Molecular Mechanisms Underlying Sexual Reward

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Dedication

This dissertation is dedicated to my friends and family who have supported me throughout my graduate training.

Abstract

Naturally motivated behaviors, such as female sexual behavior, activate neural circuits that in turn mediate the rewarding aspects of the behavior. Activation of the mesocorticolimbic dopamine system by repeated sexual experience leads to long-term changes in behavioral, cellular, and structural plasticity. Specifically, we have found that a uniquely stable transcription factor, ΔFosB, accumulates in the core of the nucleus accumbens (NAc) following repeated sexual experience. To further determine the role of ΔFosB in sexual reward, we virally overexpressed ΔFosB in the NAc of female Syrian hamsters and tested them in a conditioned place preference paradigm. Overexpression of ΔFosB in the NAc resulted in enhanced sexual reward and improved copulatory efficiency with a male compared to control females overexpressing green fluorescent protein. Having established a role for this transcription factor in sexual reward, we next wanted to analyze how repeated sexual experience affected the gene expression of targets of ΔFosB mediated transcription in a limbic circuit. Repeated sexual experience resulted in a significant increase in mRNA expression of only one target of ΔFosB mediated transcription, the GluA2 subunit of the AMPA receptor in the NAc. Interested in the implications of increased GluA2 expression in the NAc on cellular excitability, we decided to pursue this research by determining whether total protein of GluA2 and GluA1 subunits of the AMPA

receptor or their trafficking was differentially affected by repeated sexual experience. In contrast to the published changes in both total protein and receptor trafficking reported in the drug addiction literature, our results indicate that neither of these measures are affected by sexual experience, possibly indicating why natural motivated behaviors are typically not addictive. These results demonstrate that ΔFosB is important in mediating the rewarding consequences of female sexual behavior, and through its transcriptional activity alters gene expression that may underlie the consequent long-term behavioral, cellular and structural plasticity.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction and Literature Review

Female Sexual Behavior

Understanding why animals engage in sexual behavior is at the heart of dissecting mechanisms that underlie motivated behavior research. The obvious assumption is that animals engage in sexual behavior for the long-term consequence of producing offspring. Over the centuries, humans have made vast efforts to dissociate sexual behavior from reproduction (e.g. through the use of family planning), which, according to a Swedish study, suggests that only 0.1% of heterosexual copulations result in fertilization (Agmo, 1999). Even in animal species, such as rodents, that have a high percentage of copulatory interactions that result in the production of offspring, there is still not an indication that pregnancy is necessarily an expected outcome of copulation.

One possibility is that female sexual behavior in rodents is simply a reflexive response to hormonal changes produced by the estrous cycle combined with male stimulation. Indeed, exposure to the proper sequence of ovarian hormones is required for the overt expression of female sexual behavior in rodents. The rodent estrous cycle is typically 4 days in length and consists of an initial increase in estradiol levels followed by a surge in progesterone circulating hormones, which then bind their respective receptors in the brain (Blaustein & Erskine, 2002, Freeman, 1994, Kow & Pfaff, 1975, Pfaff *et al.*, 1994, Sterner *et al.*, 1992). The sequence of these changes in hormone levels is critical to produce the tightly coupled outcomes of ovulation and sexual responsiveness. A large body of

research has focused on how these circulating hormones in turn affect neural pathways to control sexual behavior (Blaustein & Erskine, 2002, Erskine, 1989, Erskine *et al.*, 1989, Freeman, 1994, Kow *et al.*, 1994, Kow & Pfaff, 1975, Pfaff *et al.*, 1977, Pfaff & Sakuma, 1979b, Pfaff *et al.*, 1994). However, an additional possible answer to why animals engage in sexual behavior could involve the neurobiology underlying the motivational control of the behavior and the resulting plasticity in that neural system. The postulate of our laboratory's research is that although the long-term consequence of engaging in sexual behavior might involve reproduction, the more immediate explanation of why animals have sex is that sexual behavior is intrinsically rewarding and activates key neural systems, which then act to drive the behavior (Meisel & Mullins, 2006).

Sexual Behavior as Model of Motivational Pathways

The majority of studies focusing on the effects of sexual experience on reward systems have been performed in rats and hamsters. Although flank stimulation in females from both species elicits the lordosis posture during a sexual interaction, the overall mating patterns of the two species differ substantially. Female rats engage in a very active mating behavior that consists of hopping in place, then darting away from the male, until stopping momentarily for the male to mount. After the male dismounts, the female continues darting around the mating area. This process has been termed "pacing", due to the ability of the

female rat to regulate, or "pace", the timing of the male's mounts (Cohen & Pfaff, 1992, Erskine, 1989, Erskine *et al.*, 1989, Pfaff & Lewis, 1974, Pfaff & Sakuma, 1979a). Female hamsters, however, engage in a far less active mating behavior (**Figure 1**). Female hamsters will maintain their sexual posture, termed lordosis, which is a characteristic dorsoflexion of the spine, for upwards of 90-95% of a 10 min test, even without tactile stimulation from the male (Murphy, 1974, Noble, 1973a, Noble, 1973b, Noble, 1979a, Noble, 1979b, Noble, 1980 Noble, 1979 #113). Rather than "pacing" the male's mounting attempts in a similar way as do female rats, female hamsters move their perineum in the direction of the male's thrusts in an effort to facilitate intromission. So although the male hamster is determining the pacing of his mounts, the female hamster has the ability to regulate whether a mounting attempt results in vaginal intromission (Bradley *et al.*, 2005b, Murphy, 1974, Noble, 1979a, Noble, 1979b, Noble, 1980). If rewarding mechanisms drive the expression of sexual behavior, ultimately resulting in pregnancy and the birth of offspring, then the neural correlates that mediate sexual reward will give insight into the mechanisms underlying motivated behaviors.

Figure 1

Figure 1. Female hamster lordosis. Pictured is a female hamster (left) engaging in sexual behavior with a male (right). The female is demonstrating the characteristic dorsoflexion of the spine, lordosis.

Mesocorticolimbic Dopamine Pathway

The mesocorticolimbic dopamine pathway mediates reward and natural motivated behaviors in the brain (Berridge, 2007, Carlezon & Thomas, 2009, Hedges *et al.*, 2010, Pierce & Kalivas, 1997a, Pierce & Kumaresan, 2006, Thomas & Malenka, 2003). Dopaminergic projection neurons of the ventral tegmental area (VTA) in the midbrain increase their firing when activated. These projections innervate the nucleus accumbens (NAc), prefrontal cortex (PFC), ventral areas of the caudate putamen (CU), as well as a number of other areas within the limbic circuit (Carlezon & Thomas, 2009, Pierce & Kumaresan, 2006). Additionally, these endpoints in turn feed back to the VTA or have separate output pathways, for instance to the ventral pallidum (VP), that can also mediate reward responses (Humphries & Prescott, 2010, Smith & Berridge, 2005, Smith *et al.*, 2009). The regulation of the mesocorticolimbic dopamine pathway is complex due to regions within the circuit that can have both excitatory and inhibitory

projections (Berridge, 2007, Berridge & Robinson, 1998, Pierce & Kumaresan, 2006). In addition, brain areas other than those found in the mesocorticolimbic dopamine pathway certainly are involved in regulating motivated behaviors.

