in the Wnt signaling pathway, frizzled (Frz) and disheveled (Dsh) proteins block the activity of Axin/Axil. When Frz and Dsh are inhibited by Wnt antagonists like secreted Frizzled-related proteins (sFRPs), Axin forms a domain with GSK3β, preventing its phosphorylation of β-catenin and thus promoting β-catenin accumulation, which, when overexpressed, can induce apoptosis by targeting tumor-suppressor genes. When left uninhibited, GSK3β blocks mechanisms underlying neuronal survival, glycogen synthesis, migration, angiogenesis, and CREB activation. It also hyperphosphorylates tau (Damalas et al, 1999; Kim et al, 2000; Kim et al, 2002; Korol et al., 2013; Rosa & Fahnestock, 2015)

Its roles in the initiation of multiple downstream cascades underlying neuroplasticity (e.g., synaptic strength, cell survival, etc.) and memory acquisition and storage render BDNF a powerful, multifunctional neurotrophin. Moreover, the functions of BDNF are complex and region-specific, as BDNF inhibition in the hippocampus results in spatial memory impairments (Linnarsson et al., 1997; Yamada & Nabeshima, 2003), whereas BDNF inhibition in the striatum results in altered social and anxiety-like behaviors (Blechkarz-Klin et al., 2018; Marais et al., 2009).

BDNF in the hippocampus and striatum. Increased expression of BDNF in the hippocampus has been linked to enhancements in hippocampus-sensitive learning and memory (Tyler et al., 2002; Yamada et al., 2002; Yamada & Nabeshima, 2003). Mutations altering BDNF expression and signaling or treatments with anti-BDNF antibodies impair performance on spatial memory tasks (Linnarsson et al., 1997;

Yamada & Nabeshima, 2003). When compared to young wildtype mice, aged mice heterozygous for a BDNF deletion demonstrated decreased BDNF expression and impaired learning in the Morris water maze, a hippocampus-sensitive task for spatial memory. Swim speed did not differ between groups (Linnarsson et al., 1997). Rats that were housed in cognitively enriching environments demonstrated improved spatial memory and increased BDNF mRNA expression when compared to those housed in cognitively non-stimulating environments when tested in the Morris water maze (Falkenberg et al., 1992; Tyler et al., 2002). This evidence suggests a connection between increased hippocampal BDNF levels and enhanced hippocampus-sensitive memory, while impairments in BDNF signaling in the hippocampus are linked to poorer performance of hippocampus-related tasks.

Decreased BDNF levels in other regions, such as the striatum, impaired social behaviors and produced depressed or anxious phenotypes. For instance, male rats born to mothers treated with paracetamol (acetaminophen) during gestation demonstrated significantly decreased BDNF levels in the striatum as well as altered social behavior, namely, reduced frequency in non-aggressive behaviors such as sniffing a conspecific and increased pinning down of conspecifics, indicating aggression and higher anxiety (Blechkarz-Klin et al., 2018). In addition, voluntary exercise (i.e., six weeks of access to running wheels) enhanced striatal BDNF levels and reduced behavioral despair phenotypes (immobility) during the forced swim test in rats with a history of stress induced by maternal separation (Marais et al., 2009). The BDNF-TrkB signaling cascade and its downstream components including GSK3β serve as key

targets for further exploration into the molecular basis of memory (Damalas et al, 1999; Kim et al, 2000; Kim et al, 2002).

GSK3β and its activation state. GSK3β is a widely expressed, cytosolic enzyme that is a key downstream target in the BDNF-TrkB signaling pathway. This enzyme is highly concentrated in the brain, both in glia and neurons (Ferrer et al., 2002; Kaidanovich-Beilin & Woodgett, 2011; Perez-Costas et al., 2010; Woodgett, 1990; Yao et al., 2002). Northern blot and *in situ* hybridization analyses demonstrate abundant expression of GSK3β mRNA in various brain regions, such as the hippocampus, the cerebral cortex (Yao et al., 2002), and the striatum (Jorge-Torres et al., 2018). Moreover, western blots confirmed the presence of the GSK3β protein in the brain (Yao et al., 2002). Immunohistochemistry combined with light and electron microscopy confirmed the presence of this protein in rough endoplasmic reticulum, free ribosomes, and mitochondria of neurons and astrocytes, as well as in the dendrites and dendritic spines (Perez-Costas et al., 2010). Phosphorylation of GSK3β at the serine-9 (ser-9) site inhibits its activity (Perez-Costas et al., 2010), and its phosphorylation state is mediated by one of the BDNF signaling pathways.

BDNF binding to the TrkB receptor induces downstream phosphorylation of GSK3β at the ser-9 site mediated by PI3K/Akt (Beurel et al., 2015). PI3K can also activate integrin-linked kinase (ILK) and/or p70 S6K, all of which phosphorylate and inhibit GSK3β downstream (Fig. 1A & B; Kim et al., 2002). Phosphorylation of GSK3β causes its N-terminal tail to act as a pre-phosphorylated substrate, or a pseudosubstrate (Beurel et al., 2015; Frame et al., 2001). The pseudosubstrate then self-associates with the primed-substrate binding pocket, which is the domain that

recruits substrates to GSK3β, thereby preventing the binding of primed substrates and hindering the phosphorylation of substrates by GSK3β (Beurel et al., 2015). Phosphorylation thereby inhibits GSK3β from binding to and acting upon substrates, preventing it from downregulating CREB or glycogen synthesis and from phosphorylating tau protein (Korol et al., 2013; Fig. 1A).

Lack of GSK3β inhibition produces synaptic loss and inhibition of neural activity, often resulting in learning and memory impairments. GSK3β disinhibition has been modeled in animals and has produced tau-related pathology and learning deficits. Experimentally-induced hyperactivity and overexpression of GSK3β in rodent models results in Alzheimer's-like pathology, including tau hyperphosphorylation, progression of neurofibrillary tangles (NFTs), neuronal apoptosis, synaptic loss, and cell death (Bhat et al., 2004; Bhat et al., 2000; de Barreda et al., 2010; Hanger et al., 1998; Liu et al., 2017; Kimura et al., 2007; Kimura et al., 2010). Transgenic mice that overexpress GSK3β exhibited LTP deficits that could be reversed by persistent treatment with lithium, which inhibits GSK3β via activation of Akt (Hooper et al., 2007). Overexpression of GSK3β produced neuroplasticity defects in this investigation that were only reversible by GSK3β inhibition, indicating the role of this kinase in neuroplasticity mechanisms like LTP. Moreover, GSK3β knock-in mice displayed impairments in novel object recognition and coordinate spatial processing, as well as deficits in hippocampal neural progenitor cell (NPC) proliferation (Pardo et al., 2016). In this experiment, genetically defective GSK3β (resistant to inhibitory phosphorylation and thus overactive) produced memory impairments and proliferation deficits, indicating the role of appropriate GSK3β inhibition in proper memory function and cell proliferation. In another experiment, MRI displayed

hyperphosphorylated tau and synapse loss preceding NFT formation in the parahippocampal areas in aged mice, resultant from GSK3β disinhibition (Kimura et al., 2007). In addition, in transgenic mice, insoluble tau aggregates led to the formation of fibrillar tau, resulting in the generation of NFTs (Kimura et al., 2010), decreased nuclear β-catenin, neuronal death, and reactive gliosis (Hernández et al., 2002). Transgenic mice that overexpressed GSK3β showed impairments in spatial memory as measured in the Morris water maze (Hernández et al., 2002). GSK3β inhibitors suppress tau phosphorylation and amyloid depositions in Alzheimer's-like transgenic mice with human mutant amyloid precursor protein (APP) and tau (Serenó et al., 2009), suggesting that tau hyperphosphorylation may serve as one avenue by which uninhibited GSK3β produces cognitive deficits. Furthermore, an adeno-associated viral vector containing a persistently active form of GSK3β in the prefrontal cortex and hippocampus of rats produced deficits in novel object recognition, spatial memory (object location), reversal learning, and associative object recognition memory (object in place); the last of those tasks requires coordination of the prefrontal cortex and ventral hippocampus (Albeely et al., 2022; Barker & Warburton, 2011). Pathogenic tau phosphorylation was heightened in these regions when GSK3β activity was elevated (Albeely et al., 2022), further suggesting a connection between GSK3β-mediated tau hyperphosphorylation and memory deficits.

Another avenue by which uninhibited GSK3β may produce neuroplastic and cognitive deficits is via inhibition of the substrate β-catenin, which has also been implicated in Alzheimer's disease (Wan et al., 2014). GSK3β is a well-known regulator of Wnt signaling, in which it prevents phosphorylation of β-catenin, promotes β-catenin

accumulation, and induces apoptosis (Fig. 1B; Damalas et al, 1999; Kim et al, 2000; Kim et al, 2002). For example, GSK3β-mediated tau hyperphosphorylation in the brains of tau-transgenic rodents was accompanied by β-catenin phosphorylation and, thus, increased apoptosis (Li et al., 2007). The interruption in this signaling is proposed to contribute to impaired neurogenesis in AD, as an amyloid β peptide treatment impaired the ability of glial progenitor cells (GPCs) to generate new cells (He & Shen, 2009). Another experiment involved using a neuroprotective acetylcholinesterase inhibitor, HupA, on transgenic mice to determine if it could trigger Wnt signaling and reduce AD-related amyloidosis. This treatment inhibited GSK3β and increased the levels of unphosphorylated β-catenin (Wang et al., 2011). β-catenin is a demonstrably important substrate by which GSK3β can influence neuroplasticity and cognition, as GSK3β disinhibition has previously altered neuroplastic mechanisms (namely, impaired neurogenesis and promoted apoptosis) via prevention of β-catenin phosphorylation.

While disinhibition of GSK3β activity impairs learning and memory, experimental inhibition of GSK3β can reverse impairments in learning and memory, thus indicating that the modulation of its activation state can shape cognition. For example, rodent models of neurological disorders like Fragile X syndrome, Huntington's disease, and stroke experience an amelioration of abnormalities in learning, memory, and neuroplasticity processes, such as LTP and LTD, when treated with GSK3β inhibitors (Beurel et al., 2015; Choi et al., 2011; Chuang et al., 2011; Franklin et al., 2014; King et al., 2014; King & Jope, 2013; Leeds et al., 2014; Scheuing et al., 2014). Fragile-X (FX) mice possess hyperactive hippocampal GSK3, which may produce locomotor hyperactivity and cognitive deficits. GSK3 inhibitors rescued LTP in the dentate gyrus

and ameliorated performance in hippocampus-sensitive cognitive tasks (Franklin et al., 2014; King & Jope, 2013), suggesting the involvement of GSK3β in the molecular underpinnings of these cognitive processes. GSK3β inhibitors rescued neuronal cell lines by reducing polyglutamine (poly(Q)) toxicity caused by the Huntington's disease genetic mutation (Carmichael et al., 2002; Scheuing et al., 2014). Inhibitors also reduced neuronal apoptosis and autophagy, measured in terms of decreased infarct volume and total tissue loss, as well as the stroke-induced dephosphorylation of GSK3β, in rats and mice with hypoxically-induced ischemia in the cortex, hippocampus, thalamus, and striatum (Li et al., 2010; Roh et al., 2005). The use of GSK3β inhibitors to ameliorate neurodegenerative disease by treating aberrant neuroplastic processes and disease symptoms demonstrates that the inhibition state of GSK3β determines its effect on neuroplastic mechanisms and cognition. GSK3β inhibition also produces enhancing effects in normal cells, which can be reversed by disinhibition. GSK3β mediates the balance of dendritic formation and degradation in an activity-dependent fashion. Namely, increased ser-9 phosphorylation occurred in hippocampal neurons, leading to increased dendritic growth and arborization; likewise, uninhibited GSK3β activity results in neuronal hyperexcitability and dendrite shrinkage (Beurel et al., 2015; Rui et al., 2013).

Experimental knockdowns or knockouts of GSK3β produce detrimental effects at the cellular and behavioral levels, indicating that the existence of GSK3β alone – regardless of its activation state – plays a role in neuroplasticity and memory. For example, GSK3β must be present and active for NMDA-receptor-mediated LTD to occur in rat hippocampal slices (Peineau et al., 2007); this form of LTD has been implicated in

memory formation in the hippocampus (Bear & Abraham, 1996). In addition, GSK3α- and GSK3β-knockdown mice exhibited significantly reduced axonal growth and branching in dissociated cultures and cortical slice preparations (Beurel et al., 2015; Kim et al., 2006). Heterozygous GSK3β knockout mice show deficits in memory, as tested by the Morris water maze and the contextual fear-conditioning test (Kimura et al., 2008; Kaidanovich-Beilin & Woodgett, 2011). Lentiviral knockdown of GSK3β in the dentate gyrus (DG) of the hippocampus impairs contextual fear memory retrieval (Chew et al., 2015) and synaptic transmission (Liu et al., 2017) in mice. GSK3β itself must therefore be present to serve crucial functions in the nervous system regarding neuroplasticity (i.e., LTD and axonal growth and branching), as well as memory. However, it is the activation (i.e., phosphorylation) state of GSK3β, not its presence alone, that seems to shape specific cognitive functions and neuroplastic processes like LTP and LTD (Beurel et al., 2015; Choi et al., 2011; Chuang et al., 2011; Franklin et al., 2014; King et al., 2014; King & Jope, 2013; Leeds et al., 2014; Scheuing et al., 2014).

BDNF-TrkB-GSK3β signaling, DHF, and physical and cognitive stimulation.

Our lab has been exploring the relationship between BDNF-TrkB-GSK3β signaling and learning that engages different neural systems, such as the hippocampus and striatum, using a place learning task and response learning task, respectively. The place learning task is hippocampus-sensitive (McDonald & White, 1995) and requires rats to locate a food reward based on visual cue configurations around the room, whereas the response learning task, which engages the striatum (Chang & Gold, 2004), requires rats to locate the reward using an egocentric body turn. We previously demonstrated that intra-striatal infusions of 7,8-dihydroxyflavone (DHF), a potent BDNF mimic (Andero et al., 2011;

Jang et al., 2010), improved response learning. Moreover, striatal DHF infusions in rats that were untrained also increased GSK3β phosphorylation in the striatum. Together, the results suggest that the BDNF-TrkB-GSK3β signaling pathway may be connected to enhancements in striatum-sensitive learning (Ambalavanar et al., 2019); however, this is contradicted by other evidence from our lab showing that morphine treatment enhances response (striatum-sensitive) learning but has no effect on BDNF or GSK3β in rats (Gardner et al., 2022), indicating that there are perhaps other pathways besides BDNF-TrkB-GSK3β that could impact learning.

In addition to enhancing learning, 7,8-DHF has been found to protect the striatum from neurodegeneration, suggesting a both protective and enhancing role of BDNF in this region. Specifically, 7,8-DHF has previously reduced dopaminergic neuron loss in the striatum and improved motor ability in an open field test in a rotenone-induced toxicity rat model of Parkinson's disease (Nie et al., 2019). This flavone also blocked further loss of dopaminergic terminals in the dorsolateral striatum and restored motor deficits in a progressive mouse model of Parkinson's disease (Sconce et al., 2015). 7,8-DHF delayed motor deficits, reversed learning impairments on the Novel Object Recognition Task (NORT), and prevented striatal loss in mouse models for Huntington's disease (García-Díaz Barriga et al., 2017). Our lab's results therefore contribute to the growing findings that BDNF signaling, including the downstream phosphorylation of GSK3β, enhances memory and motor functions associated with the striatum.

Other work in our lab showed that regular physical activity and acute cognitive stimulation prior to testing on striatum-sensitive response tasks enhance learning via the BDNF-TrkB signaling cascade (Scavuzzo, 2014). Rats were given either three

weeks of access to a running wheel or 20 minutes of working memory testing on a spontaneous alternation task one hour prior to training on place and response mazes. Extracellular BDNF levels were measured in real time in the hippocampus or striatum during maze testing using microdialysis, in which a cannula is inserted into the brain regions of interest to sample soluble molecules via a semipermeable membrane (Ketharanathan et al., 2022). Rats with prior cognitive stimulation in the form of a spontaneous alternation maze demonstrated a significantly lower trials to criterion (TTC), and thus faster learning, than did controls in both response and place learning tasks (Scavuzzo, 2014). Rats with prior physical exercise demonstrated similar enhancements. Treating rats with the TrkB inhibitor K252a attenuated the enhancements from prior cognitive and physical activity on response and place learning, (Scavuzzo, 2014). Therefore, prior cognitive and physical stimulation enhanced cognition in young adult male rats via the BDNF-TrkB signaling cascade, as this enhancing effect was attenuated when TrkB was inhibited by K252a (Scavuzzo, 2014). This conclusion is supported by prior evidence in which exercise-induced enhancements in memory (Lin et al., 2012) have been attenuated by injection of K252a into the dorsal hippocampus or basolateral amygdala (Lin et al., 2012).

The Met allele and BDNF. Since our lab has speculated that activity-dependent boosts in BDNF levels are linked to improvements in learning and memory, then our next step is to directly modify BDNF release and observe memory changes. Our lab is therefore investigating CRISPR-mediated alterations in the pro-domain of the *Bdnf* gene. A single-nucleotide polymorphism (SNP) in the BDNF gene in humans (rs6265) has been found to modify risks for certain neural disorders, including anxiety,

depression, and neurocognitive disorders such as Alzheimer's disease. The SNP occurs at codon 66 in humans (Val66Met) and produces a substitution of methionine (Met) in the place of valine (Val) in the pro-domain of the *Bdnf* gene (Fig. 2C). Approximately 15-20% of the worldwide human population possesses either one or two Met alleles (Mercado et al., 2021; Petryshen et al., 2009). Those who are homozygous for the allele comprise only about 4% of the US population, and little information exists about them due to their rarity (Bath & Lee, 2006; Shimizu et al., 2004). The high prevalence of this allele and its presumptive role in dampening activity-dependent BDNF release (Fig. 3B) necessitates further investigation into how BDNF signaling deficits contribute to neural function and dysfunction related to various Met-related psychiatric and neurocognitive disorders.

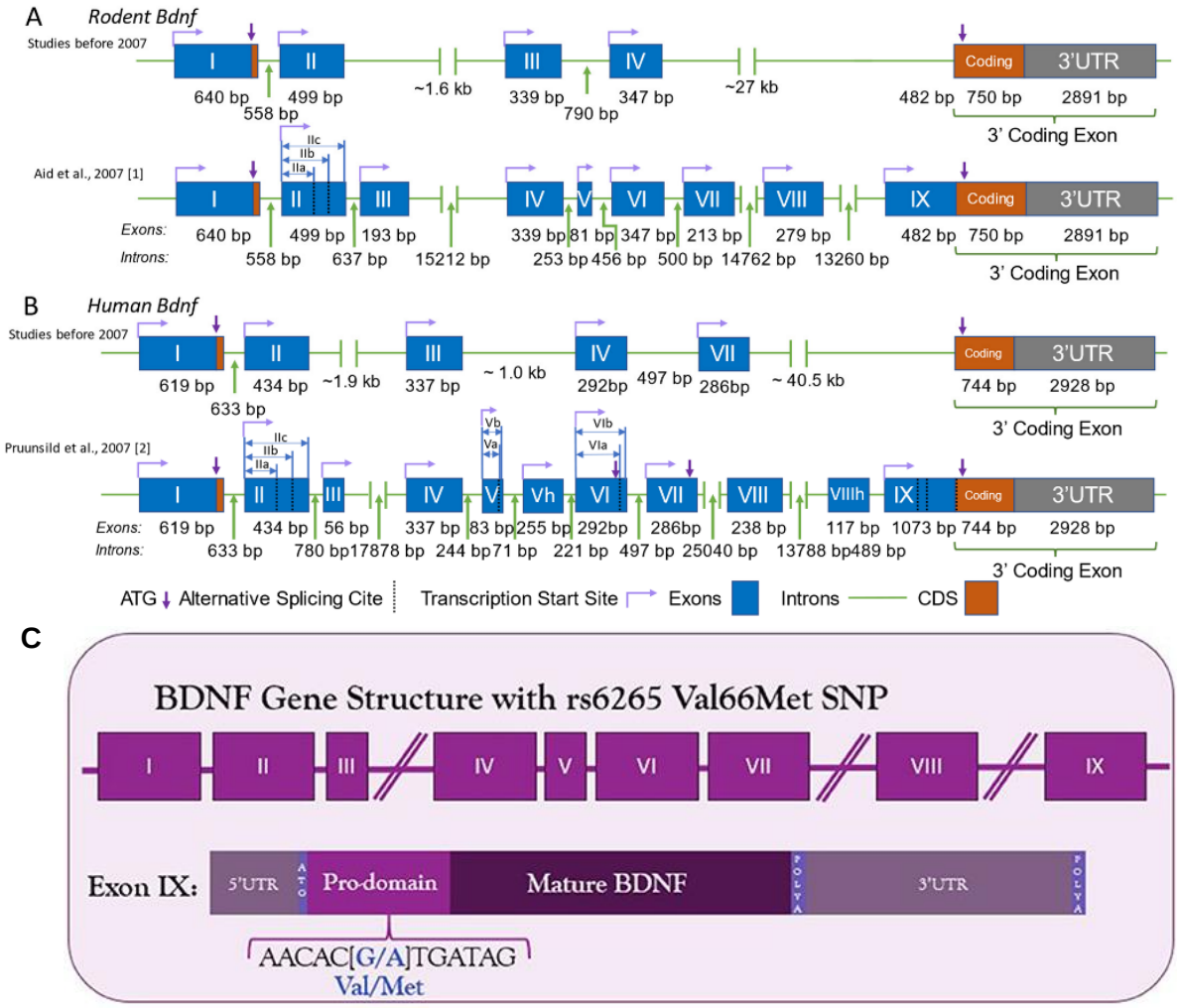


Fig. 2. *BDNF structure and splice variants in humans and rodents, and location of Met SNP*. (A) Genomic structure and splicing of BDNF in humans, in which blue boxes denote exons, green lines denote introns, purple arrows point to the translation start codon (ATG), dashed black lines show alternative splice sites, and bent arrows show the transcription start sites. The lengths of exons and introns are provided. In human BDNF, there 10 non-coding exons and one 3' coding exon for a total of 11 exons. Exons are labeled from I and IX, with 'h'-suffixed exons being unique to humans (You & Lu, 2023). (B) The homologous visual for genomic structure and splicing of BDNF in rodents (You & Lu, 2023). (C) The location of the Met variant in BDNF. The Met SNP occurs in codon 66 in humans and 68 in rats (Jaehne et al., 2022), in the pro-domain in exon IX, resultant from a guanine to adenine substitution (McGregor & English, 2019). Rats have two additional threonines at positions 57 and 58, thus rendering the rat Val68Met SNP equivalent to the human Val66Met SNP (Jaehne et al., 2022).

This allele has implications in cognitive health, particularly in the context of disease risk and decline. For instance, carrying this allele is associated with a steeper cognitive decline – particularly in episodic memory, verbal memory, and executive functioning – in individuals with increased Alzheimer's disease risk (Boots et al., 2017). The Met allele (in both heterozygotes and homozygotes) interacts with age to predict changes in cortical thickness in the entorhinal cortices and temporal gyri, white matter tract connectivity to the medial temporal lobe, and in episodic memory performance (Voineskos et al., 2011). This allele is also associated with reduced hippocampal volume and activity in homozygotes and heterozygotes (Andero et al., 2014; Banner et al., 2011; Bueller et al., 2006; Chen et al., 2008; Frodl et al., 2007; Pezawas et al., 2004; Szezko et al., 2005) and poorer performance on hippocampus-sensitive tasks (Chen et al., 2008; Egan et al., 2003; Hariri et al., 2003). Proposed reasons for these differences include decreased dendritic complexity, fewer overall neurons and glia, increased neuronal death, decreased neurogenesis (Bath & Lee, 2006), and impaired dendritic arborization (Andero et al., 2014; Cao et al., 2007; Chen et al., 2005; Chen et al., 2006; Spencer et al., 2010; Yu et al., 2009), as BDNF-TrkB signaling pathways are known to underlie these processes.