Importantly, although this pathway has the common function of mediating the motivational system that is responsible for natural reinforcing behaviors such as eating, drinking, maternal behavior, pair bonding, and sexual behavior (Bradley *et al.*, 2005a, Frohmader *et al.,* Hedges *et al.,* Meisel & Mullins, 2006, Numan, 2007, Olausson *et al.*, 2006, Pitchers *et al.,* Pitchers *et al.,* Stolzenberg & Numan, 2011, Werme *et al.*, 2002, Young *et al.*, 2005), this pathway has mostly been studied with regard to its reaction to drugs of abuse. A consequence of intermittently activating this system with drugs of abuse is a sensitized increase in dopamine neurotransmission, potentially leading to the structural and behavioral plasticity associated with addiction. If we borrow what is known about dopaminergic plasticity and signaling from the extensive drug literature and assume that some of the plasticity following stimulation from drugs of abuse normally mediates natural motivated behaviors, we can apply that knowledge to gain a more complete understanding of female sexual behavior.

Sexual Behavior Sensitizes Dopamine Release in the NAc

Due to the known involvement of dopamine sensitization within the mesocorticolimbic pathway following administration of drugs of abuse, it follows that dopamine could similarly be modulated as a result of experience with natural motivated behaviors, such as female sexual behavior. In vivo microdialysis has been used to measure increases in NAc extracellular dopamine release in female rats and hamsters during sexual behavior (Becker *et al.*, 2001, Jenkins & Becker, 2003a, Kohlert & Meisel, 1999, Kohlert *et al.*, 1997, Meisel *et al.*, 1993, Mermelstein & Becker, 1995, Pfaus *et al.*, 1995). Specifically, increases in extracellular dopamine concentrations during mating are dependent on the intromission stimulation provided by the male (Kohlert *et al.*, 1997). Not only does mating in female hamsters increase NAc extracellular dopamine, but this increase becomes sensitized with repeated sexual experience (Kohlert & Meisel, 1999). Further, through the use of an elegant experimental design that paired Fluoro-Gold administration through the microdialysis probes with immunohistochemical staining for tyrosine hydroxylase, it was determined that the number of dual labeled midbrain dopaminergic neurons was positively correlated to basal dopamine levels. This indicates that the dopamine levels reported through these microdialysis studies is due to the number of dopaminergic neurons terminating near the microdialysis probe (Kohlert *et al.*, 1997).

Studies performed in female rats also have demonstrated increases in extracellular dopamine levels during mating. Female rats permitted to pace their sexual interactions with a male show greater increases in dopamine release compared to females unable to pace their sexual interactions (Mermelstein & Becker, 1995). Further, by matching microdialysis samples to specific behaviors during mating, it was determined that the increase in extracellular dopamine levels in the NAc occurs directly before stimulation from the male (Jenkins & Becker, 2003a), highlighting the anticipatory role of the NAc in sexual behavior, rather than control of the overt expression of lordosis. This sensitized increase in dopamine within the NAc following sexual experience may then serve to underlie behavioral, cellular, and structural plasticity, modulating further behavioral interactions.

Long-term Behavioral Plasticity Following Repeated Sexual Experience

As mentioned, female hamsters can move their perineum in the direction of a male's thrusts to ultimately help facilitate intromission, a way in which the female controls her copulatory interaction with a male. This behavior is modulated through repeated sexual interactions with a male, and has been shown to improve as the female becomes more experienced (Bradley *et al.*, 2005b, Noble, 1980). Due to the difficulty in measuring the subtle perineal movements of females during copulation, copulatory efficiency often is measured indirectly

through the male's sexual behavior. The proportion of mounts that result in an intromission and/or ejaculation, termed "hit rate", is determined and used to infer changes in the female's perineal movements.

When females are given 6 weeks of repeated sexual experience, they are able to refine their perineal movements in a way that increases the hit-rate of their naïve male partners when compared to non experienced females paired with naïve males. Furthermore, these changes in the female's sexual behavior are long-lasting and have been shown to be maintained up to 6 weeks without further sexual stimulation (Bradley *et al.*, 2005b). To determine whether long-term behavioral plasticity such as this was a result of dopaminergic neurotransmission within the NAc, an experiment was performed that depleted dopamine in this brain region to look at the resulting effects on behavior. Female hamsters were given bilateral 6-hydroxy-dopamine lesions (which damages catecholamine neurons) in the NAc prior to a 6 week regimen of repeated sexual experience. Interestingly, the ability of the female hamsters to improve the copulatory efficiency of their naïve male partners following repeated sexual experience was abolished, though baseline responding was not affected (Bradley *et al.*, 2005b). This study demonstrated that dopaminergic neurotransmission in the NAc is necessary for the ability of the female to learn to refine her perineal movements to improve the copulatory efficiency observed following repeated sexual experience. By demonstrating that the long term behavioral plasticity resulting from repeated

sexual behavior in female hamsters is mediated through sensitization of the mesocorticolimbic dopamine circuit, we can begin to dissect the molecular mechanisms that underlie sexual reward and behavior, and the associated neural and behavioral plasticity.

Reward Associated with Sexual Experience

Sexual interactions have been shown to be rewarding in both female hamsters (Meisel & Joppa, 1994) and rats (Oldenburger *et al.*, 1992), presumably though the activation of the mesocorticolimbic dopamine system. To determine the reward associated with sexual behavior in female rodents, a conditioned place preference (CPP) paradigm was used. In this behavioral test, an apparatus is used that usually consists of two compartments that are differentiated by hue and tactile information. Animals are initially given a pre-test in which they establish their preferences for both chambers before training. During training, females are repeatedly allowed to copulate with a male in one chamber of the apparatus (the conditioning chamber) and are placed alone in the opposite chamber. Lastly, a post-test is given to determine if the female increases the amount of time she spends in the conditioning chamber in the absence of a male or sexual stimulation. This behavioral test measures the reinforcing properties of the sexual experience conditioning, so when the female increases the amount of time spent in the sexual conditioning chamber it is used as a measurement of the rewarding consequences

of sexual behavior. This test has been used successfully to measure the reward associated with sexual behavior in both female hamsters (Meisel & Joppa, 1994, Meisel *et al.*, 1996) and female rats (Gonzalez-Flores *et al.*, 2004, Jenkins & Becker, 2003b, Martinez & Paredes, 2001, Oldenburger *et al.*, 1992, Parada *et al.*, 2010, Paredes & Martinez, 2001).

Through the use of dopamine antagonists in conjunction with this behavioral test, it was determined that the reward associated with drugs of abuse was dependent on forebrain dopamine (Tzschentke, 1998). Naturally, it follows that similar experiments with sexual behavior could determine the importance of dopamine in sexual reward. Systemic administration of either of two dopamine 2 receptor (D2) antagonists, raclopride and sulpiride, prior to sexual conditioning resulted in blocking the formation of a conditioned place preference in female Syrian hamsters (Meisel *et al.*, 1996). Therefore, not only is dopamine important in producing the long-term behavioral plasticity following repeated sexual experience as measured through changes in copulatory efficiency, but it is also required for the rewarding consequences of that behavior.

Long-term Cellular Plasticity Following Repeated Sexual Experience

Given the necessity of dopamine in both long-term behavioral plasticity following sexual experience as well as sexual reward, the resultant effects of this increase in dopamine were examined with respect to post-synaptic signaling.

There are well-established signaling cascades that have been described with respect to motivated behaviors. The sensitized increase in dopamine within the NAc following repeated stimulation from sexual experience will presumably cause an increase in post-synaptic dopamine signaling through D1 and D2 receptor mediated pathways. The way in which sexual experience might affect changes in intracellular signaling is still largely unknown. However, we have made strides in determining some of these changes in intracellular signaling. For instance, the induction of Fos family immediate early genes is increased following sexual experience (Bradley & Meisel, 2001, Joppa *et al.*, 1995, Meisel & Mullins, 2006, Wallace *et al.*, 2008). Specifically, an acute sexual behavior test induces c-Fos activity within the NAc core of female hamsters, an increase that is sensitized following repeated sexual experience (Bradley & Meisel, 2001, Joppa *et al.*, 1995). These results support the hypothesis that not only does repeated sexual experience sensitize dopamine release, but it also sensitizes post-synaptic mechanisms within the NAc. Additionally, FosB staining is elevated following repeated sexual experience (Meisel & Mullins, 2006), further demonstrating the post-synaptic consequences of repeated stimulation by natural motivated behaviors.

To examine the possible dopamine receptor mediated post-synaptic mechanisms activated by repeated sexual experience a study was performed looking directly at the accumulation of the second messenger, cyclic AMP. NAc

homogenates from sexually experienced and sexually naïve female hamsters were stimulated with various compounds to look at the resultant accumulation of cAMP. No differences in cAMP accumulation were detected between NAc homogenates from sexually experienced and sexually naïve females following either G-protein or forskolin stimulation (Bradley *et al.*, 2004). Interestingly, however, homogenates from sexually experienced females showed an increase in cAMP accumulation over that of naïve females when stimulated with a D1 agonist (Bradley *et al.*, 2004). This demonstrates that repeated sexual experience produces a post-synaptic change in NAc neurons, possibly through an increased coupling of the D1 receptor with its G-protein to cause a sensitized increase in cAMP accumulation. This conclusion is supported by our unpublished data showing no difference in D1 or D2 receptor binding densities or protein levels following 6 weeks of repeated sexual experience. This further supports that the accumulation of cAMP through D1 receptor activation is a result of increased coupling of the D1 receptor to its G-protein rather than changes in receptor expression or binding.

To further determine how sexual experience in female hamsters may affect the consequent activation of downstream target proteins, a gene expression approach was used. A microarray study was conducted to compare NAc gene expression between sexually experienced and naïve female hamsters. Although sensitivity issues inherent to microarray do not exclude the possibility of

additional changes in gene transcription, the results from this study indicated changes in a number of genes involved in regulation of signal transduction, ion channels, receptors, neurotransmission, transcription factors, and cytoskeletal/cell adhesion genes (Bradley *et al.*, 2005a). It follows, that changes in gene expression lead to changes in behavioral, cellular, and structural plasticity.

Structural Plasticity Following Repeated Sexual Experience

In addition to behavioral and intracellular changes observed following repeated sexual experience, structural changes in the form of increased dendritic spines are also observed. Dendritic spines are dynamic morphological structures consisting of small protrusions off of the dendritic shaft, which occur undoubtedly as the result of various signaling events within medium spiny neurons in the NAc (**Figure 2**) and are believed to form mostly excitatory glutamatergic synapses (Holtmaat & Svoboda, 2009, Robinson & Kolb, 1997, Robinson & Kolb, 2004, Sala *et al.*, 2008). The morphology of individual dendritic spines is believed to reflect their physiology. Thin fillopodial spines are believed to be the least mature and the most dynamic, whereas larger mushroom spines are postulated to be the most mature and also form the most stable synapses (Holtmaat & Svoboda, 2009, Sala *et al.*, 2008). Various experiences are believed to cause plastic changes in dendritic spine formation. Synapses that are created through the formation of dendritic spines are believed to ultimately have effects on cellular excitability and

synaptic plasticity (Holtmaat & Svoboda, 2009, Maze & Russo, Robinson & Kolb, 1997, Robinson & Kolb, 2004).

Through Golgi staining it has been observed that stimulation by natural motivated behaviors causes changes in dendritic morphology (Kinsley *et al.*, 2006, Meisel & Mullins, 2006, Pitchers *et al.*, 2010a, Salmaso *et al.*, 2011). Specifically, repeated sexual experience increases dendritic spine density in the NAc of female Syrian hamsters (Meisel & Mullins, 2006). This stimulation from sexual behavior produces highly stable dendritic spines, which can be maintained for long periods of time following cessation of stimulation (Meisel & Mullins, 2006). The formation of these stable dendritic spines following repeated sexual experience may underlie the behavioral plasticity and reward associated with sex behavior. The molecular mechanisms upstream of these changes in dendritic spine density have been studied following administration of drugs of abuse, and are likely shared with natural motivated behaviors. This dissertation will attempt to gain a more complete understanding of possible cellular mechanisms that underlie these long-term structural changes that are consequent of sexual behavior.