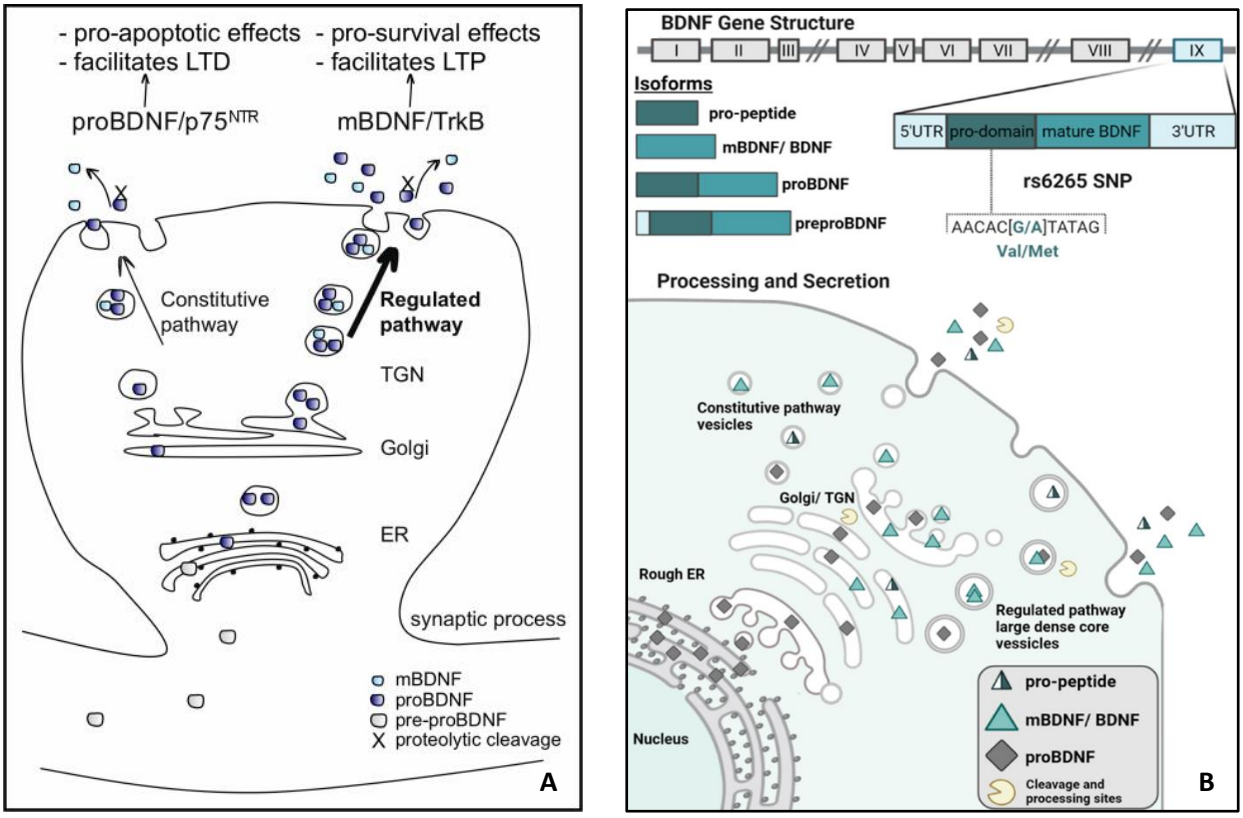


Fig. 3. *Processing, packaging, and constitutive and regulated release of BDNF in Val versus Met carrier individuals*. (A) In Val carriers, BDNF is translated as a pre-pro-BDNF protein. It moves to the endoplasmic reticulum (ER), wherein its pre-sequence is cleaved off, leaving pro-BDNF. This 32-kDa protein then passes through the trans-Golgi network via the Golgi apparatus, where it is secreted in either a regulated or constitutive fashion, depending on the kind of secretory vesicle generated. Pro-BDNF in either type of vesicle can be either cleaved to be secreted as mature BDNF (mBDNF), or more predominately released as pro-BDNF to be cleaved in the extracellular space. Both pro-BDNF and mBDNF can be packaged into vesicles to be released via the regulated pathway. Once in the extracellular space, pro-BDNF binds to pan-neurotrophin receptor p75NTR to promote LTD and apoptosis, whereas mBDNF binds preferentially to TrkB, triggering signaling cascades that promote LTP and cell survival (Cunha et al., 2010). (B) In Met carriers, the process is similar, but regulated release is stunted by the inability of the altered pro-domain of BDNF to bind with sortilin, a protein that drives the sorting of BDNF into the larger, denser vesicles to be specifically released via the regulated pathway; thus activity-dependent release of BDNF is dampened in Met carriers (Chen et al., 2005; Szarowicz et al., 2022)

The Met allele alters the structure of the BDNF pro-domain, thereby affecting its trafficking to the regulated release pathway (Fig. 3). Specifically, it has been proposed that the Met SNP results in cell trafficking deficits because the substitution of valine with methionine in the BDNF pro-domain interferes with interaction between BDNF and sortilin, a protein that sorts BDNF into its regulated secretory pathway (Chen et al, 2008; Chen et al, 2005; Fig. 3B). The presence of the Met allele in mouse models ($BDNF^{Met}$) produces a similar phenotype to that observed in humans (Chen et al., 2008; Chen et al., 2006; Dincheva et al., 2012), including decreased hippocampal volume (Chen et al., 2006) and decreased dendritic complexity in dentate gyrus in homozygotes and heterozygotes. Said model also shows deficient regulated release of $BDNF^{Met}$ protein in homozygotes (Chen et al., 2006; Dincheva et al., 2012). Met/Met rats demonstrated significant impairments in fear memory when compared to Val/Met rats and Val/Val controls (Jaehne et al., 2022). Many behavioral functions are impaired in Met carriers, including fear extinction, declarative memory, spatial learning, working memory, and verbal recall (Andero et al., 2014; Bueller et al., 2006; Cerasa et al., 2010; Gasic et al., 2009; Hariri et al., 2003; Molendijk et al., 2012; Soliman et al., 2010; Schofield et al., 2009). Interestingly, increased expression of TrkB and BDNF mRNA has been observed in the hippocampi of Met rodents, possibly to compensate for the reduced regulated BDNF signaling (Spencer et al., 2010). Furthermore, enhanced glutamatergic neurotransmission was observed in the dorsolateral striata of homozygous $BDNF^{Met/Met}$ mice, even though both LTP and LTD were impaired in this region (Jing et al., 2017). As previously mentioned, prior physical activity spiked extracellular BDNF levels in the hippocampi during the place learning task and increased basal extracellular BDNF

levels in the striata of rats (Scavuzzo, 2014), indicating that physical exercise enhances BDNF synthesis, release, or both. However, it remains unclear whether physical exercise, or any other BDNF regulator, would produce the same enhancing effect on extracellular BDNF levels in a Met carrier in either region. Other BDNF regulators, including steroids like estrogen, have increased BDNF-TrkB signaling and enhanced learning and memory (Bimonte-Nelson et al., 2004; Fortress et al., 2014; Frick, 2015; Luine & Frankfurt, 2013; Pan et al., 2010; Scharfman et al., 2007), though it is not yet clear if steroids show the same effects in Met carriers.

Estrogens and BDNF. Estrogens can modulate various types of learning and memory in rats in a dose-, task-, and age-dependent manner, generally enhancing hippocampus-sensitive cognition while impairing striatum-sensitive cognition (Korol & Kolo, 2002; Pisani et al., 2012; Wang et al., 2008; Zurkovsky et al., 2006; Zurkovsky et al., 2007; Zurkovsky et al., 2011). Estradiol (E_2), the predominant estrogen in reproductive female humans and rodents, can interact with the BDNF-TrkB-GSK3β signaling pathway to promote various forms of learning and memory (Luine & Frankfurt, 2013). The estrogen receptor alpha (ERα) is coupled with the Src family kinases, which increase phosphorylation and activation of TrkB (Arpino et al., 2008; Barletta et al., 2004; Cikla et al., 2016; Huang & McNamara, 2010). In gonadally intact female mice, TrkB phosphorylation peaks at proestrus, when circulating estrogen levels are highest (Spencer et al., 2008; Spender-Segal et al., 2011). Treatment of hippocampal slices with exogenous estradiol also increased TrkB activation (Wang et al., 2016), indicating that estrogens transactivate TrkB. Moreover, 17βE_2 increases histone H3 acetylation at the *Bdnf* gene promoters pII and pIV in the dorsal hippocampus and decreases histone

deacetylase 1 and 2 (HDAC 1 and 2) activity, thereby boosting mature and pro-BDNF expression (Kiss et al., 2012; Luine & Frankfurt, 2013; Pan et al., 2010) in the hippocampus. It is proposed that E_2 enhances object recognition and spatial memory in female mice through its regulation of TrkB signaling (Fortress et al., 2014; Frick, 2015; Luine & Frankfurt, 2013; Pan et al., 2010).

Estrogens can promote BDNF-TrkB signaling by enhancing activity-dependent BDNF release, independent of BDNF synthesis. Estrogen treatments for 16 days increased BDNF levels in the entorhinal and perirhinal cortices in ovariectomized middle-aged rats (Bimonte-Nelson et al., 2004). Estrogen treatments also significantly increased BDNF levels in the hippocampi of ovariectomized middle-aged rats and improved object recognition and spatial memory (Scharfman et al., 2007). The evidence suggests that estrogens enhanced these forms of learning and memory by enhancing BDNF-TrkB signaling. Estrogen induced synaptogenesis in cultured hippocampal neurons by boosting BDNF release, which was determined when treatment with K252a – a TrkB inhibitor – suppressed this mechanism. The mechanism was not, however, prevented by ICI182,780 (ICI), a potent nuclear estrogen receptor (nER) antagonist (Sato et al., 2007), indicating that estrogen induces synaptogenesis by enhancing BDNF in a nER-independent fashion. Blocking BDNF-TrkB signaling, but not estrogen signaling, attenuates estrogen-mediated boosts in BDNF and resultant synaptogenesis, thus indicating that estrogens promote BDNF signaling and enhance synaptogenesis via the BDNF-TrkB signaling cascade. Removal of endogenous estrogens through ovariectomy can impair BDNF-TrkB signaling (Luine & Frankfurt, 2013), indicating the role of estrogen as a positive regulator on this cascade. Estrogen deprivation reduced

BDNF mRNA expression throughout the rat hippocampus (Singh et al., 1995); this deficit can be reversed with estrogen treatment (Singh et al., 1995; Sohrabji & Lewis, 2006). When estrogens are experimentally deprived, BDNF is suppressed in the hippocampus, thus providing further evidence for the modulatory role of estrogen on BDNF-TrkB signaling in this region. In contrast, in a Parkinson's disease mouse model, E_2 treatment suppressed BDNF upregulation triggered by MPTP in the striatum, though dopaminergic cell density was unaffected (Tripanichkul et al., 2010).

Subcutaneous estrogen injections impaired striatum-sensitive memory in a response learning task but enhanced hippocampus-sensitive memory in a place learning task in ovariectomized rats (Korol & Kolo, 2002). E_2 treatment improved performance in the Morris water maze (a hippocampus-sensitive task), reduced immobility in the forced swim test, and increased hippocampal BDNF levels in female rats (Kiss et al., 2012), suggesting a link between estrogen-mediated BDNF boosts in the hippocampus and improvement of memory associated with that region. Bilateral intra-striatal infusions of estradiol also impaired response learning (Zurkovsky et al., 2011), indicating that estrogens can produce striatum-sensitive learning impairments. However, it is not yet clear how estrogen-mediated regulation of learning and memory would be affected by a BDNF-related mutation. Thus, our lab seeks to understand how estrogens interact with BDNF-TrkB signaling when there are disruptions in this cascade, like those observed with the Met substitution.

The Met allele, estrogen, and BDNF-TrkB-GSK3β signaling. We have been fortunate to obtain rats with CRISPR-cas9 modifications of the *Bdnf* gene from our colleagues at Michigan State University, who created Val/Val (dominant variant,

modeling the wildtype variant) and Met/Met (nondominant variant, with the SNP) homozygote rats to reduce breeding costs, though strain differences were possible as siblings were not compared due to the two lines being separate. Because the homozygous Met/Met genotype rarely occurs naturally in humans (Bath & Lee, 2006), animal models in which any genotype can be produced are useful tools to understand the impact of the Met allele on learning, memory, and the underlying mechanisms. These rats were involved in three studies, including 1) the interaction of estrous cycle status and genotype in ovary-intact young adult Val/Val and Met/Met rats on exploratory behaviors; 2) the interaction of estradiol status and genotype in young adult ovariectomized Val/Val and Met/Met rats on recognition learning and stress phenotypes; and 3) the interaction of estradiol status and genotype in middle-aged ovariectomized Val/Val and Met/Met rats on cognition and stress phenotypes. The latter study was the focus of my project, in which I evaluated how estradiol treatment and genotype could specifically impact phosphorylation (i.e., activation status) of GSK3β as a measure of BDNF-TrkB signaling. If BDNF release is impaired in rats with the Met allele, and estrogens modulate learning and memory through BDNF signaling, then the Met/Met rats should not respond to the modulatory effects of estrogens. However, if rats with the Met allele have upregulated TrkB receptors, then they may respond strongly to estrogen treatment, and we may observe normal, or even heightened, estradiol-induced modulation of learning and memory (Spencer et al., 2010).

To understand how the Met substitution interacts with E_2 to modulate object recognition memory, our lab ovariectomized middle-aged female Val/Val and Met/Met rats to control for endogenous hormones and treated both groups with either sesame oil

(vehicle) or 17β-estradiol benzoate (EB) prior to testing them on a striatum-sensitive double object recognition (dOR) task (Gardner et al., 2020; Korol et al., 2019; Kundu et al., 2018). dOR entails placing a rat in an open, high-walled arena with two distinct objects in fixed locations. For three training trials, called study sessions, the rat is permitted to explore the arena space and the objects. Typically, exploration of the objects decreases throughout the training trials, as the rat becomes more familiar with them. This is a measure of learning referred to as habituation (Coelho et al., 2011; Vianna et al., 2000). In the testing phase, both objects are replaced with new objects in the same fixed location in the arena. Because rodents typically prefer novelty (Berlyne, 1950), it is expected that wild-type rats will exhibit an increase in object recognition index, meaning that they will explore the novel objects in the testing phase more than the familiar ones in the third study session (Korol et al., 2019; Kundu et al., 2018). We hypothesized that EB treatment would impair object recognition index in Val/Val rats, based on prior evidence of striatal memory being inhibited by estradiol. Based on the proposed Met-related dampening in BDNF-TrkB secretion, we also predicted that the Met/Met rats would not be sensitive to the striatum-impairing effects of the EB treatment. Furthermore, if activity-dependent BDNF signaling is necessary for optimal performance on the dOR task, we hypothesized that compared to Val/Val rats, Met/Met rats would show poorer recognition memory. However, if compensatory increases in BDNF signaling through the TrkB receptor or activity-dependent BDNF signaling is not necessary for dOR, we would expect recognition memory in Met/Met rats to match or perhaps exceed that seen in Val/Val rats. There were no consistent group differences in object recognition index based on genotype or treatment in the dOR experiment;

however, Met/Met rats did demonstrate statistically different behaviors, such as reduced exploration of the arena, indicating higher stress susceptibility (Bath et al., 2012).

Given these behavioral findings, my project aimed to investigate whether Met carriers have up- or down-regulated BDNF signaling. One approach to this is to examine the impact of the CRISPR-mediated Met substitution on striatal GSK3β levels and phosphorylation status. **Based on the notion that Met/Met rats may compensate for loss of regulated BDNF secretion by upregulating BDNF and TrkB levels, I propose that Met/Met rats will demonstrate enhanced downstream GSK3β phosphorylation when compared to Val/Val rats. In contrast, if GSK3β phosphorylation is decreased in Met/Met rats, then compensatory boosts in BDNF and TrkB are not occurring. Furthermore, since estrogens promote activated BDNF release, Met/Met rats should be insensitive to EB treatment in terms of downstream GSK3β phosphorylation, given their deficits in regulated BDNF release.** Of note, a considerable confound exists in the fact that all the rats whose samples underwent biochemical analysis also were previously trained on the dOR task; therefore, GSK3β phosphorylation may indeed reflect behavioral experiences in training (e.g., arousal, locomotion, stress, learning, exploration, etc.) or their interactions with genotype and hormone status, making it impossible to isolate the main effects. This confound cannot be fully controlled for in semiquantitative measurements of GSK3β phosphorylation without using samples from untrained counterparts to EB vs. vehicle-treated Met/Met or Val/Val rats.

Methods

Animals. Twenty-four ovariectomized female middle-aged (12-14 months old) CRISPR-cas9 rats (11 Val/Val rats and 13 Met/Met rats) were obtained from Michigan State University and housed separately in a 12-hour light-dark cycle, where they were given food and water ad libitum. After being quarantined for six weeks, the rats were staged for estrous cycle for 15 days prior to being ovariectomized to determine their reproductive status by Prakapenka et al. (2021). Five Val/Val rats and six Met/Met rats were treated with vehicle (sesame oil), and six Val/Val rats and seven Met/Met rats were treated with a standard 45 µg/kg dose of 17β-estradiol benzoate (EB) prior to behavior testing, in a two-by-two experimental design with two different genotypes, each receiving one of two different treatments, creating four groups: Val/Val-veh, Val/Val-EB, Met/Met-veh, and Met/Met-EB. Immediately after behavioral testing, rats were euthanized with Fatal-Plus (0.22 µL/g; Medvet International; Mettawa, IL) and decapitated. Brains were dissected, and striata were extracted and flash-frozen on dry ice. All treatments and dissections were performed by Prakapenka et al. (2021). Of note, some rats lacked information regarding extraction procedures, as the samples were taken by Alesia Prakapenka's (2021) novice mentees and therefore lacked standard notes typical of experiments: MOR47 and MOR58 (vehicle-treated Val/Val); MOR09 (vehicle-treated Met/Met); MOR65 (EB-treated Val/Val); and MOR10 and MOR12 (EB-treated Met/Met). Extraction information missing for these samples included time of Fatal-Plus injection relative to end of behavioral (dOR) testing, time of decapitation, time from decapitation to flash-freezing, or notes on any unusual brain features (i.e., lesions). The samples were then stored at -80 ˚C until semiquantitative

western blotting for total GSK3β and phosphorylated GSK3β was performed about a year later.

Double object recognition (dOR) behavioral testing. All rats underwent double object recognition (dOR) testing by Prakapenka et al. (2021) prior to euthanasia and, subsequently, my performing the western blotting. Rats were placed in an opaque, high-walled, plexiglass arena with two distinct objects in fixed locations. For three study sessions (S1, S2, and S3), the rats were allowed to explore the arena space and the objects. Between each session, the arena and objects were cleaned with an ethanol solution to eliminate residual odor cues. In the testing phase, the objects were replaced with entirely new objects, in the same fixed location as the previous objects in the arena.

The behavioral experiments were videotaped and analyzed. Object exploration – defined as sniffing, whisking, or looking at the object (Kundu et al., 2018; Prakapenka et al., 2021) was digitally timed and recorded. When a rat explored a novel object significantly more than a familiar one, it is described as having a higher recognition index (Prakapenka et al., 2021). Object recognition index (ORI) was calculated as the time (seconds) spent exploring novel objects in the testing (T) phase minus the time (seconds) exploring familiar objects in the third study session (S3) (Prakapenka et al., 2021). Other behaviors were also recorded: Number of object approaches, time spent rearing on hind legs, time spent idle (not moving), time spent exploring the arena, and time spent self-grooming (Prakapenka et al., 2021).

Semiquantitative western blotting for total (pan) GSK3β and phospho-GSK3β. After Prakapenka et al. (2021) performed the dOR task and brain dissections, I

conducted the western blot assay based on an adaptation of the protocol as described by Gardner et al. (2022). Frozen striatal samples (~20 mg) were pulverized on dry ice using a pestle and mortar and suspended in 80 µL 1X Lysis buffer (1 mM EGTA, 1 mM EDTA, 20 mM Tris [pH 7.4], 1 mM sodium pyrophosphate tetrabasic decahydrate, protease cocktail inhibitor, and phosphatase cocktail inhibitor), and homogenized on ice using the Polytron PT 1600 E at maximum speed for approximately 10 seconds. Samples were then centrifuged at 5700 G for five minutes at 4˚C. Protein concentration of the supernatant was quantified using the Pierce BCA assay. The supernatant was diluted using 1X Lysis buffer and then mixed in a 3:1 ratio with protein loading buffer (LI-COR Biosciences; Lincoln, NE) containing 10% β-mercaptoethanol. The final protein concentration of each sample was 3 µg/µL. The samples were heated to 95 ˚C for 10 minutes in preparation for gel electrophoresis.

Samples (20 µg total protein, 6.67 µL), along with Precision Plus Protein™ All Blue Standards (4 µL; Bio-Rad Laboratories, Hercules, CA), were loaded into 10% Mini-PROTEAN® TGX™ precast gels (Bio-Rad Laboratories; Hercules, CA), and electrophoresis was run at 200 V for one hour. Each gel included six experimental samples plus three pooled striatal samples containing 10, 20, and 30 µg total protein for interblot normalization and intrablot determination of linearity of immunoblotting. For each of the three technical replicates performed, biological replicates were distributed across four different gels (to eventually be transferred to blots, named “Blot 1”, “Blot 2”, “Blot 3”, and “Blot 4”) with each experimental group represented in at least one sample / gel to control for interblot differences in electrophoresis or immunoblotting.

Proteins were then transferred via wet transfer (100 V, 2 hours, 4˚C) to polyvinylidene difluoride (PVDF) membranes (Immobilon-Fl, Millipore, Billerica, MA) and dried overnight to ensure proper retention and immobilization of transferred proteins. Membranes were rehydrated in methanol for 30 seconds and then rinsed in TBS for five minutes with gentle shaking. After a brief rinse in deionized water, membranes were incubated in REVERT 700 Total Protein Stain (LI-COR Biosciences) for five minutes. The membranes were then rinsed twice for 30 seconds in REVERT 700 Wash Solution. After another quick rinse in deionized water, the membranes were then imaged at 700 nm on an Odyssey near-infrared imaging system (LI-COR Biosciences) as an internal loading control to normalize for protein loading differences between lanes, as well as for confirmation of protein transfer. Membranes were then rinsed again with deionized water and de-stained for 9-10 minutes using REVERT Destaining Solution. A final rinse with deionized water was performed. Membranes were immediately incubated in LI-COR TBS Odyssey blocking buffer for one hour at room temperature and then placed in primary antibody cocktail overnight at 4 ˚C, consisting of anti-GSK3β mouse monoclonal antibody (1:1,500; #9832, Cell Signaling, Rockford, IL) and anti-phospho-GSK3β (ser-9) rabbit monoclonal antibody (1:1,000; #9323, Cell Signaling). The next morning, membranes were washed four times (eight minutes each) in TBS-T and then incubated in a secondary antibody cocktail for one hour at room temperature: IRDye 800 CW goat anti-rabbit (1:10,000; LI-COR Biosciences) and IRDye 680 RD goat anti-mouse (1:10,000; LI-COR 129 Biosciences), both diluted in blocking buffer (Li-Cor TBS Odyssey blocking buffer, with 0.1% Tween 20 and 0.1% sodium dodecyl sulfate, or

SDS). The membranes were washed four times (eight minutes each) using TBS-T, followed by a two-minute rinse in deionized water.