Figure 2

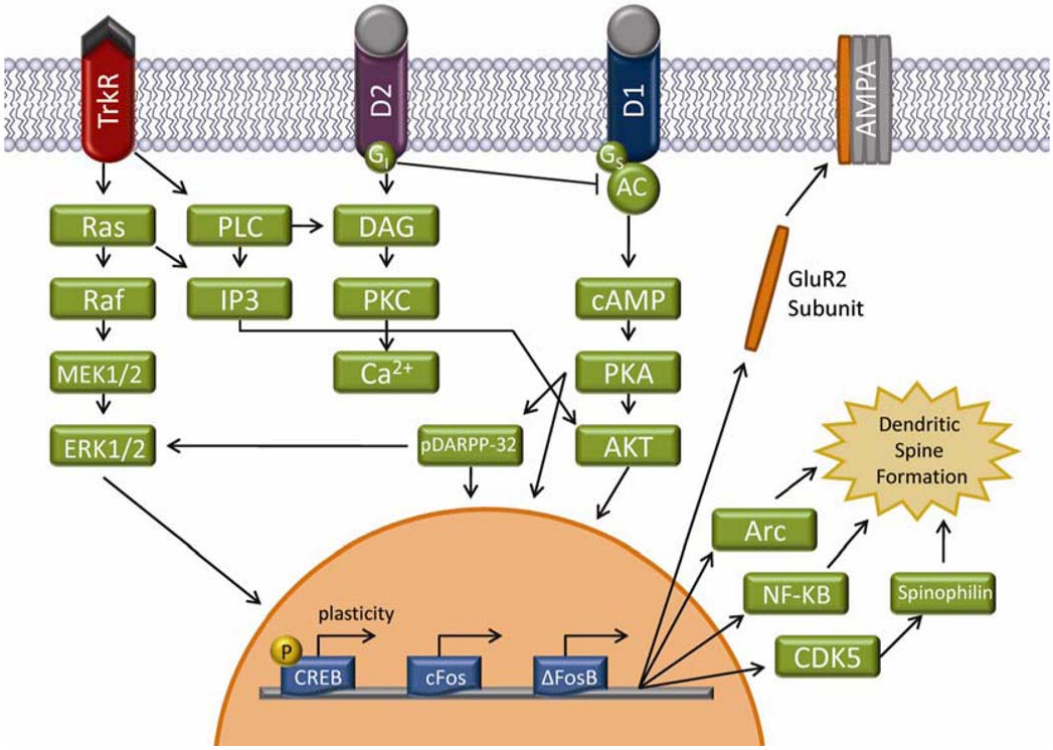


Figure 2. Schematic representation of select cell signaling pathways that ultimately leads to changes in structural plasticity. Activation of cell membrane receptors, such as D1 and Trk receptors, results in activation of multiple intracellular signaling cascades. Ultimately, activation of these intracellular signaling cascades results in gene transcription within the nucleus of the cell. Two of the many downstream gene targets of such signaling are CREB and ΔFosB. Whereas CREB phosphorylation is believed to mediate some of the behavioral plasticity resulting from drugs of abuse, the accumulation of the stable transcription factor ΔFosB is postulated to underlie drug induced structural plasticity. The targets of ΔFosB transcription in turn can affect cellular excitability through the insertion of GluR2 (GluA2) subunits into AMPA receptors, as well as structural changes through dendritic spine plasticity via Cdk5 activation. Importantly, D2 receptor activation counteracts D1 receptor activation (Hedges *et al*. 2010).

Gene Transcription Underlying Plasticity

Due to the long-term behavioral responses and structural changes resulting from natural motivated behaviors that remain even after cessation of the stimulation, it is reasonable to consider that differential gene expression is ultimately causing alterations of neural pathways in the form of synaptic plasticity. Following stimulation, various intracellular pathways can be excited and thus cause activation of transcription factors within the nucleus of the cell (Carlezon *et al.*, 2005, Chen *et al.*, 2009, Lu *et al.*, 2006, Mcdaid *et al.*, 2006, Nestler, 2001, Nestler, 2004, Wolf *et al.*, 2004). By binding to DNA, these transcription factors have the ability to either increase or decrease the rate of gene transcription, which may be the underlying cause of the long-term behavioral and structural plasticity observed following motivated behaviors.

A plethora of research has now focused on the role of differential gene expression resulting from long-term plasticity (Bradley *et al.*, 2005a, Hope *et al.*, 1992, Link *et al.*, 1995, Mcclung & Nestler, 2003, Mcclung & Nestler, 2008, Moga *et al.*, 2004, Myers *et al.*, 1999, Nestler, 2004, Nestler *et al.*, 1999, Valjent *et al.*, 2006). If we again borrow from the drug literature, a variety of potential transcription factors can be identified. Of these transcription factors, two that have been highly studied are cAMP-response-element-binding protein (CREB) (Carlezon *et al.*, 2005, Mcclung & Nestler, 2003, Nestler, 2004) and ΔFosB, proteins which are oppositely regulated (Mcclung & Nestler, 2003, Mcclung &

Nestler, 2008). Although these transcription factors have been extensively studied with respect to drug abuse regimens, how naturally rewarding motivated behaviors affect both CREB and ΔFosB is less known. For my dissertation I have chosen to focus on the transcription factor ΔFosB, which is now believed to play a large part in the persistent neural and behavioral consequences of drugs of abuse (Alibhai *et al.*, 2007, Ang *et al.*, 2001, Brenhouse & Stellar, 2006, Chen *et al.*, 1997, Chen *et al.*, 1995, Chen *et al.*, 2009, Colby *et al.*, 2003, Doucet *et al.*, 1996, Harris *et al.*, 2007, Hope *et al.*, 1992, Hope *et al.*, 1994, Kelz *et al.*, 1999, Maze & Russo, 2010, Mcdaid *et al.*, 2006, Mumberg *et al.*, 1991, Nakabeppu & Nathans, 1991, Perrotti *et al.*, 2008, Winstanley *et al.*, 2007, Zachariou *et al.*, 2006).

ΔFosB

Acute exposure to drugs of abuse induces the activation of numerous transcription factors from the Fos family that are transient in nature and have a signal that is activated immediately and decays quickly with time (Nestler, 2001, Nestler *et al.*, 1993a, Nestler *et al.*, 1993b, Nestler *et al.*, 1999). Conversely, it has been shown that chronic stimulation or administration leads to high levels of induction of ΔFosB, an alternative splice variant of the immediate early gene FosB (**Figure 3**) (Berton *et al.*, 2007, Chen *et al.*, 1997, Chen *et al.*, 1995, Doucet *et al.*, 1996, Hedges *et al.*, 2009, Hope *et al.*, 1992, Hope *et al.*, 1994, Mcclung *et*

al., 2004, Mumberg *et al.*, 1991, Nakabeppu & Nathans, 1991, Nestler, 2004, Nestler *et al.*, 1999).

Figure 3

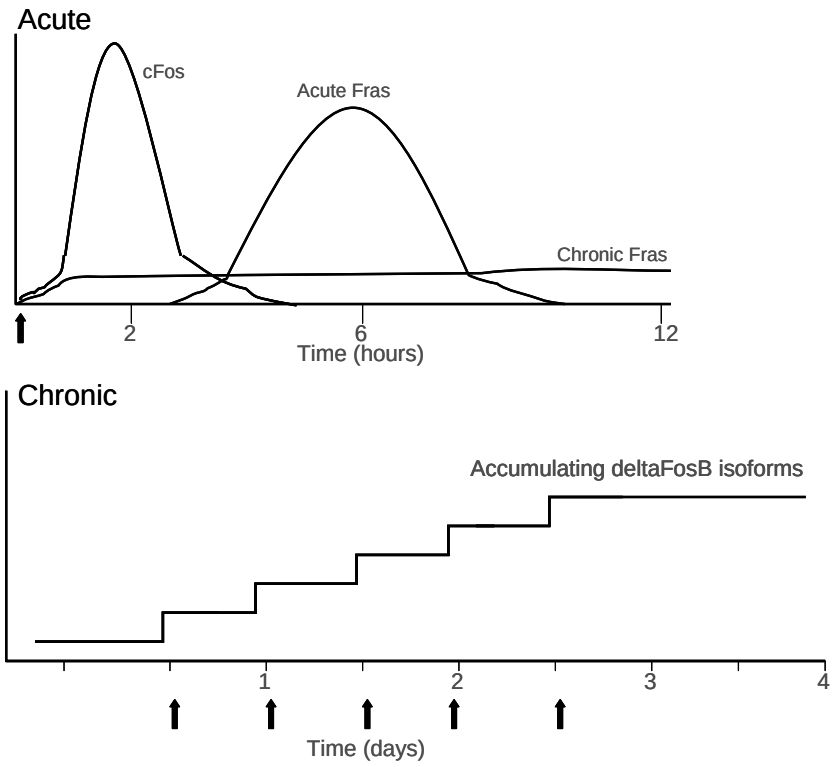


Figure 3. Time course of immediate early gene activation. **Top**. An acute stimulus produces transient increases in cFos, FosB, and Fos related antigens (Fra), with very low activation of ΔFosB isoforms. **Bottom**. Chronic stimulation, however, causes ΔFosB to accumulate to high levels. This accumulated protein can then remain elevated for long periods of time. Figure is adapted from (Nestler, 2001).

The full length FosB protein (45-48 kDa) consists of 5 exons with a 140 nucleotide intron that is expressed between exons IV and V. Although this intron is relatively short, it still contains the same motifs as the larger introns including a 5' splice site, a branch point, a polypyrimidine tract, and a 3' splice site (Carle *et al.*, 2007). The expression of an intron in the final sequence of the FosB protein is an example of intron retention, which is when an intron is in the open reading frame of mRNA that codes for protein. Both ΔFosB and FosB contain a DNA binding domain as well as a basic leucine zipper domain in which they bind to Jun proteins, specifically JunD and to a lesser extent JunB, forming heterodimers that form activator protein-1 (AP-1) complexes that bind the AP-1 regions on genes to regulate transcription (Carle *et al.*, 2007, Hope *et al.*, 1992, Hope *et al.*, 1994, Jorissen *et al.*, 2007).

Unique to ΔFosB is its ability to remain elevated for weeks even in the absence of stimulation (Mcclung *et al.*, 2004, Nestler, 2004, Nestler *et al.*, 1999, Olausson *et al.*, 2006). Importantly, the elevation of ΔFosB protein is not due to an increase in mRNA levels, but rather the stability of the protein (Alibhai *et al.*, 2007). The unusual stability of ΔFosB seems to be mediated by two important post translational mechanisms. The ΔFosB protein exists at various molecular weights. Initially, ΔFosB is 33 kDa but increases its size to 35-37 kDa over time. The 33 kDa form does not exhibit the same unique stability as does the 35-37 kDa form (Alibhai *et al.*, 2007). This molecular weight difference, as well as some of

the difference in stability between the two proteins seems to be due in part to phosphorylation of Ser27 by casein kinase 2, which has no effect on the full length FosB protein (Alibhai *et al.*, 2007, Ulery & Nestler, 2007, Ulery *et al.*, 2006). This phosphorylation event also does not affect the ability of ΔFosB to translocate to the nucleus of the cell or its ability to bind the AP-1 region of genes (Ulery & Nestler, 2007).

The majority of the unique stability of ΔFosB can be attributed to the truncation of the protein by alternative splicing. As mentioned previously, the full length FosB protein normally retains an intron in the C-terminal transactivation domain in the final sequence of the protein. In the full-length FosB protein this sequence contains two degron domains that target the protein for rapid proteasomal degradation (Carle *et al.*, 2007). The premature stop codon in ΔFosB generated through alternative splicing results in the deletion of nearly the entire C-terminal transactivation domain, most notably, the aforementioned degron domains. Hence, the absence of these degron domains protects ΔFosB from degradation. Additionally, it has also been found that ΔFosB has the ability to homodimerize after it accumulates to a threshold level, which has been postulated to also contribute to its unique stability (Jorissen *et al.*, 2007).

Although the upstream signal that ultimately causes the alternative splicing event that leads to the accumulation of ΔFosB protein is unknown, research has shown that this accumulation seems to be dependent on dopamine

receptor mediated events. Overexpression of ΔFosB in only the dynorphin-containing, and not the enkephalin-containing population of neurons within the NAc has been shown to be important in mediating the rewarding properties of both drugs of abuse (Colby *et al.*, 2003, Kelz *et al.*, 1999, Zachariou *et al.*, 2006), as well as natural rewards (Brown *et al.*, 1996, Christiansen *et al.*, 2011, Hedges *et al.*, 2010, Pitchers *et al.*, 2010a, Pitchers *et al.*, 2010b, Wallace *et al.*, 2008, Werme *et al.*, 2002). Further, increased ΔFosB protein was only observed following chronic administration of D1-like agonists, and not D2-like agonists in this population of cells (Doucet *et al.*, 1996). Spine density in the NAc has been found to be most stable in the D1 dopamine receptor expressing neurons (Lee *et al.*, 2006). Thus, the conclusion is that the activation of D1 receptor-mediated signaling cascades results in the accumulation of stable ΔFosB isoforms. Then, through its transcriptional activity, ΔFosB affects changes in gene expression to affect neural plasticity, including producing stable increases in dendritic spines.

Due to the long-term stability of ΔFosB protein following chronic stimulation, it is optimally situated to act as what has been termed a "molecular switch" to facilitate the transition between casual drug use and a drug addicted state (Mcclung *et al.*, 2004, Nestler *et al.*, 1999). Rather than focusing on how ΔFosB mediates drug abuse, we assert that ΔFosB has a more common role of mediating plasticity within natural reward systems. Due to its stability and because ΔFosB is a transcription factor, it also has the ability to independently

affect a variety of genes which may ultimately contribute to the long-term behavioral, cellular, and structural plasticity observed following repeated stimulation from sexual behavior that persists following cessation of stimulation.

Down Stream Targets of ΔFosB Regulated Transcription

A limited set of targets of ΔFosB regulated transcription have been identified through microarray and overexpression studies (Kelz *et al.*, 1999, Mcclung & Nestler, 2003), but it remains unclear if these targets are serially related to ΔFosB, or if they are simply affected by activity within the mesocorticolimbic dopamine circuit. Relatively few targets of ΔFosB transcription have been studied in terms of their effects on plasticity, and include the GluA2 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptor, cylcin dependent kinase 5 (Cdk5), and nuclear factor κB (NF-κB). The GluA2 subunit of the AMPA receptor has been verified as a target of ΔFosB regulated transcription, and has been shown to be increased following accumulation of ΔFosB (Kelz *et al.*, 1999, Peakman *et al.*, 2003). Interestingly, the GluA2 promoter has an AP-1 site that directly binds to ΔFosB (Brene *et al.*, 2000, Chen *et al.*, 1995, Myers *et al.*, 1999). Unlike the other glutamate receptor subunits, the GluA2 subunit is unique in that it undergoes RNA editing, changing an adenine to a guanine by oxidative deamination that results in a decrease in the overall current within channels that contain the GluA2 subunit, as well as making

the channel resistant to Ca^{2+} ions (Bredt & Nicoll, 2003). This increase in GluA2 subunits as opposed to other AMPA subunits could have an important physiological consequence of increasing sensitivity to rewards and motivated behaviors (Kelz *et al.*, 1999).

Another target of ΔFosB regulated transcription, that also increases following chronic drug administration, is cyclin Cdk5, a serine/threonine kinase (Bibb, 2003, Mcclung & Nestler, 2003, Taylor *et al.*, 2007, Teegarden *et al.*, 2009). Unlike other Cdk's that have a role in regulation of the cell cycle, Cdk5 has important functions in neuronal survival plus a notable role in synaptic plasticity, specifically by increasing dendritic spine formation (Benavides *et al.*, 2007, Bibb, 2003, Norrholm *et al.*, 2003). When Cdk5 is pharmacologically blocked it leads to a decrease in dendritic spine formation (Norrholm *et al.*, 2003).