The membranes were dried under cover overnight and imaged dry the next morning on an Odyssey near-infrared imaging system at a scanning intensity of 3 (LI-COR Biosciences). Target bands were identified by comparison to All Blue protein ladder and fitted to measure integrated signal intensity using Studio software. Specifically, a box was placed around bands that were straight and uniform to measure the protein signal intensity. However, bands that were crooked or slanted could not be easily or accurately measured using the method of drawing a uniform box around them without excessive background that could skew the signal measurement. Therefore, measurement shapes were hand-drawn around these bands to determine the signal intensity (Fig. 2B & C). Background was determined via the average local background method, which computes the average intensity of pixels in a border around the shape and subtracts the background from the shape (LI-COR Biosciences, 2015). Relative levels of total GSK3β and phospho-GSK3β were determined via Image Studio software (LI-COR Biosciences) and were represented by integrated signal intensity, which is the sum of the pixel intensity over all pixels in an object being measured (MetaXpress® and MetaMorph®: Relationship between Average Intensity and Integrated Intensity, 2020). Equal-sized boxes were drawn around each lane from 20 to 100 kDa (according to the All Blue protein ladder) using Studio software to measure REVERT total protein signal at 700 nm using the Li-Cor Odyssey (Fig. 2A).

The REVERT 700 Total Protein was employed as an internal loading control to account for differences in protein loading amounts or potentially different protein

concentrations between lanes. To account for between-blot variability due two between-blot immunoblotting differences, standard curves of REVERT 700 total protein signals, GSK3β protein signals, and phospho-GSK3β protein signals were created based on signals of pooled samples for each of the four blots in a technical replicate. After normalization to their respective REVERT-loaded lane signals, GSK3β sample signals were normalized to GSK3β signals of the 20-µg-loaded pooled samples for each blot. The same pooled normalization process was performed for the phospho-GSK3β protein signals. This step was employed to control for variability between blots within and across technical replicates, which can occur due to blot-specific differences in immunoblotting conditions (i.e., one blot may not have been fully submerged in antibody cocktail during the incubation process, or one technical replicate may involve issues in terms of antibody preparation resulting in a concentration too low or too high, etc.).

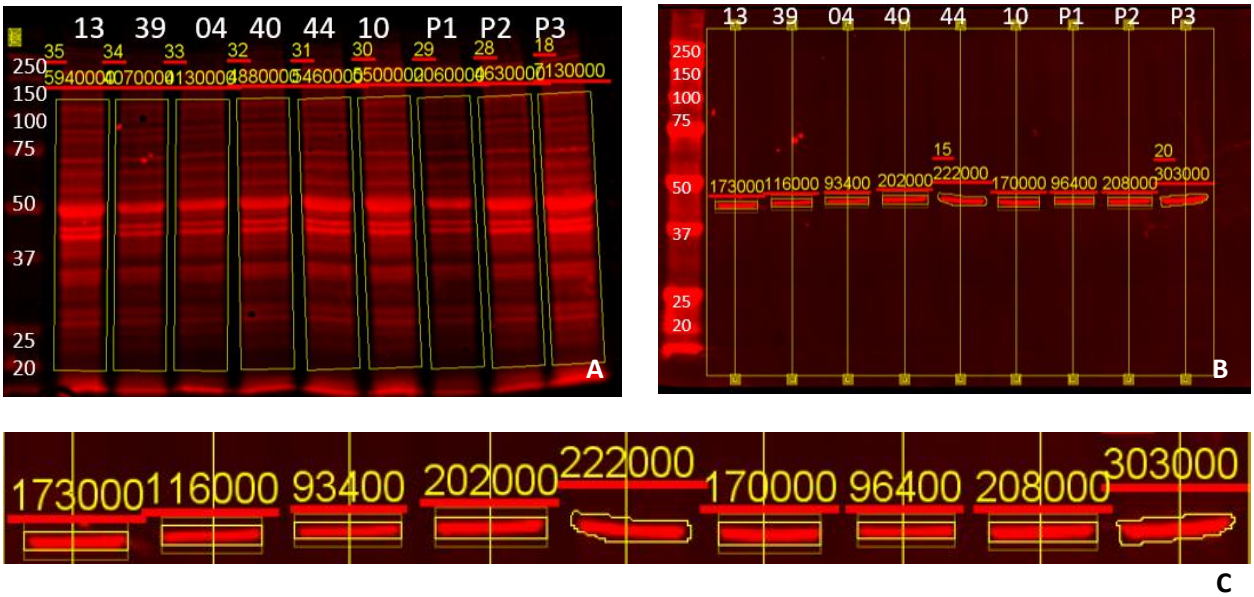


Fig 4. *Example of measurement method used for REVERT 700 Total Protein Signal and pan-GSK3β on the Li-Cor Odyssey system*. (A) A subset of samples measured for REVERT 700 Total Protein Signal, measured at 700 nm. Equal-sized boxes were placed on each lane from 20-100 kDa (according to the molecular weight ladder) to measure total protein signal. (B) A subset of samples measured for pan-GSK3β, measured at 700 nm. (C) Inset of bands as described in Panel B.

Statistical analysis. Even though three technical replicates were run, only the first and second replicates yielded blots containing bands that were visibly bright enough to be accurately measured. Standard curve graphs for target bands and for REVERT 700 total protein were produced based on the integrated intensities of the pooled samples (P1, P2, P3) and were used to gauge between-blot variability based on linearity according to the correlation coefficient. The integrated intensities of total GSK3β and phospho-GSK3β were normalized to the REVERT 700 total protein signals (loading control) in Microsoft Excel. Averages per group for normalized GSK3β protein signals, normalized phospho-GSK3β protein signals, and the phospho/pan-GSK3β ratios were computed in Microsoft Excel. The correlation between phospho-GSK3β-to-

total-GSK3β ratios and arena exploration was evaluated with Pearson correlation coefficients. Two-way analyses of variance (ANOVAs) and Tukey-Kramer's *post hoc* analyses were performed in IBM SPSS Statistics 27 software and RStudio to determine main effects of genotype, main effects of treatment, and their interaction on normalized GSK3β and phospho-GSK3β integrated intensity signals, and on phospho-GSK3β-to-total-GSK3β integrated intensity ratios. Statistical significance for between-groups differences in ANOVAs and pairwise comparisons was set at $\alpha = 0.05$. Grubbs tests for outliers were conducted based on the assumption of normality, which was determined using Shapiro-Wilk tests. Both the Grubbs and Shapiro-Wilk tests were performed using the XLSTAT add-on in Microsoft Excel.

Results

To determine if the BDNF Met allele and estradiol treatment interact to alter the inhibition (i.e., phosphorylation) state of GSK3β in the striatum, phospho-GSK3β and total GSK3β protein signals were measured using semiquantitative western blotting on striatal homogenates from ovariectomized female Met/Met and Val/Val rats treated with either vehicle or EB. Phospho-GSK3β protein signals were divided by total GSK3β signals to calculate the phosphorylation ratios for each sample. Phosphorylation ratios were compared between groups via two-way ANOVAs and Tukey-Kramer's *post hoc* analyses to determine the effects of genotype and treatment on GSK3β phosphorylation. Phospho-GSK3β and total GSK3β protein signals were both normalized to REVERT 700 total protein (loading control) measurements for each lane and compared between groups.

Three technical replicates were run, but due to apparent protein degradation over time, only the first two replicates yielded protein signal bands that were bright enough to be accurately measured in the imaging process. The first two replicates are analyzed separately and compared in this section, as their results differ slightly. The replicates are then averaged, and the combination of this data is also analyzed here.

<u>First Technical Replicate (Run 1)</u>

Genotype alone alters phosphorylation status of GSK3β. For the phospho-GSK3β-to-total-GSK3β signal ratios, there was a significant main effect of genotype (Fig. 5) - supported by *post hoc* analysis (adjusted p = 0.030) - but not treatment (Fig. 5). The interaction between treatment and genotype did not significantly alter

phosphorylation ratios (Fig. 5). These effects were driven ostensibly by low levels of GSK3β phosphorylation in the vehicle-treated Val/Val rats. Specifically, *post hoc* analyses revealed that vehicle-treated Met/Met rats showed remarkably greater phospho-GSK3β-to-total-GSK3β signal ratios than did vehicle-treated Val/Val rats (Fig. 5). Furthermore, EB-treated Met/Met rats demonstrated significantly higher phosphorylation ratios than did vehicle-treated Val/Val rats (Fig. 5). However, there were no differences in phosphorylation ratios between vehicle- and EB-treated Met/Met groups (Fig. 5) or between vehicle- and EB-treated Val/Val groups (Fig. 5). No outliers were found or excluded.

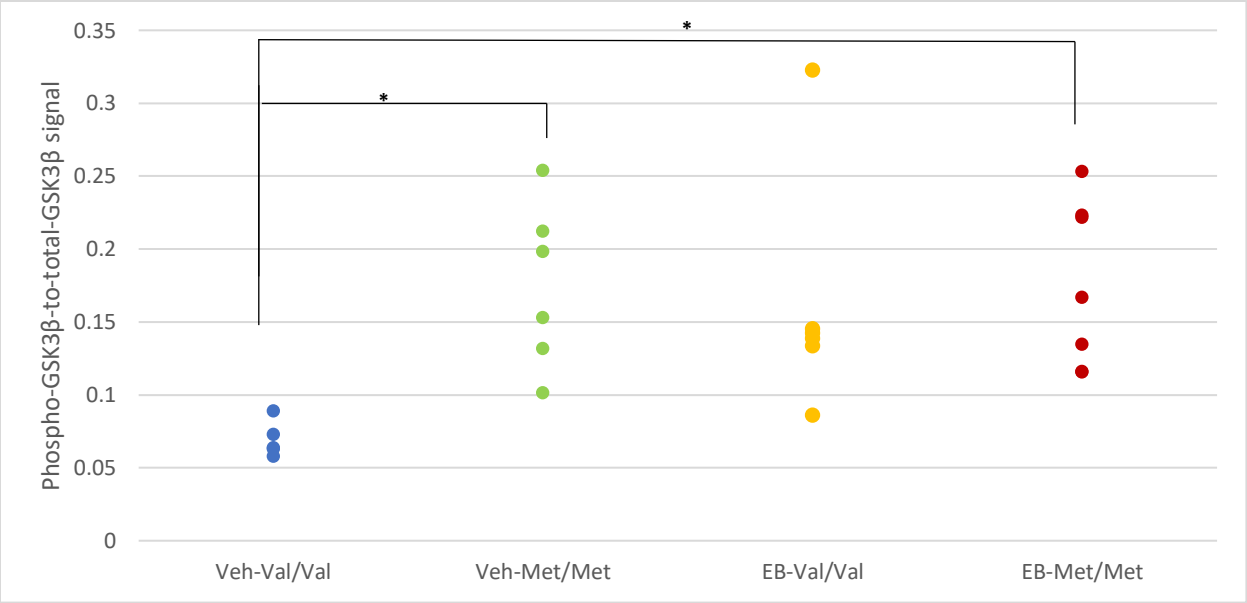


Fig. 5. *Ratio of phospho-GSK3β to total GSK3β signals in the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB.* The ratio of phospho-GSK3β signals to pan-GSK3β signals in the striatum was altered by CRISPR genotype alone ($F_{1,23}$ = 6.169; p = 0.022; p-adj = 0.030). Vehicle-treated Val/Val rats demonstrated lower phosphorylation ratios than vehicle-treated Met/Met rats and EB-treated Met/Met rats (df = 1, * indicates $p < 0.05$). Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 5, vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7.

Two-way ANOVAs and *post hoc* analyses revealed that treatment (Fig. 6A) and genotype (Fig. 6A) both produced significant differences between experimental groups in normalized phospho-GSK3β signals (normalized to REVERT 700 total protein signals). Interactions between genotype and treatment, however, did not significantly alter phospho-GSK3β signals (Fig. 6A). *Post hoc* analyses revealed that vehicle-treated Met/Met rats showed higher normalized phospho-GSK3β signals than did vehicle-treated Val/Val rats (Fig. 6A). Moreover, EB-treated Met/Met rats demonstrated higher normalized phospho-GSK3β signals than did vehicle-treated Val/Val rats (Fig. 6A). Example bands from technical replicate 1, blot 3 are provided for each experimental group, and vehicle-treated Met/Met, EB-treated Val/Val, and EB-treated Met/Met bands are all visually brighter than vehicle-treated Val/Val (Fig. 6B). Blot 3 from technical replicate 1 scanned at 800 nm for phospho-GSK3β (46 kDa) is also shown (Fig. 6C). No outliers were found or excluded.

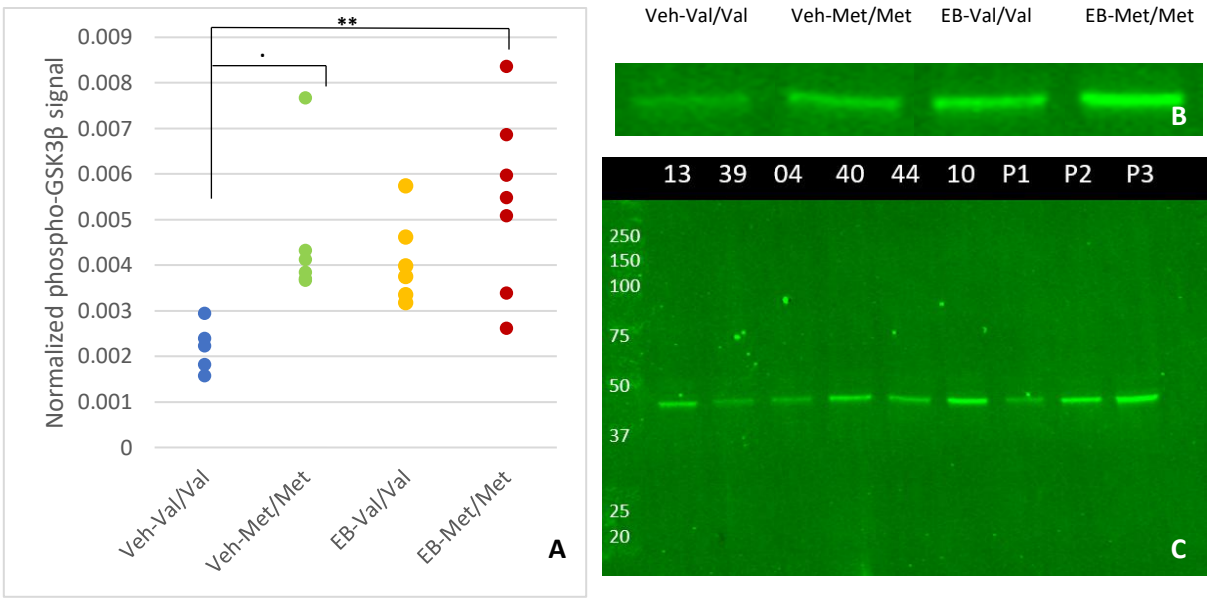


Fig. 6. *Levels of phospho-GSK3β in the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB.* (A) Phospho-GSK3β normalized signals were significantly affected by EB treatment ($F_{1,23}$ = 5.487; p = 0.030; p-adj = 0.036) and by CRISPR genotype ($F_{1,23}$ = 9.726; p = 0.005; p-adj = 0.0063). Vehicle-treated Val/Val rats demonstrated lower phospho-GSK3β normalized signals than EB-treated Met/Met rats; trends indicate that vehicle-treated Met/Met rats showed stronger phospho-GSK3β normalized signals than did vehicle-treated Val/Val rats (df = 1, ** indicates $p < 0.01$, . indicates $p \geq 0.05$). (B) Representative western blot bands for phospho-GSK3β are provided for each treatment group. (C) A subset of lanes scanned at 800 nm for phospho-GSK3β (46 kDa). MOR40 is EB-treated Val/Val, MOR04 and 10 are EB-treated Met/Met, MOR39 is vehicle-treated Val/Val, and MOR13 and 44 vehicle-treated Met/Met. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 5, vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7.

Genotype and estradiol status do not change total GSK3β protein signal.

Pan-GSK3β signals were not significantly different between experimental groups based on genotype (Fig. 7A) and treatment independently (Fig. 7A). There was also no significant interaction between genotype and treatment on GSK3β signal intensity (Fig. 7A). Example bands from technical replicate 1, blot 3 are provided for each experimental group, showing visually similar brightness (Fig. 7B). Replicate 1, blot 3 scanned at 700 nm for total GSK3β (46 kDa) is shown (Fig. 7C). No outliers were found or excluded.

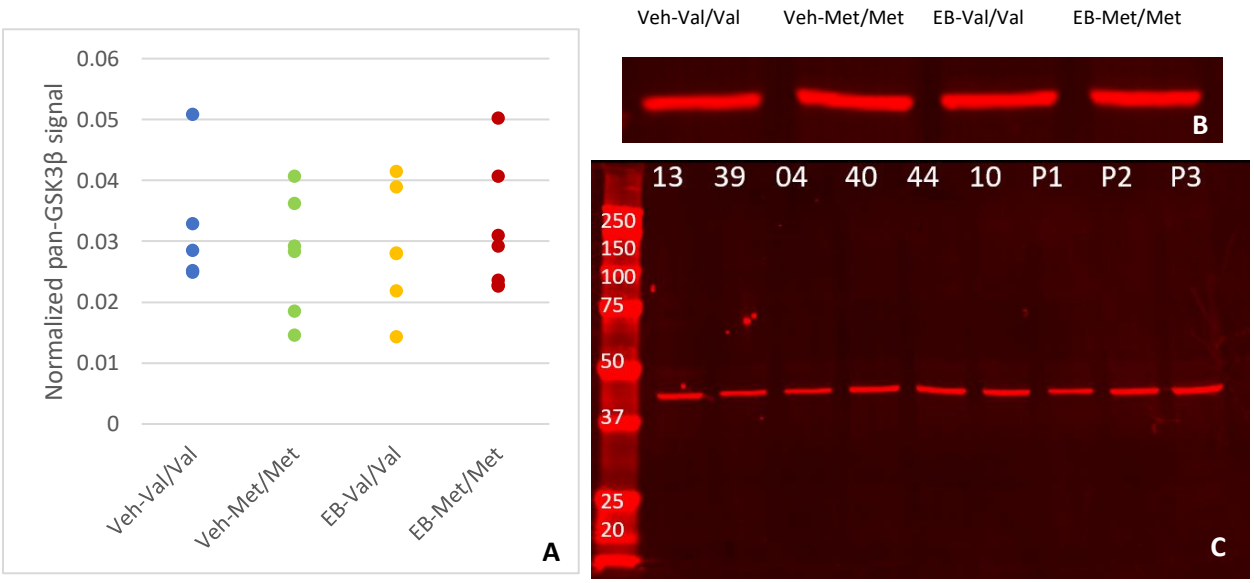


Fig. 7. *Levels of total GSK3β in the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB.* (A) Total GSK3β signal intensity was not significantly affected by CRISPR genotype ($F_{1,23}$ = 0.048; p = 0.828), treatment ($F_{1,23}$ = 0.000; p = 0.985), or the interaction between them ($F_{1,23}$ = 0.724; p = 0.405). (B) Representative western blot bands for pan-GSK3β are provided for each treatment group. (C) A subset of lanes scanned at 700 nm for pan-GSK3β (46 kDa). MOR40 is EB-treated Val/Val, MOR04 and 10 are EB-treated Met/Met, MOR39 is vehicle-treated Val/Val, and MOR13 and 44 vehicle-treated Met/Met. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 5, vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7.

Second Technical Replicate (Run 2)

Genotype alone alters phosphorylation status of GSK3β. ANOVAs revealed significant between-groups differences in phospho-GSK3β-to-total-GSK3β ratio based on genotype (Fig. 8), but not treatment (Fig. 8) or the interaction between genotype and treatment (Fig. 8). Tukey's *post hoc* analyses revealed no specific pairwise differences (Fig. 8).

MOR47 (vehicle-treated Val/Val) and MOR12 (EB-treated Met/Met) were excluded as outliers based on the Grubbs test.

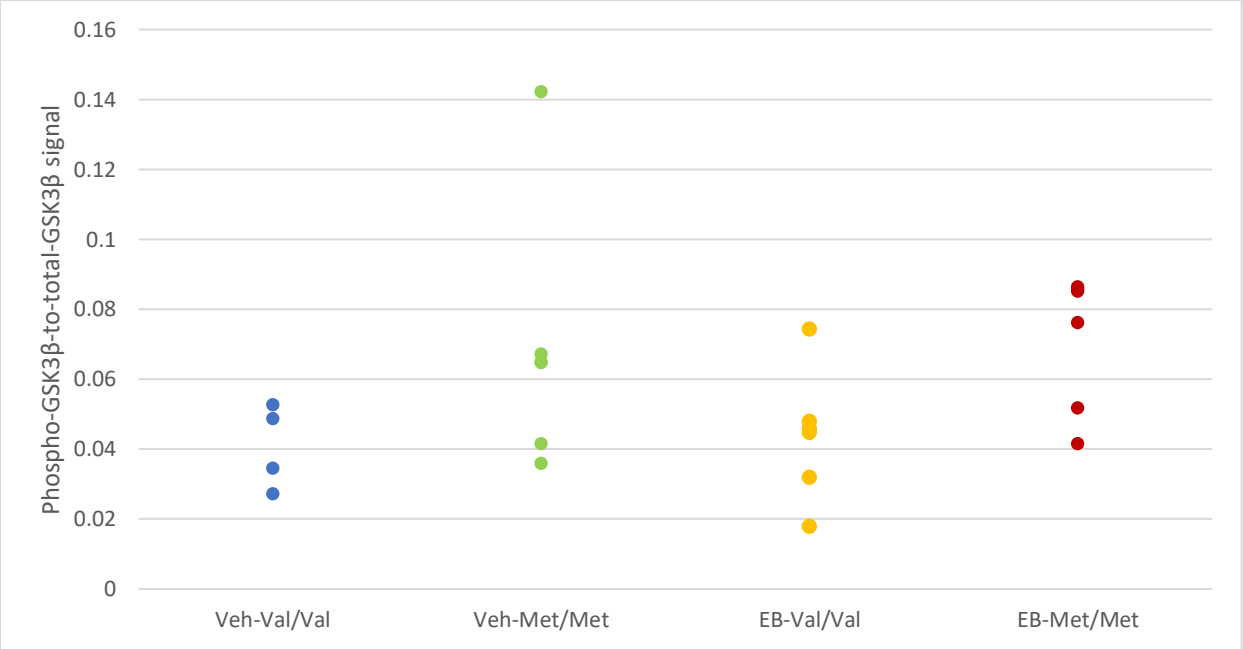


Fig. 8. *Ratio of phospho-GSK3β to total GSK3β signals in the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB.* GSK3β phosphorylation ratios in the striatum were altered by CRISPR genotype ($F_{1,21}$ = 6.661; p = 0.0188; p-adj = 0.019), but not treatment ($F_{1,21}$ = 0.002; p = 0.963) or the interaction between them ($F_{1,21}$ = 0.004; p = 0.5322). Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR47 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 6 (MOR12 excluded, Grubbs p = 0.004).

Normalized phospho-GSK3β protein signal intensity did not differ significantly between groups based on genotype (Fig. 9A), treatment (Fig. 9A), or the interaction between genotype and treatment (Fig. 9A). Due to lack of significant group differences found in the two-way ANOVAs, *post hoc* analyses were not performed.

Example bands from technical replicate 2, blot 3 are provided for each experimental group, showing little difference in visual brightness (Fig. 9B). Replicate 2, blot 3 scanned at 700 nm for total GSK3β (46 kDa) is shown (Fig. 9C).

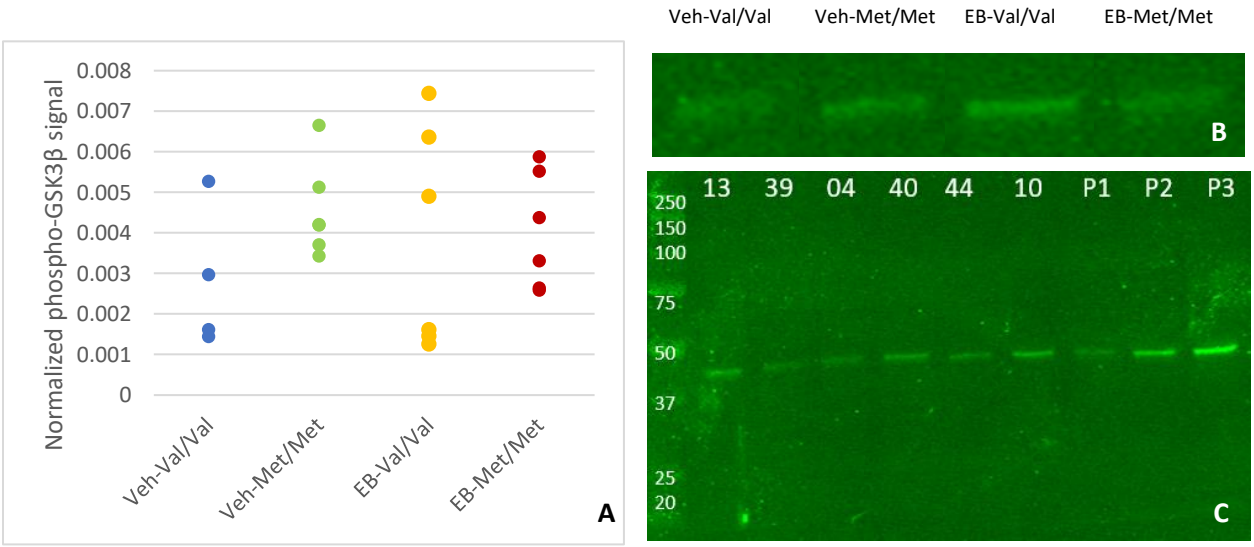


Fig. 9. *Levels of phospho-GSK3β in the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB.* (A) Phospho-GSK3β normalized signals were not significantly affected by treatment ($F_{1,21}$ = 0.011; p = 0.919), CRISPR genotype ($F_{1,21}$ = 1.19; p = 0.290), or the interaction between them ($F_{1,21}$ = 0.861; p = 0.366). (B) Representative western blot bands for phospho-GSK3β are provided for each treatment group. (C) A subset of lanes scanned at 700 nm for phospho-GSK3β (46 kDa). MOR40 is EB-treated Val/Val, MOR04 and 10 are EB-treated Met/Met, MOR39 is vehicle-treated Val/Val, and MOR13 and 44 vehicle-treated Met/Met. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR47 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 6 (MOR12 excluded, Grubbs p = 0.004).

Genotype and estradiol status do not change total GSK3β protein signal.

Pan-GSK3β signals were not significantly different between experimental groups based on genotype (Fig. 10A) and treatment independently (Fig. 10A). There was also no significant interaction between genotype and treatment on GSK3β signal intensity (Fig. 10A). Example bands from technical replicate 2, blot 3 are provided for each experimental group, showing visually similar brightness (Fig. 10B). Replicate 2, blot 3 scanned at 700 nm for total GSK3β (46 kDa) is shown (Fig. 10C).

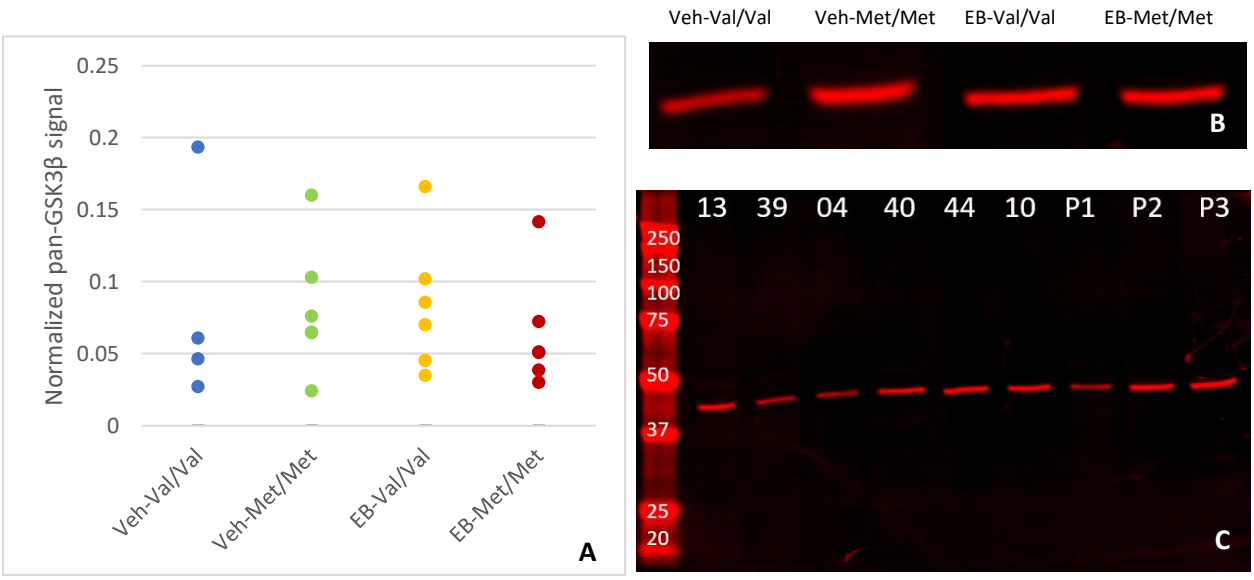


Fig. 10. *Levels of pan-GSK3β in the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB.* (A) Total GSK3β signal intensity was not significantly affected by CRISPR genotype ($F_{1,21}$ = 0.253; p = 0.621), treatment ($F_{1,21}$ = 0.135; p = 0.717), or the interaction between them ($F_{1,21}$ = 0.206; p = 0.655). (B) Representative western blot bands for pan-GSK3β are provided for each treatment group. (C) A subset of lanes scanned at 700 nm for pan-GSK3β (46 kDa). MOR40 is EB-treated Val/Val, MOR04 and 10 are EB-treated Met/Met, MOR39 is vehicle-treated Val/Val, and MOR13 and 44 vehicle-treated Met/Met. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR47 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 6 (MOR12 excluded, Grubbs p = 0.004).

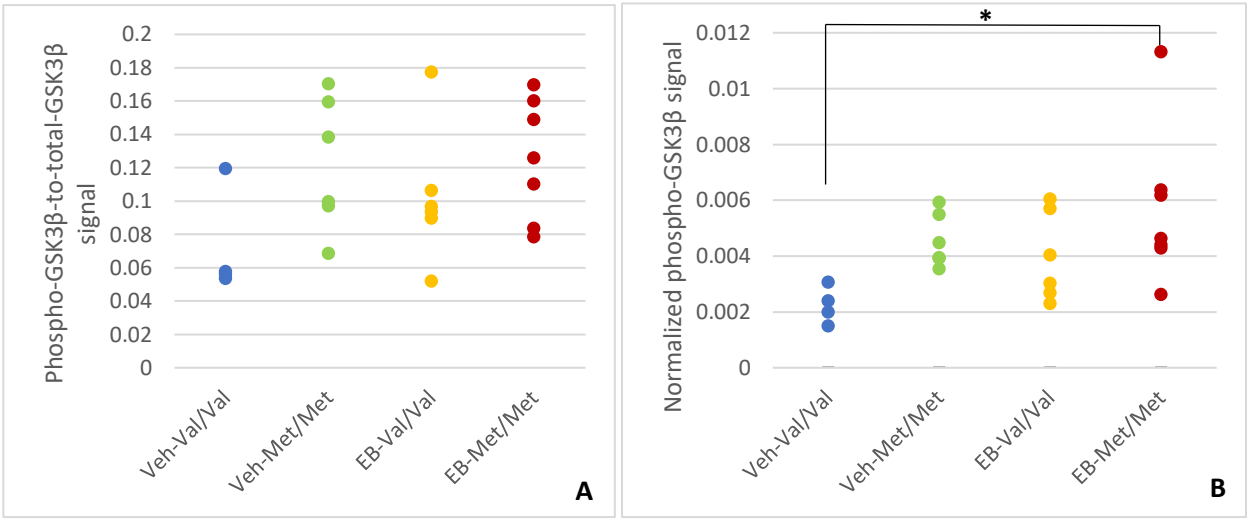
Averaging and Combining Replicates 1 and 2

Genotype alone alters phosphorylation status of GSK3β. For the GSK3β phosphorylation ratios, wherein phospho-GSK3β signal intensities were divided by those of total-GSK3β, there was a significant main effect of genotype (Fig. 11A) - supported by *post hoc* analysis (adjusted p = 0.044) - but not treatment (Fig. 11A). The interaction between treatment and genotype did not significantly alter phosphorylation ratio (Fig. 11A). *Post hoc* analysis revealed no significant pairwise differences between groups. The analysis also demonstrated that both genotypes are insensitive to EB in terms of GSK3β phosphorylation, evidenced by no significant differences when comparing vehicle-treated versus EB-treated Met/Met rats (Fig. 11A) or vehicle-treated versus EB-treated Val/Val rats (Fig. 11A). MOR51 (vehicle-treated Val/Val) was excluded as an outlier based on the Grubbs test.

Differences between groups in terms of phospho-GSK3β protein signals alone (not divided by total GSK3β protein signals) were also explored. Two-way ANOVAs and *post hoc* analyses revealed that genotype significantly impacts normalized phospho-GSK3β protein signals (Fig. 11B). There are no significant between-groups differences based on treatment (Fig. 11B) or the interaction between genotype and treatment (Fig. 11B). *Post hoc* analysis revealed that EB-treated Met/Met rats show significantly higher phospho-GSK3β signal intensities than do vehicle-treated Val/Val rats (Fig. 11B). There were no other significant differences in pairwise comparisons.

Genotype and estradiol status do not change total GSK3β protein signal. Pan-GSK3β signals did not differ between experimental groups based on genotype (Fig.

11C) and treatment independently (Fig. 11C). There was also no significant interaction between genotype and treatment on GSK3β signal intensity (Fig. 11C).



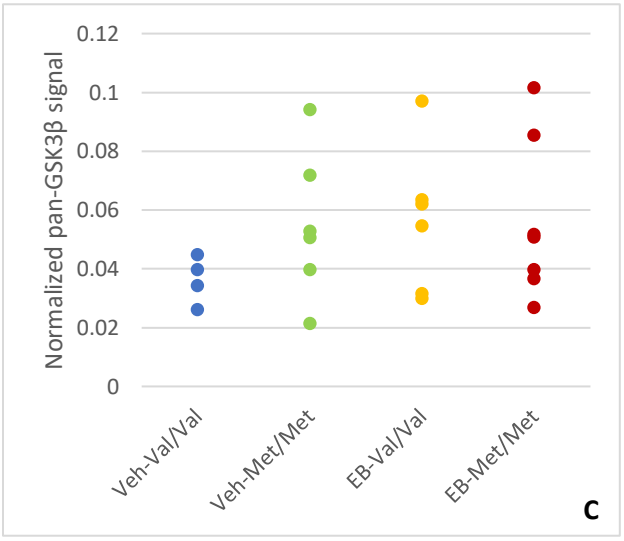


Fig. 11. *Phosphorylation ratios, phospho-GSK3β signals, and pan-GSK3β from the striata of CRISPR Val/Val and Met/Met female rats treated with either vehicle or EB*. (A) Met rats demonstrated significantly higher phosphorylation ratios overall than did Val rats ($F_{1,22}$ = 4.679; p = 0.0435). There were no pairwise group differences, and both genotypes seemed insensitive to EB treatment. (B) Met rats also overall exhibited stronger phospho-GSK3β signal intensities than did Val rats ($F_{1,22}$ = 6.39; p = 0.0205). EB-treated Met/Met rats had higher phospho-GSK3β signal intensities than vehicle-treated Val/Val rats (* indicates p < 0.05). (C) Neither genotype ($F_{1,22}$ = 0.601; p = 0.448) nor treatment ($F_{1,22}$ = 0.749; p = 0.398) nor the interaction between them ($F_{1,22}$ = 0.881; p = 0.360) affected GSK3β signal intensities. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR51 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7.

Rat ID	Genotype	Treatment
MOR19	Val/Val	Veh
MOR58	Val/Val	Veh
MOR47	Val/Val	Veh
MOR39	Val/Val	Veh
MOR51	Val/Val	Veh
MOR09	Met/Met	Veh
MOR01	Met/Met	Veh
MOR05	Met/Met	Veh
MOR13	Met/Met	Veh
MOR44	Met/Met	Veh
MOR55	Met/Met	Veh
MOR20	Val/Val	High EB
MOR65	Val/Val	High EB
MOR38	Val/Val	High EB
MOR40	Val/Val	High EB
MOR48	Val/Val	High EB
MOR27	Val/Val	High EB
MOR41	Met/Met	High EB
MOR21	Met/Met	High EB
MOR02	Met/Met	High EB
MOR04	Met/Met	High EB
MOR10	Met/Met	High EB
MOR56	Met/Met	High EB
MOR12	Met/Met	High EB

Table 1. All 24 striatal samples by genotype (Val/Val and Met/Met) and treatment type (Vehicle or High EB).

Comparison of Technical Replicates in Addressing Both Hypotheses

Hypothesis 1: Met carriers will experience augmented BDNF release – and thus, increased GSK3β phosphorylation – in compensation for altered regulated BDNF signaling when compared to Val carriers.

Do the technical replicates support the hypothesis? Replicate 1 supports the hypothesis that Met rats demonstrate compensatory increases in BDNF levels, resulting in increased downstream GSKβ phosphorylation. In this technical replicate, ANOVAs revealed that Met rats overall demonstrate significantly higher GSKβ phosphorylation ratios than do Val rats (Fig. 5). *Post hoc* comparisons showed that both vehicle- and EB-treated Met/Met rats had higher GSKβ phosphorylation levels than did vehicle-treated Val/Val rats (Fig. 5), further supporting the hypothesis of Met-related compensation.

Similarly, replicate 2 also supports the Met compensation hypothesis, as ANOVAS revealed a main effect of genotype on GSKβ phosphorylation (Fig. 8). However, there were no pairwise differences determined *post hoc* (Fig. 8).

Furthermore, both runs, when averaged and combined, support the Met-related compensatory hypothesis. In this case, ANOVAs show a main effect a genotype in which Met rats show overall greater GSKβ phosphorylation ratios than Val rats (Fig. 11A), suggesting that Met rats demonstrate Met-related BDNF compensation.

How do the replicates address the biological question being tested? The biological question being tested in this hypothesis is as follows: Do Met carriers experience a compensatory BDNF boost in the striatum due to altered regulated BDNF

signaling? The technical replicates test this question by analyzing phospho-GSKβ to total-GSKβ signal ratios (i.e., how much of the protein is inhibited versus how much total exists in the sample) in Val/Val and Met/Met homozygotes. Protein signals are determined using western blotting and fluorescent imaging. The objective is to compare phosphorylation ratios between the two genotypes to determine if Met rats show greater GSKβ phosphorylation ratios than Val rats, which would indicate boosts in BDNF levels upstream.

What results would I expect if the data supported this hypothesis? For this hypothesis, I would at least expect to find a main effect of genotype in the ANOVAs, with Met rats showing higher GSK3β phosphorylation levels, as measured by the phospho-GSK3β-to-pan-GSK3β ratios. Ideally, some *post hoc* pairwise comparisons should support these results. For instance, vehicle-treated Met/Mets should show greater phosphorylation than the vehicle-treated Val/Val rats, thus further corroborating that Mets show compensatory BDNF enhancements resulting in elevated downstream GSK3β inhibition independent from EB treatment.

In any case, I would expect the total GSK3β protein signal intensities to be the same regardless of genotype, as the amount of said protein (pan protein) should not differ significantly between lanes loaded with the same volume. Both runs showed no differences in total GSK3β signal intensities between groups (Figs. 7 & 10). In addition, no between-groups differences could be determined when the runs were combined (Fig. 11C).

Hypothesis 2: Met carriers will show no sensitivity to the BDNF-enhancing effects of estradiol when compared to Val carriers.

Do the technical replicates support the hypothesis? Neither replicate supports the hypothesis that Met rats are more insensitive to the historically BDNF-enhancing effects of estradiol when compared to their Val counterparts. In replicate 1, pairwise comparisons show there were no differences in GSKβ phosphorylation between EB- and vehicle-treated Met/Met rats or between EB- and vehicle-treated Val/Val rats; this evidence that both genotypes are insensitive to estrogen thus refutes the Met-related estrogen insensitivity hypothesis (Fig. 5). Similar results are shown in replicate 2 (Fig. 8).

When the runs are combined and averaged, they still do not corroborate this hypothesis. *Post hoc* analyses show no pairwise differences in GSKβ phosphorylation ratios between any two groups. In particular, no differences could be determined between vehicle- and EB-treated Val/Val rats or between vehicle- and EB-treated Met/Met rats, indicating that both genotypes are insensitive to estrogens (Fig. 11A). Thus, the replicates – combined or separate - provide no evidence supporting the Met-related estrogen insensitivity hypothesis.

How do the replicates address the biological question being tested? The biological question being tested here is, are Met carriers insensitive to the BDNF-enhancing qualities of estrogens? This question is addressed by treating both genotypes with either vehicle or EB (an estrogen variant) and then making pairwise comparisons between EB- versus vehicle-treated Met/Met rats and EB- versus vehicle-treated Val/Val rats in terms of GSKβ phosphorylation ratios in their striata to find

differences between EB- versus vehicle-treated animals of each genotype and to thereby determine the sensitivity of each genotype to estrogen treatment.

What results would I expect if the data supported this hypothesis? For the Met-related estrogen insensitivity hypothesis, I would examine the *post hoc* pairwise comparisons and expect to observe that EB-treated Met/Met rats would not show any significant differences in GSK3β phosphorylation from vehicle-treated Met/Mets***.*** In contrast, I would expect to observe increased phosphorylation in EB-treated Val/Val rats when compared to vehicle-treated Val/Val rats, as estrogens have historically augmented BDNF release in wildtype/control individuals. I have predicted, due to altered or perhaps even saturated release in Met allele carriers, that the Met rats would therefore not show enhanced GSK3β phosphorylation when treated with EB. While Met carriers indeed did not show EB-associated enhancements, neither did the Val carriers, so both genotypes were insensitive. Perhaps, since the rats were previously behaviorally trained, rats of both genotypes likely already experienced increases in regulated BDNF in response to the striatum-based learning. This regulated release may have reached the “saturation” point wherein it could not be notably enhanced by estrogen treatment.

Technical issues with the replicates and how to fix them

In replicate 1, the standard curve for pan-GSK3β is not strongly or significantly linear for blot 2. All other blots show significantly linear standard curves for both signals (Data not shown). Standard curves for protein signals are used to examine between-blot variability. All blots have small, insignificant artifacts for both red and green images that do not seem to interfere with band measurements (Appendix A & B). This is indicative of some minor contamination that would may mitigated in the future by cleaning equipment properly and washing the membranes sufficiently.

The standard curves and appearances of blots in replicate 2, however, indicated far greater problems than for replicate 1. For example, standard curves for both GSK3β and phospho-GSK3β signals are not as strongly linear and are not significant in blots 2 and 4 of replicate 2 (Data not shown). There is also some background for pan-GSK3β (red) images (Appendix E), but somewhat less than with Replicate 1. Background is significantly worse in Replicate 2 than in Replicate 1 for phospho-GSK3β, and there are significant artifacts – most notably in blots 1 and 2 - that overlap with the bands and may therefore interfere with the measurement of the protein signals (Appendix D). Blot 2 shows the worst artifacts out of all blots. Artifacts could be caused by contaminants on blots from the blocking reagent or even nonspecific binding of the antibodies to the blocking reagent (Mahmood & Yang, 2012). Contaminants should be filtered out of the blocking reagent, and special care should be taken in future experiments when handling the membrane to ensure that no contaminants interfere with the signal. Equipment (e.g. forceps, incubation containers, etc.) should be cleaned thoroughly and gloves should be replaced often to reduce the risk of contaminated blots and disrupting the signals.

The phospho-GSK3β band signals are also weak for blots 1 and 2 in replicate 2 (Appendix D). It is possible that the antigen levels were insufficient and not as much protein was loaded into these gels. Upon observing the REVERT total protein stains at 700 nm, it appears that blots 1 and 2 have slightly weaker total protein signals than blots 3 and 4 (Appendix F), suggesting a possible loading inconsistency. Care will have to be taken in the future to ensure more consistent loading across wells. Measures to prevent protein degradation must also be taken, including keep samples consistently cold and distributing them into single-use aliquots. In addition, since the phospho-GSK3β signal on only two out of the four blots in this replicate was weak for this replicate, it is also possible that antibody incubation may have been inconsistent between blots, with some membranes not being fully submerged in antibody cocktails or getting stuck to the sides of containers during incubation. Care will be taken in the future to ensure that blots remain at the bottom of the container and properly submerged in cocktail during the incubation process. Shaking during incubation should also ensure even distribution of the antibody across each membrane.

Discussion

Hypothesis 1: Met carriers will experience augmented BDNF release – and thus, increased GSK3β phosphorylation – in compensation for altered regulated BDNF signaling. The results are somewhat similar between technical replicates, with some differences. In the first technical replicate alone, there was a significant main effect of genotype on GSK3β phosphorylation status, with Met carriers exhibiting higher phosphorylation ratios than Val carriers. Specifically, vehicle-treated Met/Met rats in this experiment demonstrated significantly greater GSK3β phosphorylation than did vehicle-treated Val/Val rats. Similar results were found for the second replicate and for the combined replicates, except that there were no pairwise differences between groups.

Prior evidence suggests that the Met substitution can trigger a compensatory increased expression of BDNF and TrkB mRNA in the rodent brain (Spencer et al., 2010), which may be reflected in the trends indicating increased downstream GSK3β phosphorylation in Met/Met rats. It has been proposed that this phenomenon occurs in compensation for the Met-allele-induced deficit in activity-dependent BDNF release and that it may either mitigate or contribute to the behavior phenotype of $BDNF_{Met}$ mice, which includes impaired object recognition (Spencer et al., 2010). This proposed compensatory molecular mechanism may thus explain the trend indicating higher levels GSK3β phosphorylation in the Met/Met ovariectomized female rats here when compared to Val/Val rats. Moreover, in human participants with generalized anxiety disorder, the Met allele was significantly associated with higher serum levels of BDNF (Bus et al., 2012; Lang et al., 2009; Moreira et al., 2015), indicating greater BDNF synthesis in these individuals. Activity-dependent secretion – not constitutive expression

– is attenuated in Met individuals (Chen et al., 2004), but the constitutive upregulation of peripheral BDNF concentrations in Met allele carriers may occur in compensation for defective intracellular protein signaling (Bus et al., 2012; Egan et al., 2003; Lang et al., 2009; Moreira et al., 2015). Furthermore, human Met-allele carriers with probable post-traumatic stress disorder (PTSD) showed significantly higher plasma BDNF levels than Val carriers with probable PTSD, as well as control Val and Met carriers (Zhang et al., 2014). Thus, Met carriers can indeed overexpress BDNF, especially in response to stress.

It therefore follows that this compensatory phenomenon would be reflected in my results, in which I note a main effect of genotype in which Met/Met rats show significantly greater phosphorylation of GSK3β, a downstream target of BDNF.

Hypothesis 2: Met carriers will show no sensitivity to the BDNF-enhancing effects of estradiol. The results presented here do not corroborate this hypothesis, regardless of the replicate being analyzed. Neither replicate showed pairwise differences in GSKβ phosphorylation between EB- and vehicle-treated Met/Met rats or between EB- and vehicle-treated Val/Val rats. The results were similar even when the replicates were averaged and combined. Thus, it appears that both genotypes in this experiment were insensitive to estradiol enhancement of the BDNF-TrkB-GSK3β signaling pathway.