Through its activity as a kinase, Cdk5 can in turn phosphorylate a variety of target proteins to affect spine density. One of these targets in the brain is the myocyte enhancer factor 2 (MEF2) family of transcription factors (Gong *et al.*, 2003, Pulipparacharuvil *et al.*, 2008). The role of MEF2 proteins in the brain are largely unknown, however, it has now been established that MEF2 activity regulates excitatory synapses (Flavell *et al.*, 2006, Shalizi *et al.*, 2007, Shalizi *et al.*, 2006, Shalizi & Bonni, 2005), in part through activity-dependent synapse elimination (Flavell *et al.*, 2006). Phosphorylation of MEF2 by Cdk5 acts to inhibit MEF2 activity, inhibition which is relieved through activity of protein

phosphatase 2B. Therefore the increased protein levels of Cdk5 following chronic administration of drugs of abuse could act to inhibit MEF2 activity to ultimately affect dendritic spine plasticity. Indeed, chronic cocaine administration increases phosphorylation of MEF2 by Cdk5, and this phosphorylation event regulates dendritic spine density within the NAc as well as cocaine-mediated behavioral plasticity (Pulipparacharuvil *et al.*, 2008). How Cdk5 activity regulates MEF2 in natural motivated behaviors is unknown, but could clearly be critical in ΔFosB dependent plasticity.

Further opening up the possibilities for ΔFosB actions is an additional transcription factor, NF- κB (Mcclung & Nestler, 2003). Although it has mostly been studied for its involvement in immune responses (Chen & Greene, 2004, Lin & Karin, 2007), NF-κB has also been implicated in synaptic plasticity and memory formation (Mattson, 2005, Mattson & Meffert, 2006, Meffert & Baltimore, 2005, Meffert *et al.*, 2003), as well as neuronal morphology consequent of cocaine administration (Ang *et al.*, 2001, Russo *et al.*, 2009). Determining how these various targets of ΔFosB mediated gene transcription play their individual roles in cellular and structural plasticity will help to dissect the ways in which ΔFosB mediates natural motivated behaviors.

ΔFosB Expression and Natural Motivated Behaviors

Numerous naturally motivated behaviors have been shown to be regulated through ΔFosB accumulation. Sexual reward in males has been demonstrated to be mediated through ΔFosB, with viral overexpression of ΔFosB causing an enhancement of the facilitation of sexual behavior which is abolished with overexpression of a competitive binding partner of ΔFosB, ΔJunD (Pitchers *et al.*, 2010a, Pitchers *et al.*). Overexpression of ΔFosB has been shown to increase motivation for food by reinforced instrumental behavior (Olausson *et al.*, 2006, Vialou *et al.*) and increased induction of ΔFosB has also been observed following a palatable high fat diet (Christiansen *et al.*, 2011) and sucrose consumption (Christiansen *et al.,* Wallace *et al.*, 2008). Wheel running, an additional motivated behavior, increases ΔFosB staining in the NAc compared to locked-wheel controls (Greenwood *et al.,* Werme *et al.*, 2002). Further, following viral overexpression of ΔFosB in dynorphin-containing cells within the NAc, mice actually increase their running over control animals overexpressing green fluorescent protein (GFP) (Werme *et al.*, 2002). (Refer to **Table 1** for a more detailed review of how ΔFosB has been implicated in the aforementioned natural motivated behaviors.) These studies exemplify that ΔFosB also regulates natural rewarding behaviors and potentially may act to mediate the plasticity following repeated sexual experience.

My dissertation will concentrate on the molecular mechanisms underlying sexual behavior and reward by focusing on the transcription factor ΔFosB. The experiments in this dissertation are designed to understand the role that ΔFosB, and its downstream transcriptional targets play in the long-term behavioral and cellular plasticity that result from repeated sexual experience.

Table 1. ΔFosB Actions in Natural Motivated Behaviors

Motivated Behavior	Summary of Research	Reference
Male Sexual Behavior	A. Repeated sexual experience in male rats resulted in accumulation of ΔFosB in various brain areas including the NAc, medial prefrontal cortex, ventral tegmental area, and caudate putamen, but not the medial preoptic nucleus. Rats with ΔFosB overexpression displayed enhanced facilitation of sexual performance with sexual experience. In contrast, overexpression of ΔJunD, a dominant negative binding partner of ΔFosB, attenuated sexual experience-induced facilitation of sexual performance and stunted long-term maintenance of facilitation compared to green fluorescent protein overexpressing controls. B. Western blotting and immunohistochemistry revealed that repeated sexual experience in male rats significantly increased ΔFosB protein in the NAc core and shell, but not the caudate nucleus. These effects were not found in control males that were exposed to hormone-treated females but not allowed to mate, or a set of olfaction-arena controls, suggesting that ΔFosB accumulation occurs as a response to sexual behavior and not social or olfactory cues. Further, viral-mediated overexpression of ΔFosB in the NAc of males significantly decreased the number of intromissions required to reach ejaculation for the initial sexual behavior test, as well as decreasing the postejaculatory interval for ΔFosB overexpressing males after their first sexual experience.	(Pitchers *et al.*) (Wallace *et al.*, 2008)
Wheel Running	A. Young adult male rats given 6 weeks of access to a running wheel found wheel running rewarding as measured through a CPP paradigm and also showed increased ΔFosB staining in the NAc, compared to males with running wheel access for 2 weeks, and locked wheel controls. B. Male Lewis rats given access to a running wheel for 30 days showed increased levels of ΔFosB in the NAc compared locked wheel controls. Mice with overexpression of ΔFosB selectively in the striatal dynorphin-containing neurons increased their daily running compared to control littermates, whereas mice	(Greenwood *et al.*, 2010) (Werme *et al.*, 2002)

	that overexpressed ΔFosB predominantly in the striatal enkephalin-containing neurons ran considerably less than control littermates.	
Food Reinforced Instrumental Behavior	Inducible overexpression of ΔFosB in the NAc and dorsal striatum of bitransgenic mice, or viral-mediated overexpression in the NAc core of rats, enhanced food-reinforced instrumental performance and progressive ratio responding.	(Olausson *et al.*, 2006)
Sucrose Intake	A. Male rats with free access to water and rat chow were given additional twice daily access to 4ml sucrose (30%) or water for 14 days. Animals were sacrificed following cessation of sucrose (1, 6, or 21 days). Sucrose consumption increased ΔFosB staining in the basolateral amygdala and in the NAc core, which persisted through 21 days following cessation of sucrose. B. Male rats given access to 10% sucrose for 10 days had increased levels of ΔFosB in the NAc core and shell, but not the dorsal striatum as measured by both Western blotting and immunohistochemistry. Further, rats with viral-mediated overexpression of ΔFosB in the NAc drank significantly more sucrose that a control group overexpressing green fluorescent protein, with no effect on the amount of water drank in either group.	A. (Christiansen *et al.*) (Wallace *et al.*, 2008)
High-fat Diet	A. Male bitransgenic mice with inducible overexpression of ΔFosB in dynorphin-positive neurons in the NAc and dorsal striatum showed decreased pCREB, CREB, and BDNF levels in the NAc. However, these effects were ameliorated following exposure to a high-fat diet (4.73 kcal/g) for six weeks. B. Mice exposed to a high-fat diet during their third postnatal week showed a significant preference for a high-fat diet as adults compared to controls. The increased intake of a high-fat diet was specific to dietary preferences because there were no changes in caloric intake or caloric efficiency. Further, mice exposed to a high-fat diet during early life displayed significantly increased levels of ΔFosB in the NAc.	(Teegarden *et al.*, 2008) (Teegarden *et al.*, 2009)
Maternal Behavior	Mating homozygous fosB knockout mice resulted in a dramatic increase in pup lethality. Western blotting showed that heterozygous mutants had significantly reduced levels of both full-length FosB protein as well as ΔFosB protein, while homozygous mutants had a	(Brown *et al.*, 1996)