These results conflict with prior evidence, which has established that estrogens can promote TrkB-BDNF signaling – and thus, likely boost downstream GSK3β phosphorylation – via transactivational and epigenetic means (Kiss et al., 2012; Luine & Frankfurt, 2013; Pan et al., 2010) in the hippocampus. Based on this concept, EB

treatments should have boosted available BDNF levels, regulated BDNF release, or kinetics of TrkB signaling in Val/Val rats, leading to higher levels of phosphorylated GSK3β. There was no difference, however, in GSK3β activation between vehicle-treated rats and EB-treated rats in either the Met/Met or Val/Val genotype, suggesting that both genotypes in my experiment were insensitive to estrogen-mediated BDNF enhancements.

Of note, Met/Met rats showed significant endocrine dysregulation, evidenced by their abnormal estrous cycles – namely, they become either acyclic or experience irregular cycles, meaning they have at least one proestrus or proestrus-to-estrus day, with less than three 4-5 day cycles – prior to ovariectomy and reduced ovarian weight and increased uterine horn weight post-euthanasia (Prakapenka et al., 2021).

Because Met carriers demonstrate deficits in regulated BDNF secretion, it follows that they would not be sensitive to estrogen-enhancing effects on TrkB-BDNF signaling when compared to Val carriers, but my findings – in which Met and Val carriers both show insensitivity to estrogen - do not support this idea and seem to conflict with prior literature. For instance, Met mice have shown better performance even than Val mice in object placement tasks during proestrus, when estrogen levels are rising (Spencer et al., 2010), indicating that Met rodents demonstrate considerable sensitivity to the enhancing effects of estrogen during hippocampus-sensitive tasks. In my experiment, it was the striatum, not the hippocampus, that was assessed for GSK3β phosphorylation in Val versus Met carriers treated with estrogen. Perhaps, estrogen sensitivity can thus be altered in a region-specific manner, with some regions (like the hippocampus) showing Met-related alterations in estrogen sensitivity and others demonstrating no

effects in this regard. If estrogen sensitivity is region-specific, I propose that I would have noted differences in GSK3β phosphorylation between vehicle- and EB-treated Met/Met rats, had I conducted by western blots instead on the hippocampus. However, I opted to study the striatum, as the dOR task performed on the rats by Prakapenka et al. (2021) was striatum-sensitive, and I therefore intended to explore connections between behavior and BDNF-TrkB-GSK3β signaling pertaining to that region.

In addition, when ovariectomized and given estradiol in their drinking water, mice heterozygous for the Met allele exhibited significantly greater anxiety-like phenotypes in the splash test and open-field tests when compared to wildtype mice given the same treatment (Marrocco et al., 2020), indicating that the Met allele carriers exhibit increased sensitivity to estrogens, which, in turn, ostensibly increased their susceptibility to stress.

Limitations. The results of this investigation show trends consistent with the hypothesis that Met rats show compensatory increases in BDNF and TrkB. However, this study is held back by some limitations. For example, I had intended to produce three technical replicates in the semiquantitative western blotting, thereby increasing statistical power and better accounting for some experimental variability for each given sample on a given day. Had I obtained more successful technical replicates, I could have had more blots containing more biological replicates, resulting in a higher confidence level. Unfortunately, upon imaging for GSK3β and phospho-GSK3β on the Li-Cor Odyssey, the third replicate showed faintness and inconsistency in the bands that rendered it far too difficult to accurately measure signal intensity (Appendix G & H), despite REVERT 700 signals for this replicate being strong (Appendix I). Because of this, I attempted a fourth replicate, in which the REVERT 700 signal revealed no protein

staining, indicating protein degradation (not shown). I therefore only ended up with two technical replicates, each consisting of four blots for a total of eight.

I examined the linearity of raw measure (i.e., GSK3β and phospho-GSK3β signals) standard curves (indicated by high correlation coefficient, ideally R^2 equal to or greater than 0.96) of these blots, as this measure indicates lower between-blot variability. Run 1, blot 2 showed a lower correlation coefficient ($R^2 = 0.9541$, data not shown) and significance value of the raw GSK3β standard curve ($p = 0.1375$, data not shown). This statistical evidence indicates a potential issue that may have occurred in the immunoblotting process for this blot, such as perhaps this blot not being fully submerged in cocktail during the incubation process or receiving lower antibody concentration due to antibody preparation error. Run 2, blots 2 and 4 showed poorer linearity of raw GSK3β standard curves ($R^2 = 0.9262$ and 0.9532, respectively, data not shown) and linear insignificance of raw phospho-GSK3β standard curves ($p = 0.1752$ and $p = 0.1388$, respectively, data not shown). Examination of run 2, blot 2 shows salient immunoblotting problems, with artifacts and bright signal speckling that may have interfered with the signal measurements of the target bands, particularly of phospho-GSK3β (Appendix D). Of note, two samples were excluded as outliers from replicate 2 (MOR47 from the Veh-Val/Val group and MOR12 from the EB-Met/Met), and one sample was excluded as an outlier when the runs were combined (MOR51 from the Veh-Val/Val group). Fixing the aforementioned experimental immunoblotting issues (most prominent in replicate 2) in future runs may mitigate signal interference and variability, thus hopefully obviating the potential need for outlier exclusions and resultant reductions in sample size.

Another experimental limitation is that the rats were behaviorally trained prior to brain dissection and use of their striata for western blotting. Behavioral training presents a confound to biochemical testing in that it produces changes in BDNF-TrkB signaling. BDNF mRNA expression is typically augmented after training in learning tasks (Cunha et al., 2010), so training in the dOR task may therefore have boosted BDNF expression and signaling in the striata of the rats, regardless of their genotype or treatment. The prior behavioral training of the rats could also explain the unexpected lack of estrogen sensitivity observed in both genotypes (as opposed to just the Met carriers). All rats have likely experienced previous increases in regulated striatal BDNF release in response to the striatum-based learning. These increases may have caused "saturation" of BDNF release, wherein the release could not be noticeably be augmented by estrogen treatment. This confound would have been eliminated if a separate group of CRISPR rats subjected to the same treatments had been dissected and used for western blotting analysis without first undergoing behavioral testing.

The complex regulation of GSK3β via other pathways besides BDNF-TrkB signaling constitutes another important confound in this experiment. For instance, GSK3β activity is also inhibited by the insulin/IGF1 and Wnt signaling pathways. When insulin or insulin-like growth factor 1 (IGF1) binds to the IGF1 receptor, Akt becomes active and phosphorylates GSK3β to suppress its activity, which entails suppression of glycogen synthesis and of eukaryotic translation initiation factor 2B (eIF2B), which regulates overall protein translation (Fogli & Boespflug-Tanguy, 2006; Souder & Anderson, 2019; Fig. 12). In addition, GSK3β activity is also regulated by the Wnt signaling pathway. A Wnt ligand binds to frizzled (Frz), and Axin forms a domain with GSK3β and

adenomatous polyposis coli (APC) (Souder & Anderson, 2019; Fig. 12). When Wnt signaling is absent, GSK3β is active, inhibiting mammalian target of rapamycin complex 1 (mTORC1, or Rheb-mTOR-Raptor) and cell growth (Souder & Anderson, 2019; Fig. 12). Likewise, GSK3β is suppressed by active mTORC1 in a negative feedback loop facilitated by ribosomal S6 kinase (S6K) (Souder & Anderson, 2019; Fig. 12). Because GSK3β activity is regulated by various other signaling pathways besides BDNF-TrkB, I therefore cannot necessarily rule out the possibility of these other pathways being activated to phosphorylate GSK3β by either the behavioral training by Prakapenka et al. (2021) or the genotype and treatment in my experiment.

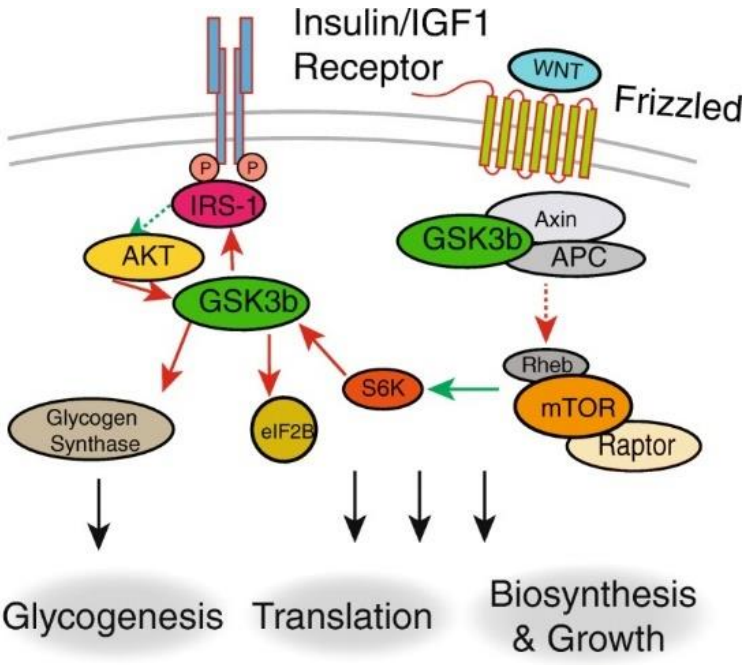


Fig. 12. *GSK3β activity is also regulated by the IGF1 and Wnt signaling pathways.* When the IGF1 receptor is activated, Akt phosphorylates and inhibits GSK3β, which would otherwise suppress glycogen synthesis and the protein eIF2B, a regulator of overall protein translation (Fogli & Boespflug-Tanguy, 2006; Souder & Anderson, 2019). Furthermore, when a Wnt ligand binds to frizzled (Frz), and Axin forms a domain with GSK3β and the protein APC. GSK3β is active in the absence of Wnt signaling, suppressing mammalian target of mTORC1 and cell growth. mTORC1 can likewise inhibit GSK3β via S6K (Souder & Anderson, 2019).

Future directions. The results from the individual and combined replicates – in which the Met carriers showed enhanced downstream GSK3β inactivation – could be attributed to the compensatory increases in BDNF and TrkB to counteract reduced BDNF bioavailability, a characteristic of this genotype. However, GSK3β phosphorylation in Met/Met rats was insensitive to the BDNF-boosting properties of estrogens, a result that did not contrast with that of the Val/Val rats in either or both replicates. I would therefore like to repeat this experiment, taking special care to avoid the various technical issues detailed in the Results section. If the results are reproduced – again showing that the striata of both Val and Met carriers are insensitive to BDNF enhancements by estradiol – then future investigations are crucial to comprehend why exactly the BDNF-TrkB signaling mechanisms denoted by downstream GSK3β activation state in the striatum are insensitive to estrogens in Met and Val carriers. I propose an experiment which, like the current experiment, involves a two-by-two design with Val/Val and Met/Met rats being treated with either vehicle or EB. I would euthanize the animals and extract the striata for semiquantitative western blot analysis for BDNF protein, as well as for TrkB and phospho-TrkB (the activated form of the receptor after BDNF binding), to determine the effects of genotype and treatment on these protein levels. Specifically, if the Met-related BDNF compensation hypothesis holds true (as previously supported by the results of the individual and combined replicates in the current investigation), then I would expect to see higher BDNF signals and phospho-to-pan-TrkB ratios (i.e., higher TrkB activation rates) in all Met carriers when compared to those of Val carriers. Furthermore, if phospho-TrkB to pan-TrkB ratios (i.e., TrkB activation levels) do not differ between vehicle- and EB-treated Met/Met rats or between

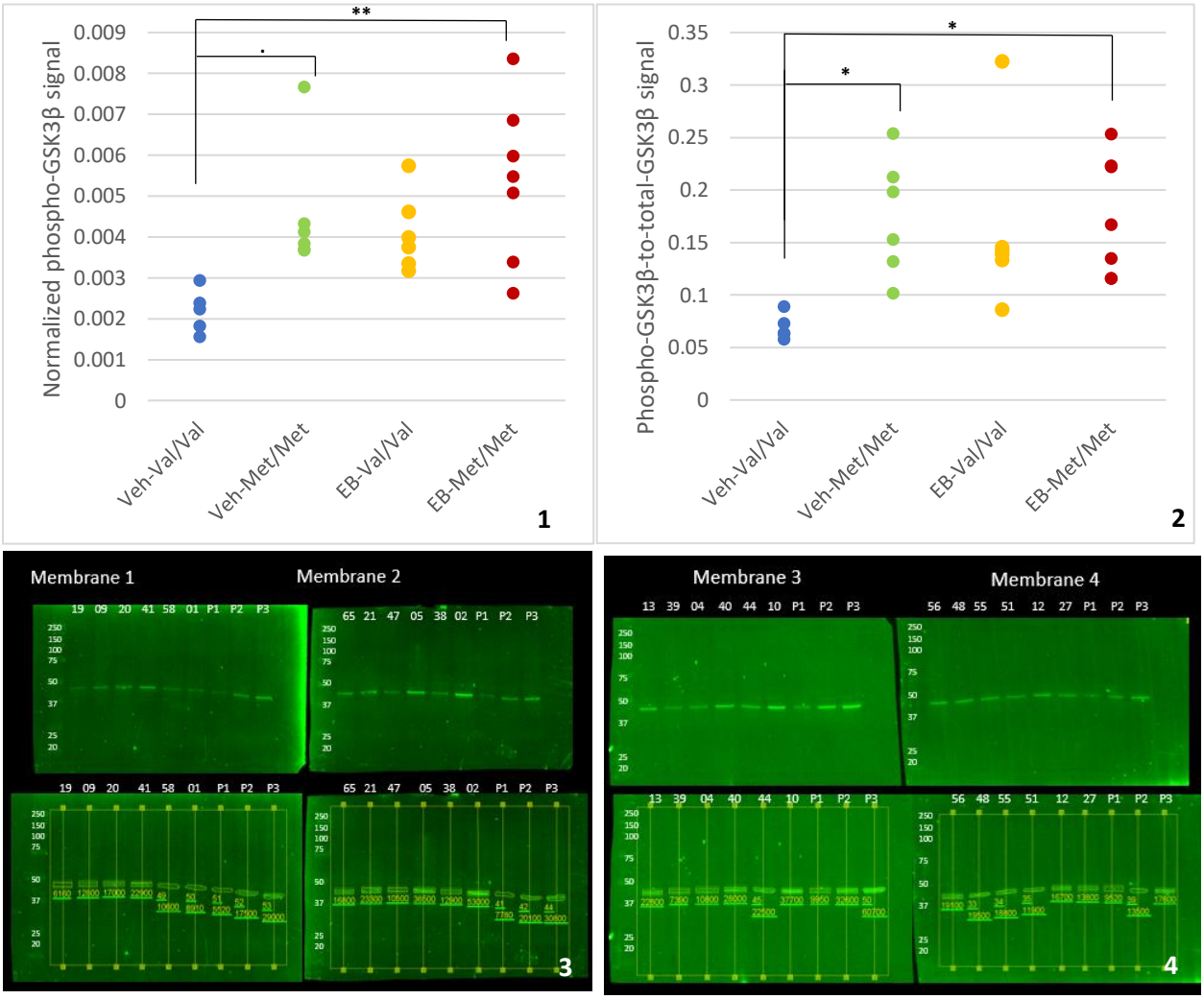
vehicle- and EB-treated Val/Val rats, then this evidence would corroborate my previous result that the striata of both Met and Val carriers are insensitive to the BDNF-enhancing effects of estrogen, indicated by lack of BDNF binding to and activating the TrkB receptor to initiate the BDNF-TrkB signaling cascade. In contrast, if EB-treated rats show higher or lower TrkB phosphorylation ratios than do vehicle-treated rats in either genotype, then I would conclude that BDNF signaling in that genotype is indeed sensitive to estrogens. I could also perform this same investigation on the hippocampus to determine if estrogen sensitivity is region-specific in either genotype, namely, if Met or Val carriers demonstrate changes in GSK3β phosphorylation when treated with EB versus with vehicle.

In any case, the Met allele poses as a risk factor for various illnesses in humans, including Alzheimer's disease, anxiety, and depression (Marrocco et al., 2020). Therefore, further exploration into the intricacies of the proposed Met-related compensatory phenomenon and estrogen sensitivity may foster a deeper understanding of the exact regulation of BDNF production and secretion in individuals carrying the Met allele, thus paving way for refinement of therapeutics and personalization of treatments based on genotype.

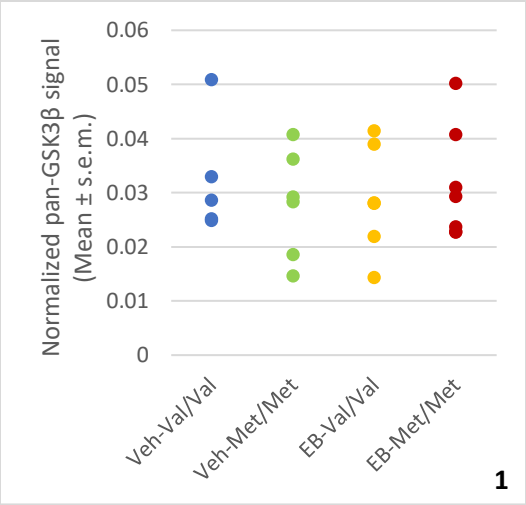
Appendices

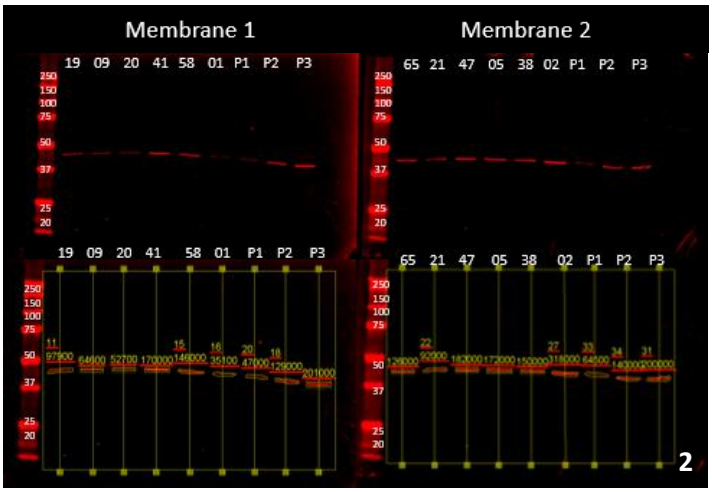
Data from the three completed runs

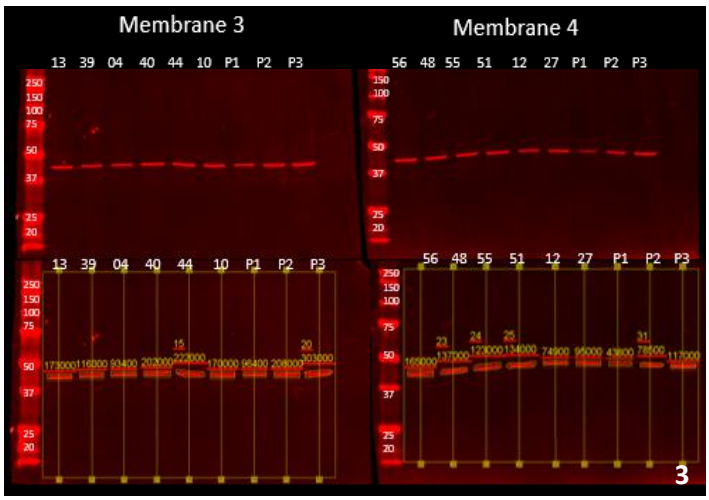
Run 1 (First technical replicate)



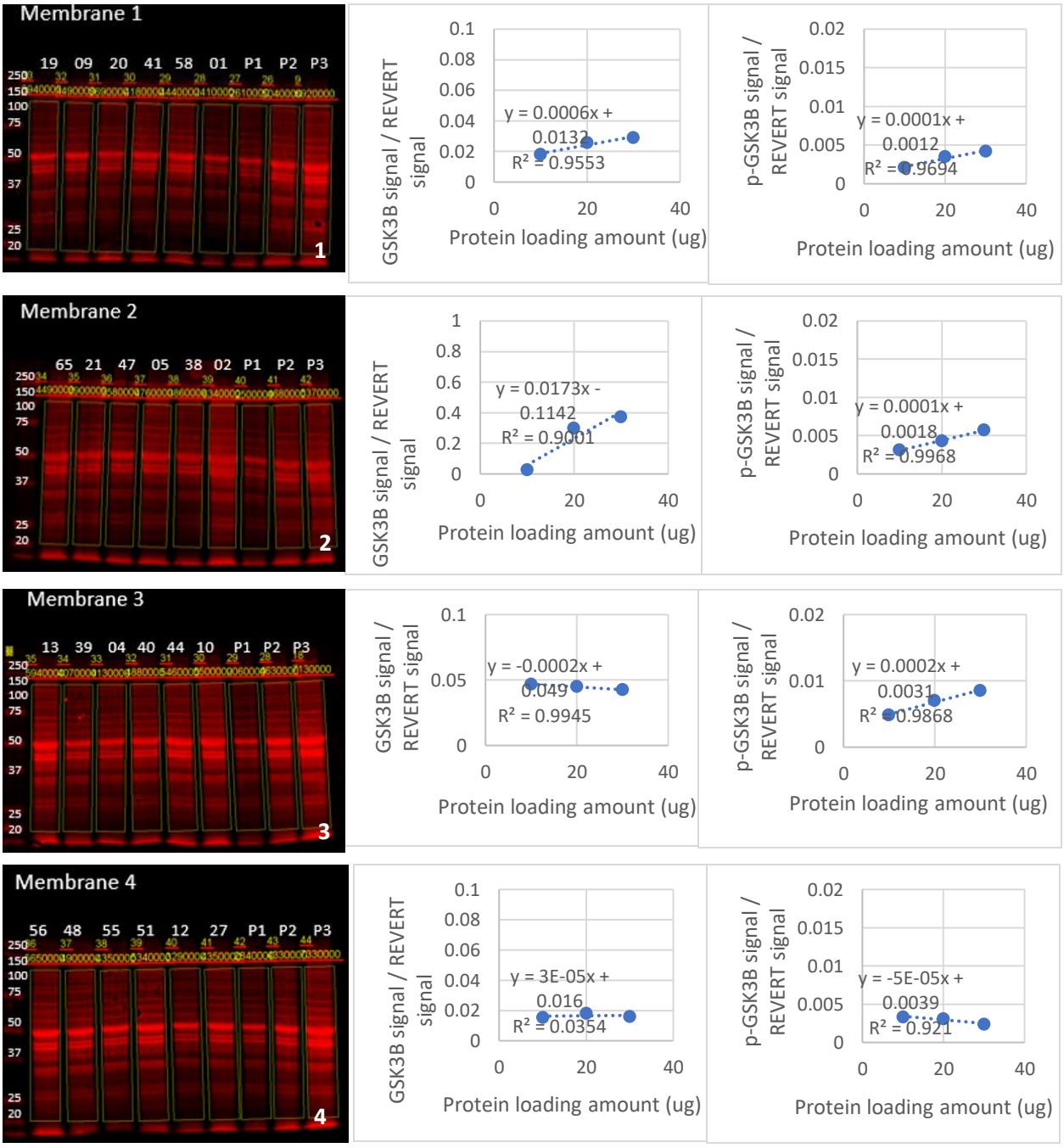
Appendix A. *Run 1 phospho-GSK3β protein signals and phosphorylation ratios.* (1) Normalized phospho-GSK3β signal is significantly affected by EB treatment ($F_{1,23}$ = 5.487; $p < 0.05$) and by CRISPR genotype ($F_{1,23}$ = 9.726; $p < 0.01$). (2) Ratio of phospho-GSK3β signals to pan-GSK3β signals in the striatum differs based on CRISPR genotype ($F_{1,23}$ = 6.169; $p < 0.05$). (3) and (4) Immunoblotting in Li-Cor Odyssey System at 800 nm wavelength for phospho-GSK3β protein signals across all four blots (membranes). Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 5, vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7.





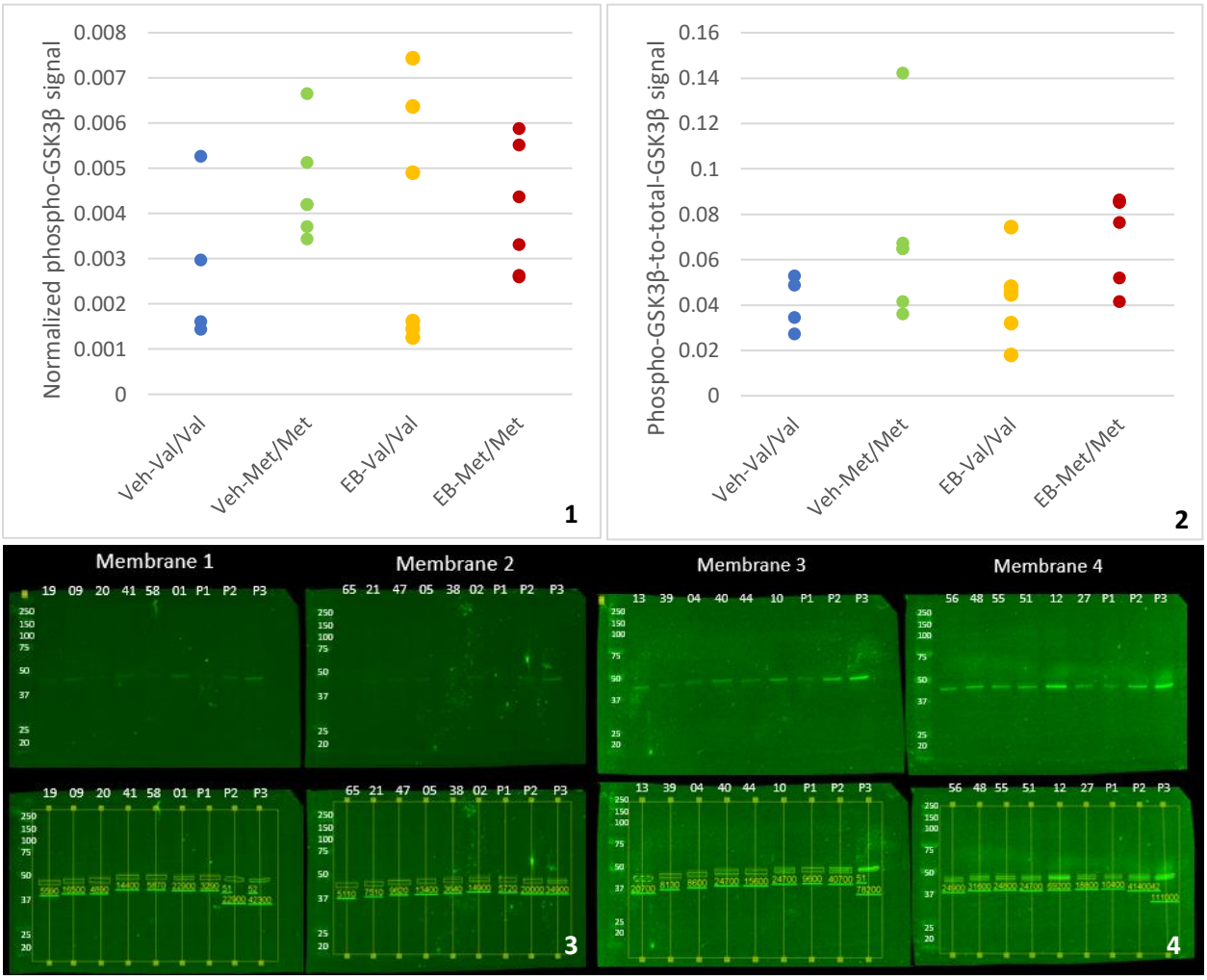


Appendix B. *Run 1 pan-GSK3β protein signals*. (1) Total GSK3β signal intensity is not significantly affected by CRISPR genotype ($F_{1,23}$ = 0.048; $p > 0.05$), treatment (i.e., vehicle or EB) ($F_{1,23}$ = 0.00; $p > 0.05$), or the interaction between them ($F_{1,23}$ = 0.724; $p > 0.05$). (2) and (3) Immunoblotting in Li-Cor Odyssey System at 700 nm wavelength for pan-GSK3β protein signals across all four blots (membranes). Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 5, vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7

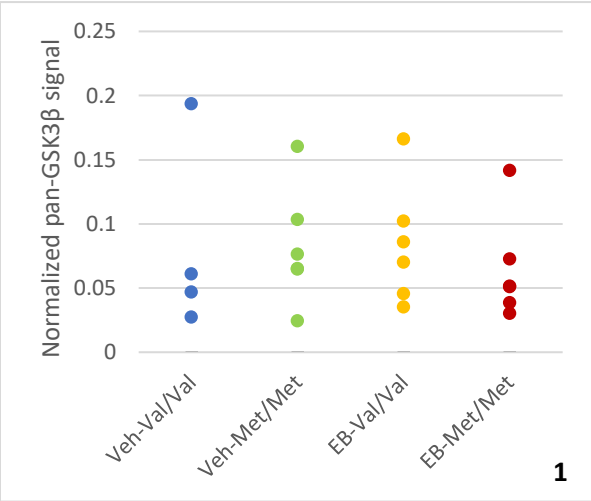


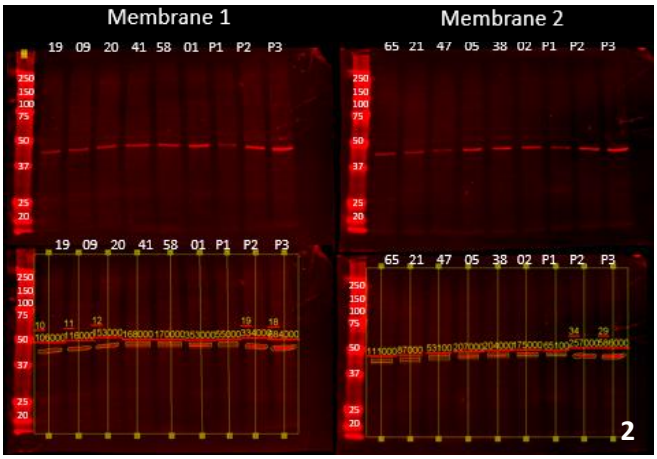
Appendix C. *Run 1 REVERT 700 total protein signals and normalized standard curves.* (1) Membrane/Blot 1 REVERT 700 total protein signals measured via Li-Cor Odyssey System at 700 nm with standard curves of pooled pan-GSK3β and phospho-GSK3β signals, each normalized to pooled REVERT total protein signals and plotted against protein loading amount (µg). (2) The same for Membrane/Blot 2, (3) Membrane/Blot 3, and (4) Membrane/Blot 4. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 5, vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 7

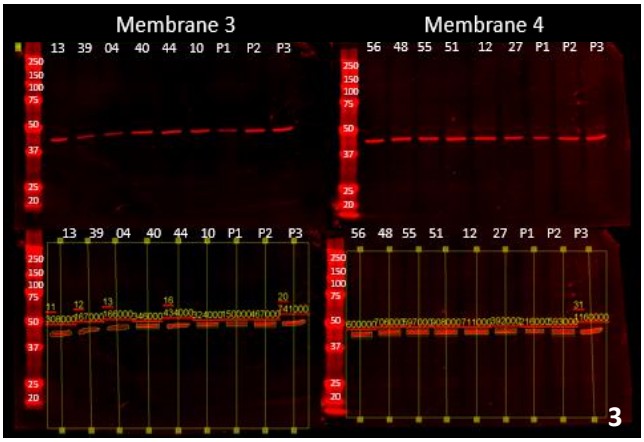
Run 2 (second technical replicate)



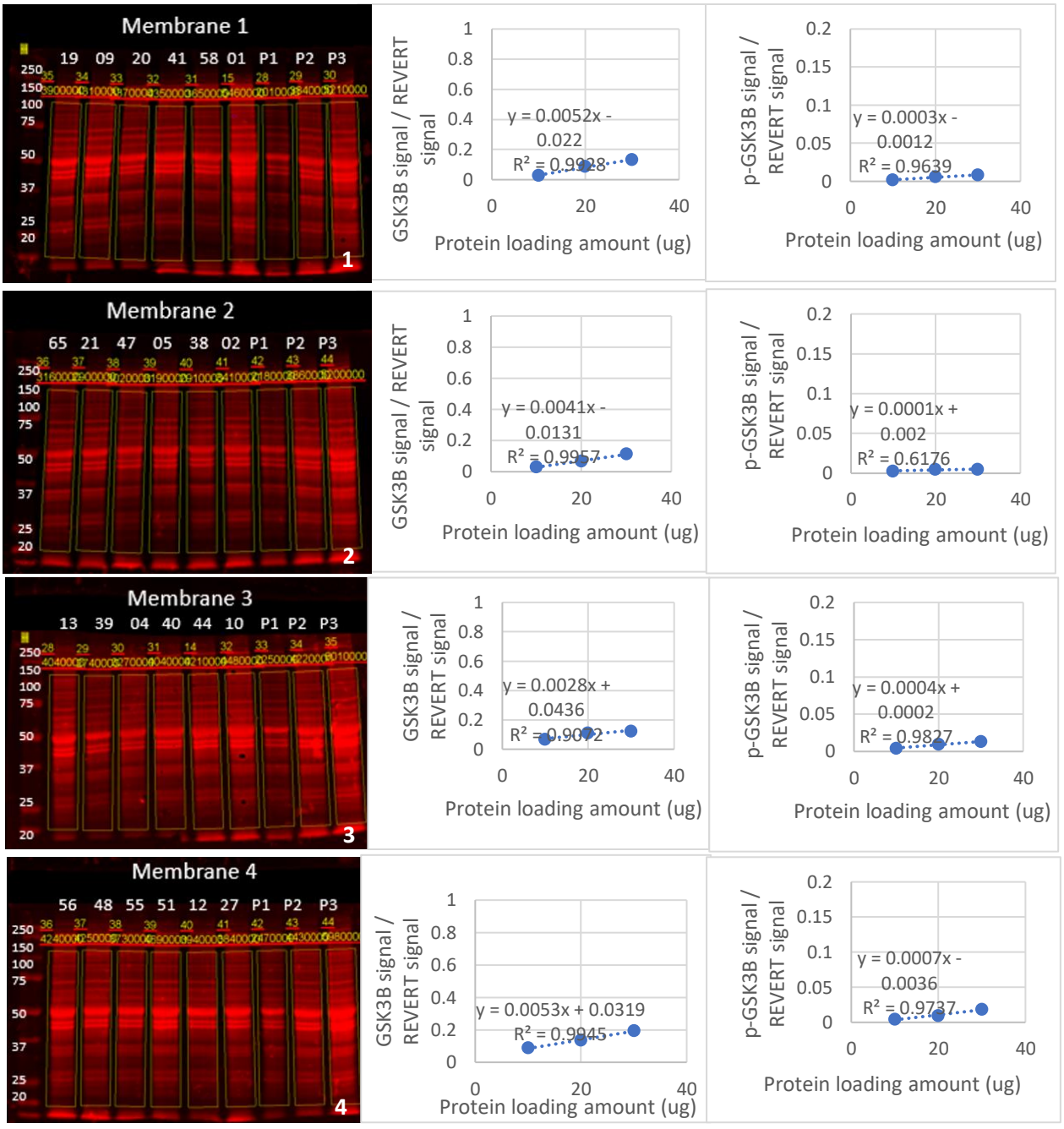
Appendix D. *Run 2 phospho-GSK3β protein signals and phosphorylation ratios*. (1) Normalized phospho-GSK3β signal is not significantly affected by genotype ($F_{1,21}$ = 1.19; $p > 0.05$) or treatment ($F_{1,21}$ = 0.011; $p > 0.05$). (2) Ratio of phospho-GSK3β signals to pan-GSK3β signals in the striatum differs between groups based on genotype ($F_{1,21}$ = 6.661; $p < 0.05$) but not treatment ($F_{1,21}$ = 0.002; $p > 0.05$). (3) and (4) Immunoblotting in Li-Cor Odyssey System at 800 nm wavelength for phospho-GSK3β protein signals across all four blots (membranes). Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR47 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 6 (MOR12 excluded, Grubbs p = 0.004).





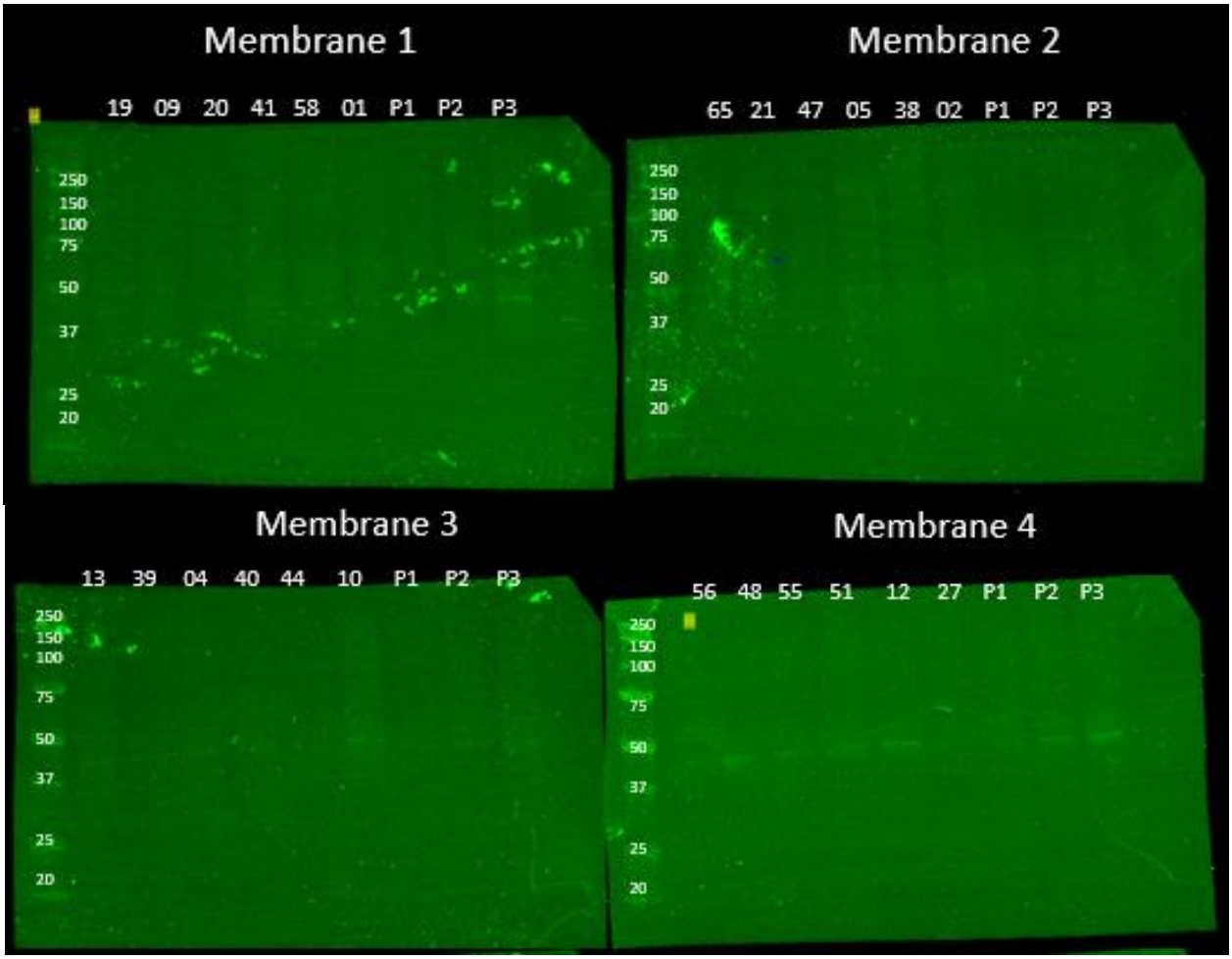


Appendix E. *Run 2 pan-GSK3β protein signals.* (1) Total GSK3β signal intensity is not significantly affected by CRISPR genotype ($F_{1,21} = 0.253$; $p > 0.05$), treatment ($F_{1,21} = 0.135$; $p > 0.05$), or the interaction between them ($F_{1,21} = 0.206$; $p > 0.05$). (2) and (3) Immunoblotting in Li-Cor Odyssey System at 700 nm wavelength for pan-GSK3β protein signals across all four blots (membranes). Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR47 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 6 (MOR12 excluded, Grubbs p = 0.004).

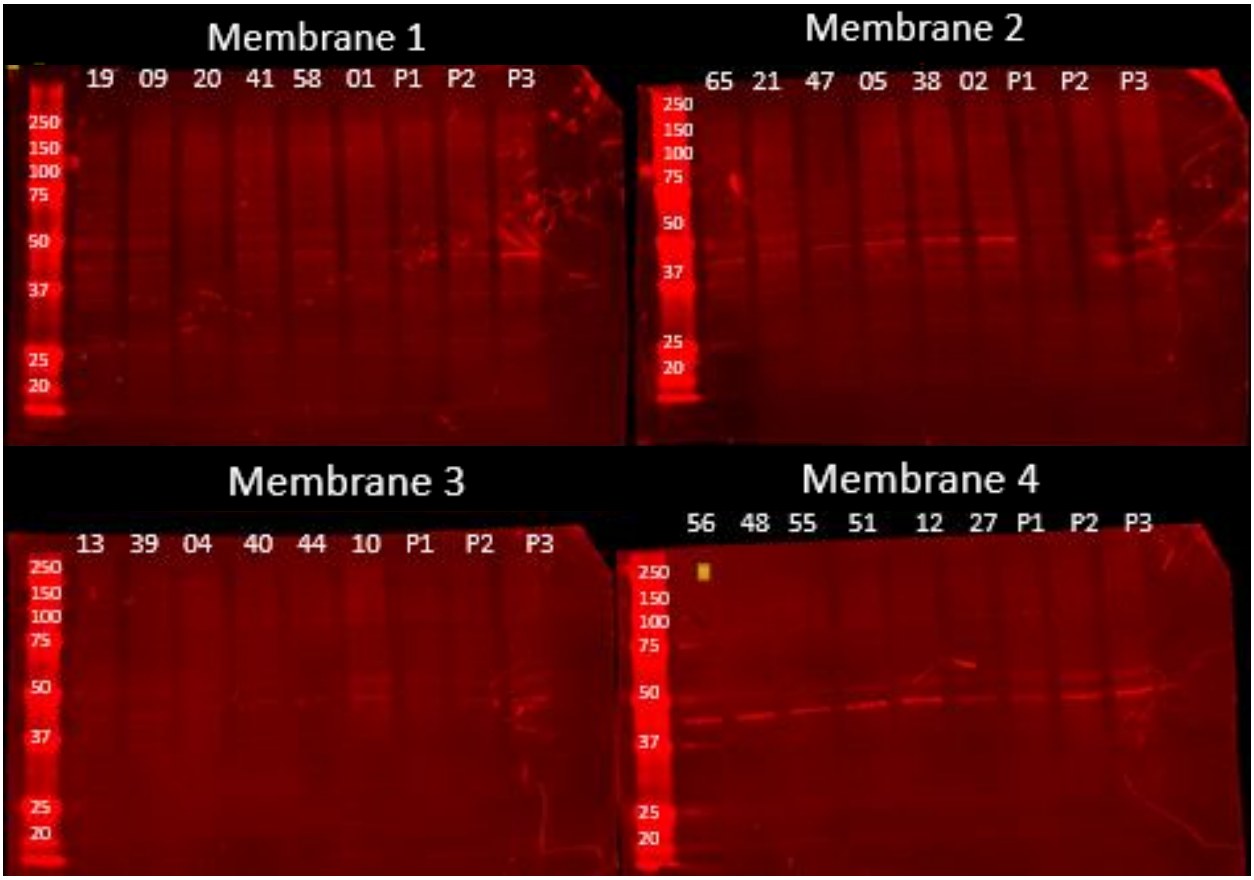


Appendix F. *Run 2 REVERT 700 Total protein signals and normalized standard curves*. 1) Membrane/Blot 1 REVERT 700 total protein signals measured via Li-Cor Odyssey System at 700 nm with standard curves of pooled pan-GSK3β and phospho-GSK3β signals, each normalized to pooled REVERT total protein signals and plotted against protein loading amount (µg). 2) The same for Membrane/Blot 2 and 3) Membrane/Blot 3 and 4) Membrane/Blot 4. Striatum: Vehicle-treated Val/Val (Veh-Val/Val) n = 4 (MOR47 excluded, Grubbs p = 0.015), vehicle-treated Met/Met (Veh-Met/Met) n = 6, EB-treated Val/Val (EB-Val/Val) n = 6, EB-treated Met/Met (EB-Met/Met) n = 6 (MOR12 excluded, Grubbs p = 0.004).

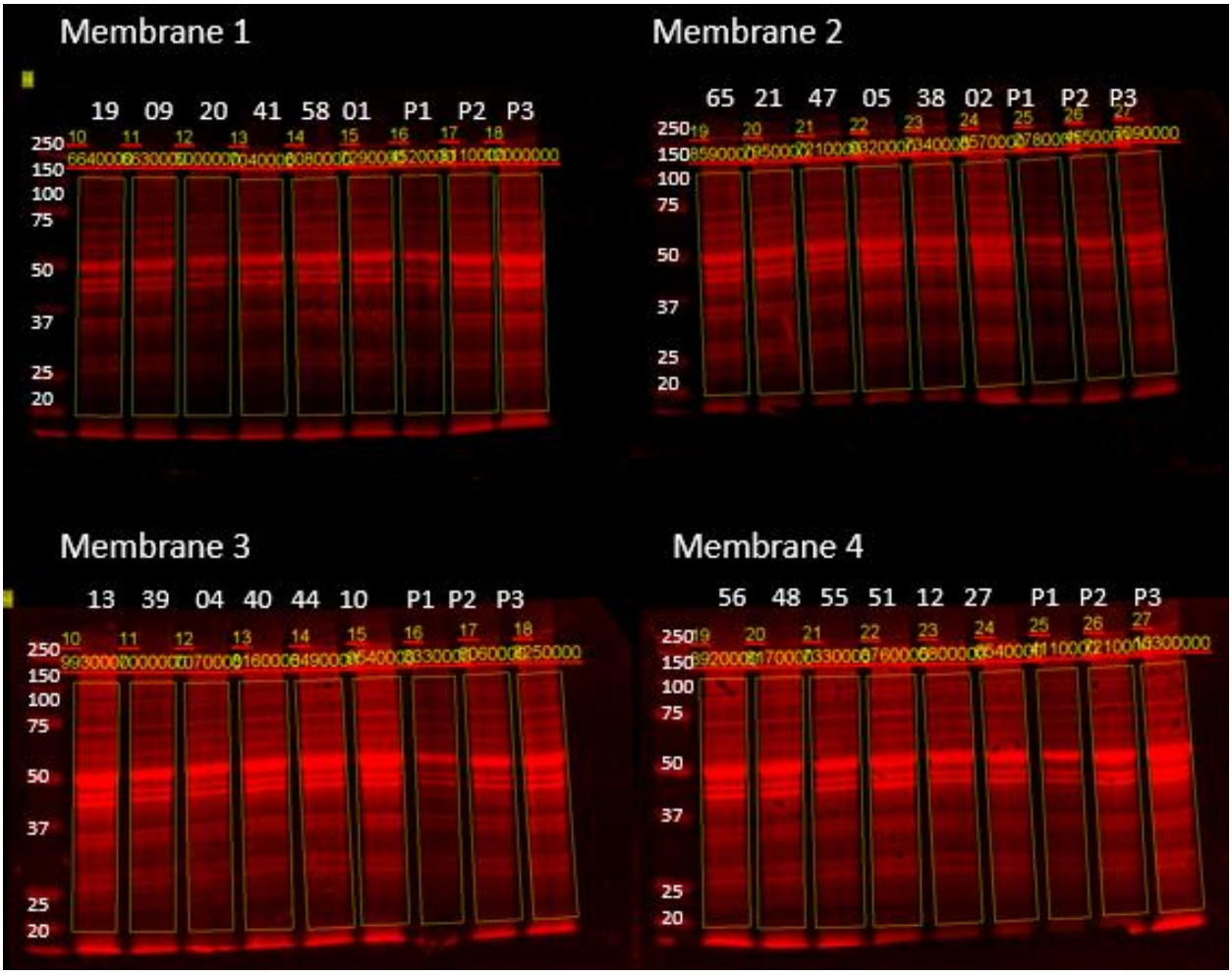
Run 3 (third technical replicate)



Appendix G. *Run 3 phospho-GSK3β protein signals.* Phospho-GSK3β protein signals were too weak and inconsistent across all four blots/membranes to adequately measure protein integrated signal intensity via the Li-Cor Odyssey System. Artifacts can be seen across Membranes 1 and 2, further interfering with signal measurement.