	complete lack of both proteins. The increased pup lethality was due to a nurturing defect present in the homozygous fosB knockout females that was not present in heterozygous mutants. Homozygous fosB mutants did not appropriately crouch over pups and pups were found scattered throughout the cage. Further, when given a pup-retrieval task, fosb knockouts took drastically longer to retrieve their pups compared to controls. Immunohistochemical analysis of wild type females exposed to pups for 6 hrs found increase FosB staining in the medial preoptic area and striatum compared to females that were not exposed to pups.	

CHAPTER 2

Overexpression of ΔFosB in the nucleus accumbens enhances sexual reward and copulatory efficiency

Rationale

Due to the known role that ΔFosB plays in the rewarding consequences of drugs of abuse as well as the underlying neural plasticity in drug addiction, I needed to establish that ΔFosB played a similar role in the rewarding consequences of natural motivated behaviors, specifically, female sexual behavior. Here we took a viral mediated overexpression approach coupled with a conditioned place preference paradigm to determine if overexpression of ΔFosB could alter sexual reward.

Introduction

Experiential plasticity that occurs as a result of drugs of abuse, motivated behaviors, wheel running behavior or instrumental learning is mediated through activation of the mesocorticolimbic dopamine system as well as persistent alterations in the nucleus accumbens (NAc) (Becker *et al.*, 2001, Bradley *et al.*, 2005a, Bradley & Meisel, 2001, Bradley *et al.*, 2004, Colby *et al.*, 2003, Di Chiara *et al.*, 1998, Harris *et al.*, 2007, Kelz *et al.*, 1999, Kohlert & Meisel, 1999, Kohlert *et al.*, 1997, Kumar *et al.*, 2005, Mcclung & Nestler, 2003, Mcclung & Nestler, 2008, Mcdaid *et al.*, 2006, Meisel *et al.*, 1993, Meisel & Mullins, 2006, Mermelstein & Becker, 1995, Nestler, 2001, Nestler, 2004, Olausson *et al.*, 2006, Perrotti *et al.*, 2008, Pfaus *et al.*, 1995, Pierce & Kalivas, 1995, Pierce & Kalivas, 1997b, Pierce & Kumaresan, 2006, Robinson & Kolb, 2004, Tzschentke, 1998,

Werme *et al.*, 2002, Wolf *et al.*, 2004, Zachariou *et al.*, 2006)). Structural changes, particularly the formation of dendritic spines, are an important component of this experience based plasticity (Allen *et al.*, 2006, Lee *et al.*, 2006, Li *et al.*, 2003, Meisel & Mullins, 2006, Norrholm *et al.*, 2003, Robinson *et al.*, 2001, Robinson & Kolb, 1997, Robinson & Kolb, 1999a, Robinson & Kolb, 1999b, Robinson & Kolb, 2004), which persists following cessation of either the behavioral experience or drug administration (Mcclung & Nestler, 2008, Meisel & Mullins, 2006, Nestler, 2001, Wolf *et al.*, 2004).

Previous research has shown that a transcription factor, ΔFosB, has molecular properties that make it a good candidate to mediate the enduring structural and behavioral modifications consequent to behavioral or drug experiences (Chen *et al.*, 1997, Chen *et al.*, 1995, Colby *et al.*, 2003, Doucet *et al.*, 1996, Hope *et al.*, 1994, Kelz *et al.*, 1999, Mcclung & Nestler, 2003, Mcclung *et al.*, 2004, Mcdaid *et al.*, 2006, Nakabeppu & Nathans, 1991, Nestler, 2004, Nestler *et al.*, 1999, Nye *et al.*, 1995, Olausson *et al.*, 2006, Perrotti *et al.*, 2008, Werme *et al.*, 2002, Zachariou *et al.*, 2006). ΔFosB is an alternative splice variant of the immediate early gene *fos*B (Mumberg *et al.*, 1991, Nakabeppu & Nathans, 1991). Unlike the full length FosB protein, the truncated ΔFosB has unusual stability resulting in accumulation of the protein following repeated stimulation (Chen *et al.*, 1997, Chen *et al.*, 1995, Hope *et al.*, 1994, Kelz *et al.*, 1999, Perrotti *et al.*, 2008, Zachariou *et al.*, 2006). Although the mechanism by which the *fos*B

gene is alternatively spliced remains unknown, the truncation of the protein along with phosphorylation protects the protein from rapid proteasomal degradation producing a greater level of transcriptional activity compared with more transiently-lived FosB family members (Carle *et al.*, 2007) (Ulery & Nestler, 2007, Ulery *et al.*, 2006). The postulate is that accumulation of ΔFosB protein produces patterns of gene expression that may underlie the effects of experience on long-term behavioral and cellular plasticity (Mcclung & Nestler, 2008).

We have used female sexual behavior in Syrian hamsters as a model of experience-based plasticity in the brain (Bradley *et al.*, 2005a, Bradley *et al.*, 2005b, Bradley & Meisel, 2001, Bradley *et al.*, 2004, Kohlert & Meisel, 1999, Kohlert *et al.*, 1997, Meisel *et al.*, 1993, Meisel & Joppa, 1994, Meisel *et al.*, 1996, Meisel & Mullins, 2006). An advantage to working with sexual behavior is the ability to control the level of an animal's experiences by having either completely sexually naïve animals, or by differentially exposing animals to varying levels of sexual experience. We have previously shown that repeated sexual experience results in sensitization of the mesocorticolimbic dopamine system, analogous to that of drugs of abuse (Bradley *et al.*, 2005b, Bradley & Meisel, 2001, Brenhouse & Stellar, 2006, Cadoni & Di Chiara, 1999, Hope *et al.*, 1992, Kelz *et al.*, 1999, Kohlert & Meisel, 1999, Pierce & Kalivas, 1995, Pierce & Kalivas, 1997a, Pierce & Kalivas, 1997b, Robinson & Kolb, 1999a). Further, like the effects of drugs, repeated sexual experience increases dendritic spines in

medium spiny neurons of the NAc (Lee *et al.*, 2006, Li *et al.*, 2003, Meisel & Mullins, 2006, Norrholm *et al.*, 2003, Robinson *et al.*, 2001, Robinson & Kolb, 1997, Robinson & Kolb, 1999a, Robinson & Kolb, 1999b, Robinson & Kolb, 2004). Additionally, we have found that ΔFosB/FosB staining is persistently elevated in the NAc following repeated sexual experience (Meisel & Mullins, 2006).