Appendix H. *Run 3 pan-GSK3β protein signals.* Pan-GSK3β protein signals were too weak and inconsistent across all four blots/membranes to adequately measure protein integrated signal intensity via the Li-Cor Odyssey System. Artifacts can be seen across Membrane 1, further interfering with signal measurement.



Appendix I. *Run 3 REVERT 700 Total protein signals.* REVERT 700 total protein signals were visually consistent and strong across Membranes/Blots 1-4, despite some dark spots and interference observed across Membrane 4.

References

Aid, T., Kazantseva, A., Piirsoo, M., & Palm, K. (2007). Mouse and Rat BDNF Gene Structure and Expression Revisited. *Journal of Neuroscience Research, 535*(April 2006), 525–535.

Albeely, A. M., Williams, O. O. F., & Perreault, M. L. (2022). GSK-3β Disrupts Neuronal Oscillatory Function to Inhibit Learning and Memory in Male Rats. *Cellular and molecular neurobiology*, *42*(5), 1341–1353. https://doi.org/10.1007/s10571-020-01020-z

Ambalavanar, M.T., Gardner, R.S., Gold, P.E., & Korol, D.L. (2019). Enhanced response learning with intrastriatal infusions of a BDNF mimic, 7,8-dihydroxyflavone. 49th Annual Meeting for the Society for Neuroscience, *Society for Neuroscience Abstracts*, 45, 513.15.

Andero, R., Choi, D. C., & Ressler, K. J. (2014). BDNF-TrkB receptor regulation of distributed adult neural plasticity, Memory formation, and psychiatric disorders. *Progress in Molecular Biology and Translational Science*, *122*, 169–192. https://doi.org/10.1016/B978-0-12-420170-5.00006-4

Andero, R., Heldt, S.A., Ye, K., Liu, X., Armario, A., & Ressler, K.J. (2011). Effect of 7,8-dihydroxyflavone, a small-molecule TrkB agonist, on emotional learning. *Am J Psychiatry, 168*(2), 163–172.

Arpino, G., Wiechmann, L., Osborne, C. K., & Schiff, R. (2008). Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocrine reviews*, *29*(2), 217-233.

Bahrami-B, F., Ataie-kachoie, P., Pourgholami, M. H., & Morris, D. L. (2014). P70 Ribosomal protein S6 kinase (Rps6kb1): an update. *J Clin Pathol, 0,* 1–7. https://doi.org/10.1136/jclinpath-2014-202560

Balkowiec, A., & Katz, D. M. (2002). Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *22*(23), 10399–10407. https://doi.org/10.1523/JNEUROSCI.22-23-10399.2002

Banner, H., Bhat, V., Etchamendy, N., Joober, R., & Bohbot, V.D. (2011). The brain-derived neurotrophic factor Val66Met polymorphism is associated with reduced functional magnetic resonance imaging activity in the hippocampus and increased use of caudate nucleus dependent strategies in a human virtual navigation task. *Eur J Neurosci. 33*(5), 968–977.

Barde, Y. A., Davies, A. M., Johnson, J. E., Lindsay, R. M., & Theonen, H. (1987). Chapter 15 Brain derived neurotrophic factor. In Seil, F. J., Herbert, E., & Carlson, B. M. (Eds.), *Progress in Brain Research: Neural Regeneration* (Vol. 71, pp. 185-189). Elsevier.

Barker, G. R., & Warburton, E. C. (2011). When is the hippocampus involved in recognition memory?. *Journal of Neuroscience*, *31*(29), 10721-10731. https://doi.org/10.1523/JNEUROSCI.6413-10.2011

Barletta, F., Wong, C. W., McNally, C., Komm, B. S., Katzenellenbogen, B., & Cheskis, B. J. (2004). Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. *Molecular endocrinology*, *18*(5), 1096-1108.

Bath, K. G., & Lee, F. S. (2006). Variant BDNF (Val66Met) impact on brain structure and function. *Cognitive, Affective and Behavioral Neuroscience*, *6*(1), 79–85. https://doi.org/10.3758/CABN.6.1.79

Bath, K. G., Chuang, J., Spencer-segal, J. L., Amso, D., Altemus, M., Mcewen, B. S., & Lee, F. S. (2012). Variant Brain-Derived Neurotrophic Factor (Valine66Methionine) Polymorphism Contributes to Developmental and Estrous Stage-Specific Expression of Anxiety-Like Behavior in Female Mice. *Biol Psychiatry, 72,* 499-504. https://doi.org/10.1016/j.biopsych.2012.03.032

Bear, M. F., & Abraham, W. C. (1996). Long-term depression in hippocampus. *Annual review of neuroscience*, *19*(1), 437-462.

Bekinschtein, P., Cammarota, M., & Medina, J. H. (2014). BDNF and memory processing. *Neuropharmacology*, *76*, 677-683.

Berlyne, D.E. (1950). Novelty and curiosity as determinants of exploratory behaviour. *Brit. J. Psychol., 41*, 68–80.

Beurel, E., Grieco, S. F., & Jope, R. S. (2015). Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacology and Therapeutics*, *148*, 114–131. https://doi.org/10.1016/j.pharmthera.2014.11.016

Bhat, R. V., Budd Haeberlein, S. L., & Avila, J. (2004). Glycogen synthase kinase 3: A drug target for CNS therapies. *Journal of Neurochemistry*, *89*(6), 1313–1317. https://doi.org/10.1111/j.1471-4159.2004.02422.x

Bhat R. V., Shanley J., Correll M. P., Fieles, W. E., Keith R. A., Scott C.W. & Lee C. M. (2000) Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. *Proc Natl Acad Sci USA, 97*, 11074–11079.

Bimonte-Nelson, H. A., Nelson, M. E., & Granholm, A. C. (2004). Progesterone counteracts estrogen-induced increases in neurotrophins in the aged female rat brain. *Neuroreport, 15*, 2659–2663.

Blechkarz-Klin, K., Wawer, A., Jawna-Zboińska, K., Pyrzanowska, J., Piechal, A., Mirowska-Guzel, D., Widy-Tyszkiewicz, E. (2018). Early paracetamol exposure decreases brain-derived neurotrophic factor (BDNF) in striatum and affects social behaviour and exploration in rats. *Pharmacology Biochemistry and Behavior, 168*, 25-32.

Boots, E. A., Schultz, S. A., Clark, L. R., Racine, A. M., Darst, B. F., Koscik, R. L., Carlsson, C. M., Gallagher, C. L., Hogan, K. J., Bendlin, B. B., Asthana, S., Sager, M. A., Hermann, B. P., Christian, B. T., Dubal, D. B., Engelman, C. D., Johnson, S.

C., & Okonkwo, O. C. (2017). BDNF Val66Met predicts cognitive decline in the Wisconsin Registry for Alzheimer's Prevention. *Neurology*, *88*(22), 2098–2106. https://doi.org/10.1212/WNL.0000000000003980

Bramham, C. R., & Messaoudi, E. (2005). BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Progress in neurobiology*, *76*(2), 99-125.

Brigadski, T., & Leßmann, V. (2020). The physiology of regulated BDNF release. *Cell and tissue research*, *382*(1), 15–45. https://doi.org/10.1007/s00441-020-03253-2

Bueller, J.A., Aftab, M., Sen, S., Gomez-Hassan, D., Burmeister, M., & Zubieta, J.K. (2006). BDNF Val66Met allele is associated with reduced hippocampal volume in healthy subjects. *Biol Psychiatry, 59*(9), 812–815.

Burkhalter, J., Fiumelli, H., Allaman, I., Chatton, J.Y., & Martin, J.L. (2003). Brain-derived neurotrophic factor stimulates energy metabolism in developing cortical neurons. *J Neurosci, 23,* 8212-8220.

Bus, B. A., Arias-Vasquez, A., Franke, B., Prickaerts, J., de Graaf, J., & Voshaar, R. C. (2012). Increase in serum brain-derived neurotrophic factor in met allele carriers of the BDNF Val66Met polymorphism is specific to males. *Neuropsychobiology*, *65*(4), 183–187. https://doi.org/10.1159/000336997

Cao, L., Dhilla, A., Mukai, J., et al. (2007). Genetic modulation of BDNF signaling affects the outcome of axonal competition in vivo. *Curr Biol, 17*(11), 911–921.

Carmichael, J., Sugars, K. L., Bao, Y. P., & Rubinsztein, D. C. (2002). Glycogen synthase kinase-3beta inhibitors prevent cellular polyglutamine toxicity caused by the Huntington's disease mutation. *The Journal of biological chemistry*, *277*(37), 33791–33798. https://doi.org/10.1074/jbc.M204861200

Cefis, M., Quirié, A., Pernet, N., Marie, C., Garnier, P., & Prigent-Tessier, A. (2020). Brain-derived neurotrophic factor is a full endothelium-derived factor in rats. *Vascular pharmacology*, *128-129*, 106674. https://doi.org/10.1016/j.vph.2020.106674

Cerasa, A., Tongiorgi, E., Fera, F., et al. (2010). The effects of BDNF Val66Met polymorphism on brain function in controls and patients with multiple sclerosis: an imaging genetic study. *Behav Brain Res, 207*(2), 377–386.

Chang, Q., & Gold, P. E. (2004). Inactivation of Dorsolateral Striatum Impairs Acquisition of Response Learning in Cue-Deficient, but Not Cue-Available, Conditions. *Behavioral Neuroscience, 118*(2), 383–388. https://doi.org/10.1037/0735-7044.118.2.383

Chen, Z. Y., Bath, K., McEwen, B., Hempstead, B., & Lee, F. (2008). Impact of genetic variant BDNF (Val66Met) on brain structure and function. *Novartis Foundation Symposium*, *289*, 180–188. https://doi.org/10.3758/CABN.6.1.79

Chen, Z.Y., Ieraci, A., Teng, H., et al. (2005). Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *J Neurosci, 25*, 6156–6166.

Chen, Z. Y., Jing, D., Bath, K. G., Ieraci, A., Khan, T., Siao, C. J., Herrera, D. G., Toth, M., Yang, C., McEwen, B. S., Hempstead, B. L., & Lee, F. S. (2006). Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science*, *314*(5796), 140–143. https://doi.org/10.1126/science.1129663

Chen, Z.Y., Patel, P.D., Sant, G., et al. (2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *J Neurosci, 24*, 4401–4411.

Chew, B., Ryu, J. R., Ng, T., Ma, D., Dasgupta, A., Neo, S. H., Zhao, J., Zhong, Z., Bichler, Z., Sajikumar, S., & Goh, E. L. K. (2015). Lentiviral silencing of GSK-3β in adult dentate gyrus impairs contextual fear memory and synaptic plasticity. *Frontiers in Behavioral Neuroscience*, *9*(JUNE), 1–12. https://doi.org/10.3389/fnbeh.2015.00158

Choi, C.H., Schoenfeld, B.P., Bell, A.J., Hinchey, P., Kollaros, M., Gertner, M.J., et al. (2011). Pharmacological reversal of synaptic plasticity deficits in the mouse model of Fragile X syndrome by group II mGluR antagonist or lithium treatment. *Brain Res, 1380*, 106–119.

Chuang, D.M., Wang, Z., & Chiu, C.T. (2011). GSK-3 as a target for lithium-induced neuroprotection against excitotoxicity in neuronal cultures and animal models of ischemic stroke. *Front Mol Neurosci 4,* 15.

Cikla, U., Chanana, V., Kintner, D. B., Udho, E., Eickhoff, J., Sun, W., Marquez, S., Covert, L., Otles, A., Shapiro, R. A., Ferrazzano, P., Vemuganti, R., Levine, J. E., & Cengiz, P. (2016). ERα Signaling Is Required for TrkB-Mediated Hippocampal Neuroprotection in Female Neonatal. *Disorders of the Nervous System, 3*(February 2016), 1–14. http://dx.doi.org/10.1523/ENEURO.0025-15.2015

Coelho, V. R., Gianesini, J., Von Borowski, R., Mazzardo-Martins, L., Martins, D. F., Picada, J. N., ... & Pereira, P. (2011). (−)-Linalool, a naturally occurring monoterpene compound, impairs memory acquisition in the object recognition task, inhibitory avoidance test and habituation to a novel environment in rats. *Phytomedicine*, *18*(10), 896-901.

Cunha, C., Brambilla, R., & Thomas, K. L. (2010). A simple role for BDNF in learning and memory?. *Frontiers in molecular neuroscience*, *3*, 865.

Damalas, A., Ben-Ze'ev, A., Simcha, I., Shtutman, M., Leal, J.F.M., Zhurinsky, J., Geiger, B. & Oren, M. (1999), Excess β-catenin promotes accumulation of transcriptionally active p53. *The EMBO Journal*, *18*, 3054-3063. https://doi.org/10.1093/emboj/18.11.3054

de Barreda, E. G., Pérez, M., Gómez-Ramos, P., et al. (2010). Tau- knockout mice show reduced GSK3-induced hippocampal degeneration and learning deficits. *Neurobiology of Disease, 37*(3), 622–629.

Dempster, E., Toulopoulou, T., McDonald, C., Bramon, E., Walshe, M., Filbey, F., ... & Collier, D. A. (2005). Association between BDNF val66 met genotype and episodic

memory. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, *134*(1), 73-75.

Dincheva, I., Glatt, C. E., & Lee, F. S. (2012). Impact of the BDNF Val66Met polymorphism on cognition: Implications for behavioral genetics. *Neuroscientist*, *18*(5), 439–451. https://doi.org/10.1177/1073858411431646

Egan, M. F., Kojima, M., Callicott, J. H., Goldberg, T. E., Kolachana, B. S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M., Lu, B., & Weinberger, D. R. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*, *112*(2), 257–269. https://doi.org/10.1016/S0092-8674(03)00035-7

Falkenberg, T., Mohammed, A. K., Henriksson, B., Persson, H., Winblad, B., & Lindefors, N. (1992). Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. *Neuroscience letters*, *138*(1), 153–156. https://doi.org/10.1016/0304-3940(92)90494-r

Ferrer, I., Barrachina, M., & Puig, B. (2002). Glycogen synthase kinase-3 is associated with neuronal and glial hyperphosphorylated tau deposits in Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. *Acta Neuropathol, 104*, 583–591.

Fogli, A., & Boespflug-Tanguy, O. (2006). The large spectrum of eIF2B-related diseases. *Biochemical Society transactions*, *34*(Pt 1), 22–29. https://doi.org/10.1042/BST20060022

Fortress, A. M., Kim, J., Poole, R. L., Gould, T. J., & Frick, K. M. (2014). 17β-Estradiol regulates histone alterations associated with memory consolidation and increases *Bdnf* promoter acetylation in middle-aged female mice. *Learning & Memory, 21,* 457–468. http://www.learnmem.org/cgi/doi/10.1101/lm.034033.113.

Frame, S., Cohen, P., & Biondi, R.M. (2001). A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Mol Cell, 7*, 1321–1327.

Franklin, A.V., King, M.K., Palomo, V., Martinez, A., McMahon, L., & Jope, R.S. (2014). Glycogen synthase kinase-3 inhibitors reverse deficits in long-term potentiation and cognition in Fragile X mice. *Biol Psychiatry, 75,* 198–206.

Frick, K. M. (2015). Hormones and Behavior Molecular mechanisms underlying the memory-enhancing effects of estradiol. *Hormones and Behavior*, *74*, 4–18. https://doi.org/10.1016/j.yhbeh.2015.05.001

Frodl, T., Schule, C., Schmitt, G., et al. (2007). Association of the Brain-Derived Neurotrophic Factor Val66Met Polymorphism With Reduced Hippocampal Volumes in Major Depression. *Arch Gen Psychiatry, 64*, 410–416.

García-Díaz Barriga, G., Giralt, A., Anglada-Huguet, M., Gaja-Capdevila, N., Orlandi, J. G., Soriano, J., ... & Alberch, J. (2017). 7, 8-dihydroxyflavone ameliorates cognitive and motor deficits in a Huntington's disease mouse model through specific activation of the PLCγ1 pathway. *Human molecular genetics*, *26*(16), 3144-3160.

Gardner, R. S., Newman, L. A., Mohler, E. G., Tunur, T., Gold, P. E., & Korol, D. L. (2020). Aging is not equal across memory systems. *Neurobiology of Learning and Memory, 172,* 1-14.

Gardner, R. S., Korol, D. L., & Gold, P. E. (2022). Hippocampus-sensitive and striatum-sensitive learning one month after morphine or cocaine exposure in male rats. *Pharmacology Biochemistry and Behavior*, *217*, 173392.

Gasic, G .P., Smoller, J. W., Perlis, R. H., et al. (2009). BDNF, relative preference, and reward circuitry responses to emotional communication. *Am J Med Genet B Neuropsychiatr Genet, 150B*(6), 762–781.

Goldberg, T. E., Iudicello, J., Russo, C., Elvevåg, B., Straub, R., Egan, M. F., & Weinberger, D. R. (2008). BDNF Val66Met polymorphism significantly affects d′ in verbal recognition memory at short and long delays. *Biological psychology*, *77*(1), 20-24.

Goodman, L. J., Valverde, J., Lim, F., Geschwind, M. D., Federoff, H. J., Geller, A. I., & Hefti, F. (1996). Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. *Molecular and cellular neurosciences*, *7*(3), 222–238. https://doi.org/10.1006/mcne.1996.0017

Guo, W., Nagappan, G., & Lu, B. (2018). Differential effects of transient and sustained activation of BDNF-TrkB signaling. *Developmental Neurobiology*, *78*(7), 647–659. https://doi.org/10.1002/dneu.22592

Guo, W., Ji, Y., Wang, S., Sun, Y., & Lu, B. (2014.) Neuronal activity alters BDNF–TrkB signaling kinetics and downstream functions. *J Cell Sci, 127*, 2249–2260.

Hanger, D. P., Betts, J. C., Loviny, T. L., Blackstock, W. P. & Anderton, B. H. (1998) New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry*. J Neurochem, 71*, 2465–2476.

Haniu, M., Montestruque, S., Bures, E. J., Talvenheimo, J., Toso, R., Lewis-sandy, S., Welcher, A. A., & Rohde, M. F. (1997). Interactions between Brain-derived Neurotrophic Factor and the TRKB Receptor. *Journal of Biological Chemistry*, *272*(40), 25296–25303. https://doi.org/10.1074/jbc.272.40.25296

Hansell, N. K., James, M. R., Duffy, D. L., Birley, A. J., Luciano, M., Geffen, G. M., ... & Martin, N. G. (2007). Effect of the BDNF V166M polymorphism on working memory in healthy adolescents. *Genes, Brain and Behavior*, *6*(3), 260-268.

Hariri, A.R., Goldberg, T.E., Mattay, V.S., et al. (2003). Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *J Neurosci, 23*, 6690–6694.

He, P., & Shen, Y. (2009). Interruption of β-catenin signaling reduces neurogenesis in alzheimer's disease. *Journal of Neuroscience*, *29*(20), 6545–6557. https://doi.org/10.1523/JNEUROSCI.0421-09.2009

Hernández, F., Borrell, J., Guaza, C., Avila, J., & Jucas, J. J. (2002). Spatial learning deficit in transgenic mice that conditionally over-express GSK-3β in the brain but do not form tau filaments. *Journal of Neurochemistry, 83,* 1529-1533.

Hooper, C., Markevich, V., Plattner, F., Killick, R., Schofield, E., Engel, T., et al. (2007). Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. *Eur J Neurosci, 25*, 81–86.

Huang, E. P. (1998). Synaptic plasticity: going through phases with LTP. *Current Biology, 8*(10), R350–R352.

Huang, Y. Z., & McNamara, J. O. (2010). Mutual regulation of Src family kinases and the neurotrophin receptor TrkB. *Journal of Biological Chemistry*, *285*(11), 8207-8217.

Jaehne, E. J., Kent, J. N., Antolasic, E. J., Wright, B. J., Spiers, J. G., Creutzberg, K. C., Rosa, F. De, Riva, M. A., Sortwell, C. E., Collier, T. J., & Buuse, M. Van Den. (2022). Behavioral phenotyping of a rat model of the BDNF Val66Met polymorphism reveals selective impairment of fear memory. *Translational Psychiatry, 12*(93), 1-11. https://doi.org/10.1038/s41398-022-01858-5

Jang, S.W., Liu, X., Yepes, M., et al. (2010). A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. *Proc Natl Acad Sci USA, 107,* 2687-2692.

Jing, D., Lee, F. S., & Ninan, I. (2017). The BDNF Val66Met polymorphism enhances glutamatergic transmission but diminishes activity-dependent synaptic plasticity in the dorsolateral striatum. *Neuropharmacology*, *112*, 84-93.

Jorge-Torres, O. C., Szczesna, K., Roa, L., Casal, C., Gonzalez-Somermeyer, L., Soler, M., ... & Esteller, M. (2018). Inhibition of Gsk3b reduces Nfkb1 signaling and rescues synaptic activity to improve the Rett syndrome phenotype in Mecp2-knockout mice. *Cell Reports*, *23*(6), 1665-1677.

Kaidanovich-Beilin, O., & Woodgett, J. R. (2011). GSK-3: Functional Insights from Cell Biology and Animal Models. *Frontiers in Molecular Neuroscience*, *4*(November), 1–25. https://doi.org/10.3389/fnmol.2011.00040

Ketharanathan, N., Rohlwink, U. K., Figaji, A. A., Wildschut, E. D., Tibboel, D., & de Lange, E. C. (2022). Brain microdialysis and applications to drug therapy in severe traumatic brain injury. In *Cellular, Molecular, Physiological, and Behavioral Aspects of Traumatic Brain Injury* (pp. 231-242). Academic Press.

Kim, H. S., Skurk, C., Thomas, S. R., Bialik, A., Suhara, T., Kureishi, Y., Birnbaum, M., Keaney, J. F., & Kenneth Walsh, S. (2002). Regulation of angiogenesis by glycogen synthase kinase-3β. *Journal of Biological Chemistry*, *277*(44), 41888–41896. https://doi.org/10.1074/jbc.M206657200

Kim, K., Pang, K. M., Evans, M., & Hay, E. D. (2000). Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. *Molecular biology of the cell*, *11*(10), 3509–3523. https://doi.org/10.1091/mbc.11.10.3509

Kim, W.Y., Zhou, F.Q., Zhou, J., Yokota, Y., Wang, Y.M., Yoshimura, T., et al. (2006). Essential roles for GSK-3 s and GSK-3-primed substrates in neurotrophin-induced and hippocampal axon growth. *Neuron, 52*, 981–996.

Kimura, T., Fukuda, T., Sahara, N., Yamashita, S., Murayama, M., Mizoroki, T., Yoshiike, Y., Lee, B., Sotiropoulos, I., Maeda, S., & Takashima, A. (2010). Aggregation of detergent-insoluble tau is involved in neuronal loss but not in synaptic loss*. J Biol Chem, 285*, 38692–38699.

Kimura, T., Yamashita, S., Fukuda, T., Park, J. M., Murayama, M., Mizoroki, T., Yoshiike, Y., Sahara, N., & Takashima, A. (2007). Hyperphosphorylated tau in parahippocampal cortex impairs place learning in aged mice expressing wild-type human tau*. EMBO J, 26*, 5143–5152.

Kimura, T., Yamashita, S., Nakao, S., Park, J. M., Murayama, M., Mizoroki, T., Yoshiike, Y., Sahara, N., & Takashima, A. (2008). GSK-3β is required for memory reconsolidation in adult brain. *PloS ONE, 3*, e3540. Doi: 10.1371/journal.pone.0003540

King, M.K., & Jope, R.S. (2013). Lithium treatment alleviates impaired cognition in a mouse model of fragile X syndrome. *Genes Brain Behav, 12*, 723–731.

King, M.K., Pardo, M., Cheng, Y., Downey, K., Jope, R.S., & Beurel, E. (2014). Glycogen synthase kinase-3 inhibitors: rescuers of cognitive impairments. *Pharmacol Ther, 141*, 1–12.

Kiss, Á., Márcia, A., Pereira, S. I. R., Carolino, R. G., Szawka, R. E., Anselmo-franci, J. A., Zanata, S. M., & Ferraz, A. C. (2012). 17β -Estradiol replacement in young, adult and middle-aged female ovariectomized rats promotes improvement of spatial reference memory and an antidepressant effect and alters monoamines and BDNF levels in memory- and depression-related brain areas. *Behavioural Brain Research*, *227*(1), 100–108. https://doi.org/10.1016/j.bbr.2011.10.047

Kleim, J. A., Chan, S., Pringle, E., Schallert, K., Procaccio, V., Jimenez, R., & Cramer, S. C. (2006). BDNF val66met polymorphism is associated with modified experience-dependent plasticity in human motor cortex. *Nature neuroscience*, *9*(6), 735-737.

Korol, D. L., Gardner, R. S., Tunur, T., & Gold, P. E. (2019). Involvement of Lactate Transport in Two Object Recognition Tasks That Require Either the Hippocampus or Striatum. *Behavioral Neuroscience*, *133*(2), 176–187. https://doi.org/10.1037/bne0000304

Korol, D. L., Gold, P. E., & Scavuzzo, C. J. (2013). Use it and boost it with physical and mental activity. *Hippocampus*, *23*(11), 1125-1135.

Korol, D. L., & Kolo, L. L. (2002). Estrogen-Induced Changes in Place and Response Learning in Young Adult Female Rats. *Behavioral Neuroscience, 116*(3), 411–420. https://doi.org/10.1037//0735-7044.116.3.411

Kundu, P., Korol, D. L., Bandara, S., Monaikul, S., Ondera, C. E., Helferich, W. G., Khan, I. A., Doerge, D. R., & Schantz, S. L. (2018). Hormones and Behavior Licorice root components mimic estrogens in an object location task but not an object recognition task. *Hormones and Behavior*, *103*(July), 97–106. https://doi.org/10.1016/j.yhbeh.2018.06.002

Lang, U., Hellweg, R., Sander, T. & Gallinat, J. (2009). The Met allele of the BDNF Val66Met polymorphism is associated with increased BDNF serum concentrations. *Mol Psychiatry, 14*, 120–122. https://doi.org/10.1038/mp.2008.80

Leeds, P.R., Yu, F., Wang, Z., Chiu, C.T., Zhang, Y., Leng, Y., et al. (2014). A new avenue for lithium: intervention in traumatic brain injury. *ACS Chem Neurosci, 5*(6), 422–433. Doi: 10.1021/cn500040g

Li, Q., Li, H., Roughton, K., Wang, X., Kroemer, G., Blomgren, K., & Zhu, C. (2010). Lithium reduces apoptosis and autophagy after neonatal hypoxia-ischemia. *Cell death & disease*, *1*(7), e56. https://doi.org/10.1038/cddis.2010.33

Li, Z., Tan, F., & Thiele, C.J. (2007). Inactivation of glycogen synthase kinase-3beta contributes to brain-derived neurotrophic factor/TrkB-induced resistance to chemotherapy in neuroblastoma cells. *Mol Cancer Ther, 6,* 3113-3121.

LI-COR Biosciences (2015). Image Studio™ Software Background Subtraction Guide. *LI-COR Biosciences, Lincoln, NE.*

Linnarsson, S., Bjuörklund, A., & Ernfors, P. (1997). Learning deficit in BDNF mutant mice. *Eur J Neurosci, 9*, 2581 – 2587.

Liu, E., Xie, A. J., Zhou, Q., Li, M., Zhang, S., Li, S., Wang, W., Wang, X., Wang, Q., & Wang, J. Z. (2017). GSK-3β deletion in dentate gyrus excitatory neuron impairs synaptic plasticity and memory. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-06173-4

Lin, T. W., Chen, S. J., Huang, T. Y., Chang, C. Y., Chuang, J. I., Wu, F. S., ... & Jen, C. J. (2012). Different types of exercise induce differential effects on neuronal adaptations and memory performance. *Neurobiology of learning and memory*, *97*(1), 140-147.

Luine, V., & Frankfurt, M. (2013). Interactions between estradiol, BDNF and dendritic spines in promoting memory. *Neuroscience*, *239*, 34–45. https://doi.org/10.1016/j.neuroscience.2012.10.019

Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences*, *4*(9), 429–434. https://doi.org/10.4103/1947-2714.100998

Marais, L., Stein, D.J. & Daniels, W.M.U. (2009). Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats. *Metab Brain Dis*, *24*, 587–597. https://doi.org/10.1007/s11011-009-9157-2

Marrocco, J., Einhorn, N. R., Petty, G. H., Li, H., Dubey, N., Hoffman, J., Berman, K. F., Goldman, D., Lee, F. S., Schmidt, P. J., McEwen, B. S. (2020). Epigenetic intersection of BDNF Val66Met genotype with premenstrual dysphoric disorder transcriptome in a cross-species model of estradiol add-back. *Mol Psychiatry, 25*, 572–583. https://doi.org/10.1038/s41380-018-0274-3

McDonald, R. J., & White, N. M. (1995). Hippocampal and nonhippocampal contributions to place learning in rats. *Behavioral Neuroscience, 109*(4), 579–593. https://doi.org/10.1037/0735-7044.109.4.579

McGregor, C. E., & English, A. W. (2018). The role of BDNF in peripheral nerve regeneration: activity-dependent treatments and Val66Met. Front Cell Neurosci 12: 522.

Mercado, N. M., Stancati, J. A., Sortwell, C. E., Mueller, R. L., Boezwinkle, S. A., Duffy, M. F., ... & Steece-Collier, K. (2021). The BDNF Val66Met polymorphism (rs6265) enhances dopamine neuron graft efficacy and side-effect liability in rs6265 knock-in rats. *Neurobiology of disease*, *148*, 105175.

MetaXpress® and MetaMorph®: Relationship between Average Intensity and Integrated Intensity. (2020, December 16). Molecular Devices Support Portal. Retrieved April 27, 2023, from https://support.moleculardevices.com/s/article/MetaXpress-and-

MetaMorph-Relationship-between-Average-Intensity-and-Integrated-Intensity#:~:text=Integrated%20intensity%20is%20the%20sum,to%20get%20the%20integrated%20intensity.

Miranda, M., Morici, J. F., Zanoni, M. B., & Bekinschtein, P. (2019). Brain-derived neurotrophic factor: a key molecule for memory in the healthy and the pathological brain. *Frontiers in cellular neuroscience*, 363.

Molendijk, M.L., van Tol, M.J., Penninx, B.W., et al. (2012). BDNF val66met affects hippocampal volume and emotion-related hippocampal memory activity. *Transl Psychiatry, 2*, e74.

Moreira, F. P., Fabião, J. D., Bittencourt, G., Wiener, C. D., Jansen, K., Oses, J. P., Ávila, L. De, Souza, L. D. M., Crispim, D., Portela, L. V, Pinheiro, R. T., Lara, D. R., Kaster, M. P., Ricardo, A., & Ghisleni, G. (2015). The Met allele of BDNF Val66Met polymorphism is associated with increased BDNF levels in generalized anxiety disorder The Met allele of BDNF Val66Met polymorphism is associated with increased BDNF levels in generalized anxiety disorder. *Psychiatric Genetics, 25*(5). https://doi.org/10.1097/YPG.0000000000000097

Nie, S., Ma, K., Sun, M., Lee, M., Tan, Y., Chen, G., ... & Cao, X. (2019). 7, 8-Dihydroxyflavone protects nigrostriatal dopaminergic neurons from rotenone-induced neurotoxicity in rodents. *Parkinson's Disease*, *2019*. https://doi.org/10.1016/j.neuroscience.2014.12.080

Pan, M., Li, Z., Yeung, V., & Xu, R. (2010). Dietary supplementation of soy germ phytoestrogens or estradiol improves spatial memory performance and increases gene expression of BDNF, TrkB receptor and synaptic factors in ovariectomized rats. *Nutrition & Metabolism, 7*(75), 1–11. http://www.nutritionandmetabolism.com/content/7/1/75

Pardo, M., Abrial, E., Jope, R. S., & Beurel, E. (2016). GSK3β isoform-selective regulation of depression, memory and hippocampal cell proliferation. *Genes, Brain and Behavior*, *15*(3), 348–355. https://doi.org/10.1111/gbb.12283

Peineau, S., Taghibiglou, C., Bradley, C., Wong, T. P., Liu, L., Lu, J., ... & Collingridge, G. L. (2007). LTP inhibits LTD in the hippocampus via regulation of GSK3β. *Neuron*, *53*(5), 703-717.

Perez-Costas, E., Gandy, J. C., Melendez- Ferro, M., Roberts, R. C., & Bijur, G. N. (2010). Light and electron microscopy study of glycogen synthase kinase-3beta in the mouse brain. *PLoS ONE, 5*, e8911. doi:10.1371/journal.pone.0008911

Petryshen, T., Sabeti, P., Aldinger, K. *et al.* Population genetic study of the brain-derived neurotrophic factor (*BDNF*) gene. *Mol Psychiatry* **15**, 810–815 (2010). https://doi.org/10.1038/mp.2009.24

Pezawas, L., Verchinski, B. A., Mattay, V. S., Callicott, J. H., Kolachana, B. S., Straub, R. E., Egan, M. F., Meyer-Lindenberg, A., & Weinberger, D. R. (2004). The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical

morphology. *Journal of Neuroscience*, *24*(45), 10099–10102. https://doi.org/10.1523/JNEUROSCI.2680-04.2004

Pisani, S. L., Neese, S. L., Doerge, D. R., Helferich, W. G., Schantz, S. L., & Korol, D. L. (2012). Acute genistein treatment mimics the effects of estradiol by enhancing place learning and impairing response learning in young adult female rats. *Hormones and Behavior*, *62*(4), 491–499. https://doi.org/10.1016/j.yhbeh.2012.08.006

Prakapenka, A. V., Roberts, S. V., Myers, D. C., Berlin, A., Gold, P. E., & Korol, D. L. (2021). The ripple effect of a single nucleotide polymorphism in the BDNF gene on estradiol-mediated cognition in middle-aged female rats. 24th Annual Meeting of the Society for Behavioral Neuroendocrinology.

Roh, M. S., Eom, T. Y., Zmijewska, A. A., De Sarno, P., Roth, K. A., & Jope, R. S. (2005). Hypoxia activates glycogen synthase kinase-3 in mouse brain in vivo: protection by mood stabilizers and imipramine. *Biological psychiatry*, *57*(3), 278–286. https://doi.org/10.1016/j.biopsych.2004.10.039

Rosa, E., & Fahnestock, M. (2015). CREB expression mediates amyloid β-induced basal BDNF downregulation. *Neurobiology of Aging, 36,* 2406-2413. https://doi.org/10.1016/j.neurobiolaging.2015.04.014

Rui, Y., Myers, K.R., Yu, K., Wise, A., De Blas, A.L., Hartzell, H.C., et al. (2013). Activity-dependent regulation of dendritic growth and maintenance by glycogen synthase kinase 3β. *Nat Commun, 4*, 2628.

Sato, K., Akaishi, T., Matsuki, N., Ohno, Y., & Nakazawa, K. (2007). β-Estradiol induces synaptogenesis in the hippocampus by enhancing brain-derived neurotrophic factor release from dentate gyrus granule cells. *Brain Research, 1150,* 108-120. https://doi.org/10.1016/j.brainres.2007.02.093

Scavuzzo, C.J. (2014). Use it and boost it: Cognitive and physical activities modulate cognition via BDNF-TrkB signaling. *Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience in the Graduate College of the University of Illinois at Urbana-Champaign.*

Scharfman, H. E., Hintz, T. M., Gomez, J., Stormes, K. A., Barouk, S., Malthankar-Phatak, G. H., McCloskey, D. P., Luine, V. N., & Maclusky, N. J. (2007). Changes in hippocampal function of ovariectomized rats after sequential low doses of estradiol to simulate the preovulatory estrogen surge. *Eur J Neurosci, 26*, 2595–2612.

Scheuing, L., Chiu, C.T., Liao, H.M., Linares, G.R., & Chuang, D.M. (2014). Preclinical and clinical investigations of mood stabilizers for Huntington's disease: what have we learned? *Int J Biol Sci, 10*, 1024–1038.

Schinder, A.F., & Poo, M.M. (2000) The neurotrophin hypothesis for synaptic plasticity. *Trends Neurosci 23*, 639 – 645.

Schofield, P.R., Williams, L.M., Paul, R.H., et al. (2009). Disturbances in selective information processing associated with the BDNF Val66Met polymorphism:

evidence from cognition, the P300 and fronto-hippocampal systems. *Biol Psychol, 80*(2), 176–188.

Sconce, M. D., Churchill, M. J., Moore, C., & Meshul, C. K. (2015). Intervention with 7, 8-dihydroxyflavone blocks further striatal terminal loss and restores motor deficits in a progressive mouse model of Parkinson's disease. *Neuroscience*, *290*, 454-471. https://doi.org/10.1016/j.neuroscience.2014.12.080

Serenó, L., Coma, M., Rodríguez, M., et al. (2009) A novel GSK- 3β inhibitor reduces Alzheimer's pathology and rescues neuronal loss in vivo. *Neurobiology of Disease, 35*(3), 359–367.

Shimizu, E., Hashimoto, K., & Iyo, M. (2004). Ethnic difference of the BDNF 196G/A (val66met) polymorphism frequencies: The pos-sibility to explain ethnic mental traits. *American Journal of Medical Genetics: Part B, 126*, 122-123.

Shiojima, I., & Walsh, K. (2002). Role of Akt signaling in vascular homeostasis and angiogenesis. *Circulation research*, *90*(12), 1243–1250. https://doi.org/10.1161/01.res.0000022200.71892.9f

Singh, M., Meyer, E. M., & Simpkins, J. W. (1995). The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology*, *136*(5), 2320–2324. https://doi.org/10.1210/endo.136.5.7720680

Sohrabji, F., & Lewis, D. K. (2006). Estrogen – BDNF interactions: Implications for neurodegenerative diseases. *Frontiers in Neuroendocrinology, 27*, 404–414. https://doi.org/10.1016/j.yfrne.2006.09.003

Soliman, F., Glatt, C.E., Bath, K.G., et al. (2010). A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science, 327*(5967), 863–866.

Souder, D. C., & Anderson, R. M. (2019). An expanding GSK3 network: implications for aging research. *Geroscience*, *41*(4), 369-382.

Spencer, J. L., Waters, E. M., Milner, T. A., & McEwen, B. S. (2008). Estrous cycle regulates activation of hippocampal Akt, LIM kinase, and neurotrophin receptors in C57BL/6 mice. *Neuroscience*, *155*(4), 1106-1119.

Spencer, J. L., Waters, E. M., Milner, T. A., Lee, F. S., & Mcewen, B. S. (2010). BDNF variant Val66Met interacts with estrous cycle in the control of hippocampal function. *PNAS, 107*(9). https://doi.org/10.1073/pnas.0915105107

Spencer-Segal, J. L., Waters, E. M., Bath, K. G., Chao, M. V., McEwen, B. S., & Milner, T. A. (2011). Distribution of phosphorylated TrkB receptor in the mouse hippocampal formation depends on sex and estrous cycle stage. *Journal of Neuroscience*, *31*(18), 6780-6790.

Szarowicz, C. A., Steece-Collier, K., & Caulfield, M. E. (2022). New frontiers in neurodegeneration and regeneration associated with brain-derived neurotrophic

factor and the rs6265 single nucleotide polymorphism. *International Journal of Molecular Sciences*, *23*(14), 8011.

Szeszko, P.R., Lipsky, R., Mentschel, C., et al. (2005). Brain-derived neurotrophic factor val66met polymorphism and volume of the hippocampal formation. *Mol Psychiatry, 10*, 631–636.

Tripanichkul, W., Gerdprasert, O., & Jaroensuppaperch, E. O. (2010). Estrogen reduces BDNF level, but maintains dopaminergic cell density in the striatum of MPTP mouse model. *International Journal of Neuroscience*, *120*(7), 489-495. https://doi.org/10.3109/00207451003721892

Troussard, A. A., Tan, C., Yoganathan, T. N., & Dedhar, S. (1999). Cell-extracellular matrix interactions stimulate the AP-1 transcription factor in an integrin-linked kinase- and glycogen synthase kinase 3-dependent manner. *Molecular and cellular biology*, *19*(11), 7420–7427. https://doi.org/10.1128/MCB.19.11.7420

Tyler, W.J., Alonso, M., Bramham, C.R. & Pozzo-Miller, L.D. (2002). From the acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learn Mem, 9*, 224 – 237.

Vianna, M. R., Alonso, M., Viola, H., Quevedo, J., De Paris, F., Furman, M., ... & Izquierdo, I. (2000). Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. *Learning & memory*, *7*(5), 333-340.

Voineskos, A. N., Lerch, J. P., Felsky, D., Shaikh, S., Rajji, T. K., Miranda, D., Lobaugh, N. J., Mulsant, B. H., Pollock, B. G., & Kennedy, J. L. (2011). The brain-derived neurotrophic factor Val66Met polymorphism and prediction of neural risk for alzheimer disease. *Archives of General Psychiatry*, *68*(2), 198–206. https://doi.org/10.1001/archgenpsychiatry.2010.194

Wan, W., Xia, S., Kalionis, B., Liu, L., & Li, Y. (2014). The role of Wnt signaling in the development of Alzheimer's disease: a potential therapeutic target?. *BioMed research international*, *2014*, 301575. https://doi.org/10.1155/2014/301575

Wang, C. Y., Zheng, W., Wang, T. Xie, J. W., Wang, S. L., Zhao, B. L., Teng, W. P., & Wang, Z. Y (2011). Huperzine A Activates Wnt/*β*-Catenin Signaling and Enhances the Nonamyloidogenic Pathway in an Alzheimer Transgenic Mouse Model. *Neuropsychopharmacol, 36*, 1073–1089. https://doi.org/10.1038/npp.2010.245

Wang, V. C., Sable, H. J. K., Ju, Y. H., Allred, C. D., Helferich, W. G., Korol, D. L., & Schantz, S. L. (2008). Effects of Chronic Estradiol Treatment on Delayed Spatial Alternation and Differential Reinforcement of Low Rates of Responding. *Behavioral Neuroscience, 122*(4), 794–804. https://doi.org/10.1037/a0012513

Wang, W., Kantorovich, S., Babayan, A. H., Hou, B., Gall, C. M., & Lynch, G. (2016). Estrogen’s effects on excitatory synaptic transmission entail integrin and TrkB transactivation and depend upon β1-integrin function. *Neuropsychopharmacology*, *41*(11), 2723-2732.

Woodgett, J. R. (1990). Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J., 9*, 2431–2438.

Yamada, K., Mizuno, M., & Nabeshima, T. (2002) Role for Brain-derived neurotrophic factor in learning and memory. *Life Sci, 70*, 735 –744.

Yamada, K., & Nabeshima, T. (2003). Current Perspective Brain-Derived Neurotrophic Factor / TrkB Signaling in Memory Processes. *J Pharmocol Sci*, *91*(4), 267–270. https://doi.org/10.1254/S1347-8613(19)32708-2

Yamada, M., Ohnishi, H., Sano, S., Nakatani, A., Ikeuchi, T., & Hatanaka H. (1997). Insulin Receptor Substrate (IRS)-1 and IRS-2 Are Tyrosine-phosphorylated and Associated with Phosphatidylinositol 3-Kinase in Response to Brain-derived Neurotrophic Factor in Cultured Cerebral Cortical Neurons. *J Biol Chem*, *272*, 30334-30339.

Yamada, M., Tanabe, K., Wada, K., Shimoke, K., Ishikawa, Y., Ikeuchi, T., Koizumi, S., & Hatanaka, H. (2001). Differences in survival-promoting effects and intracellular signaling properties of BDNF and IGF1 in cultured cerebral cortical neurons. *J Neurochem, 78*, 940-51

Yao, H. B., Shaw, P. C., Wong, C. C., & Wan, D. C. (2002). Expression of glycogen synthase kinase-3 iso- forms in mouse tissues and their transcription in the brain. *J Chem Neuroanat, 23*, 291–297.

You, H., & Lu, B. (2023). Diverse Functions of Multiple Bdnf Transcripts Driven by Distinct Bdnf Promoters. *Biomolecules*, *13*(4), 655.

Yu, H., Wang, Y., Pattwell, S., et al. (2009). Variant BDNF Val66Met polymorphism affects extinction of conditioned aversive memory. *J Neurosci, 29*(13), 4056–4064.

Yu, H., Wang, D. D., Wang, Y., Liu, T., Lee, F. S., & Chen, Z. Y. (2012). Variant brain-derived neurotrophic factor Val66met polymorphism alters vulnerability to stress and response to antidepressants. *Journal of Neuroscience*, *32*(12), 4092–4101. https://doi.org/10.1523/JNEUROSCI.5048-11.2012

Zhang, L., Benedek, D.M., Fullerton, C.S., Forsten, R.D., Naifeh, J.A., Li, X.X., Hu, X.Z., Li, H., Jia, M. & Xing, G.Q. (2014). PTSD risk is associated with BDNF Val66Met and BDNF overexpression. *Molecular Psychiatry, 19*, 8-10. https://doi.org/10.1038/mp.2012.180

Zurkovsky, L., Brown, S. L., Boyd, S. E., Fell, J. A., & Korol, D. L. (2007). Estrogen modulates learning in female rats by acting directly at distinct memory systems. *Neuroscience*, *144*(1), 26–37. https://doi.org/10.1016/j.neuroscience.2006.09.002

Zurkovsky, L., Brown, S. L., & Korol, D. L. (2006). Estrogen modulates place learning through estrogen receptors in the hippocampus. *Neurobiology of Learning and Memory*, *86*(3), 336–343. https://doi.org/10.1016/j.nlm.2006.07.008

Zurkovsky, L., Serio, S. J., & Korol, D. L. (2011). Intra-striatal estradiol in female rats impairs response learning within two hours of treatment. *Hormones and Behavior*, *60*(5), 470–477. https://doi.org/10.1016/j.yhbeh.2011.07.014

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