Given that sexual experience can produce long-lasting expression of FosB family members, the purpose of this study was to manipulate ΔFosB expression to mimic the behavioral consequences of repeated sexual experience. Following viral-mediated overexpression of ΔFosB in the NAc, female Syrian hamsters were tested for enhanced conditioned place preference and also increased copulatory efficiency with naïve male hamsters, two endpoints that have previously been shown to be affected by repeated sexual experience (Bradley *et al.*, 2005b, Meisel & Joppa, 1994, Meisel *et al.*, 1996, Meisel & Mullins, 2006).

Materials and Methods

Experimental Subjects. Male and female Syrian hamsters were delivered at approximately 60 days of age from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Females were housed individually in plastic cages (50.8 cm long x 40.6 cm wide x 20.3 cm high), while the male stimulus animals were group-housed in identical cages in numbers of three or four. The animal room was

maintained at a controlled temperature of 22 °C with a 14:10 hr light-dark schedule (lights off between 1:30 and 11:30 p.m.). Food and water were available to the animals *ad libitum*.

All the procedures used in this experiment were in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Purdue Animal Care and Use Committee.

Surgery. Female hamsters were bilaterally ovariectomized by a small flank incision under sodium pentobarbital anesthesia (Nembutal; 8.5 mg per 100 gram body weight, i.p.). The muscle was then sutured and skin stapled. Females were then given supplemental anesthetic and underwent bilateral stereotaxic surgery for the delivery of viral vectors. During stereotaxic surgery, the head was shaven and the skin and muscle retracted. A small hole was drilled in the skull and a 5 μL Hamilton syringe was lowered to the level of the NAc (AP +2.2, ML +/- 1.8, DV -5.9 from Bregma) from a 2º lateral angle to ensure clearance of the lateral cerebral ventricles. The syringe was kept in place for 5 min prior to injections and then either adeno-associated virus (AAV)-GFP or AAV- ΔFosB (0.7 μL) was delivered into the NAc over 7 min, to minimize damage, with the syringe then kept in place for an additional 5 min. This procedure was repeated for the contralateral side of the brain. Females were allowed to recover for 3 weeks prior to behavioral testing.

Viral Vectors. Adeno-associated Virus (AAV) is characterized by its ability to efficiently transfect neurons as well as to maintain specific transgene expression for long periods of time (Chamberlin *et al.*, 1998). AAV vectors exist in different serotypes based on the characterization of their capsid protein coat. This experiment utilized an AAV2 (serotype 2) from Stratagene with a titer of over 10^8/μl expressing green fluorescent protein (AAV-GFP) as well as an AAV vector that had constructs for both ΔFosB and GFP (AAV-ΔFosB-GFP). The viral vectors were injected into the NAc at least 3 weeks prior to behavioral testing to allow for ΔFosB overexpression to develop. These AAV vectors mediate transgene expression in rats and mice that becomes maximal within 10 days of injection and then persists at this level for at least 6 months (Winstanley *et al.*, 2007, Zachariou *et al.*, 2006). Importantly, the vectors infect neurons only and produce no toxicity greater than vehicle infusions alone. Details of the production and use of these vectors are provided in earlier publications (Winstanley *et al.*, 2007, Zachariou *et al.*, 2006).

Sexual Experience. All ovariectomized female hamsters were primed for sexual experience once a week by giving two daily subcutaneous injections of estradiol benzoate (10 μg in 0.1 ml of cottonseed oil) approximately 48 hr and 24 hr prior to the sexual behavior test followed by a subcutaneous injection of

progesterone (500 μg in 0.1 ml of cottonseed oil) 4-6 hr prior to the sexual behavior test. Females that received sexual experience were presented with a sexually experienced male hamster for a 10 min session 4-6 hr after the progesterone injection. Each male and female was only paired once during the duration of the sexual experience tests.

Conditioned Place Preference. A biased conditioned place preference paradigm was utilized in this experiment (Tzschentke, 1998). Our conditioned place preference apparatus (Meisel & Joppa, 1994, Meisel *et al.*, 1996) consists of one white and one gray compartment (60 x 45 x 38 cm) connected by a clear central compartment (37 x 22 x 38). The main compartments were further differentiated by aspen bedding (Harlan Laboratories, IN) in the gray compartment and corncob bedding (Harlan Laboratories, IN) in the white compartment. The ovariectomized female hamsters were hormonally primed prior to the pre-test, sexual conditioning sessions, and the post-test. During the pretest the animal was placed in the clear central chamber and was free to roam the different compartments for 10 min to establish an initial preference for each compartment. As all animals showed an initial preference for the white chamber, conditioning was performed in the gray chamber. The hormone priming was repeated during the 2 (Groups 2-5) or 5 weeks (Group 1) of conditioning. During conditioning, females were given sexual experience with a male in the gray

compartment for 10 min, with female copulatory parameters measured (lordosis latency and total lordosis duration). One hr following the sexual experience test, the female was placed alone in the white chamber for 10 min. A control group of females that did not receive sexual experience were hormonally primed but placed alone in each chamber for 10 min. Following the 2 or 5 weeks of conditioning, animals were given a post-test in which they again were free to roam the chambers for 10 min. Regardless of group, all post-tests were done seven weeks post-stereotaxic surgery, and therefore all animals were sacrificed with the same level of viral expression. There were 5 groups of animals in this experiment: A positive control group of animals received bilateral AAV-GFP and given 5 weekly sexual behavior pairings with a male (Group 1, n=8), which has been previously shown to by the necessary amount of sexual experience to elicit a place preference to sex behavior. Two negative control groups were not given any sexual conditioning for 2 weeks, and received either AAV-ΔFosB (Group 2, n=5) or AAV-GFP (Group 3, n=4). Lastly, there were animals that received 2 weeks of sexual behavior pairings with a male with a bilateral injection of either AAV-ΔFosB (Group 4, n=7) or AAV-GFP (Group 5, n=7).

Naïve Male Experiment. Previous research has shown that sexually experienced female hamsters can improve the copulatory efficiency of interactions with their sexually naïve male partners (Bradley *et al.*, 2005b). This

test was given approximately one week following the conditioned place preference post-test to the two groups of animals that received 2 weeks of sexual conditioning (Groups 4 and 5). Females were hormonally primed for sexual experience as described. During the 10 min test, a sexually naïve male hamster was introduced to the female's home cage and the test session was videotaped for later analysis. The number of mounts and intromissions (including ejaculations) by the male as well as the proportion of total mounts that included intromission (hit rate) were determined from the videotape.

Immunohistochemistry. Immunostaining was performed on all animals to verify both virus injection location and anatomical extent of protein expression. Females were given an overdose of Sleepaway (0.2 ml i.p., active ingredient: sodium pentobarbital, Fort Dodge Laboratories, Fort Dodge, IA) and intracardially perfused with 25 mM phosphate buffered saline (PBS) for 2 min (approximately 50 ml) followed by 4% paraformaldehyde in 25 mM PBS for 20 min (approximately 500 ml). The brains were removed and post-fixed for 2 hr in 4% paraformaldehyde then placed in a 10% sucrose solution in PBS overnight at 4ºC. Animals that received only bilateral AAV-GFP had serial coronal sections (40 µm) cut from frozen tissue into 25 mM PBS + 0.1% bovine serum albumin (BSA) (wash buffer) then mounted directly onto slides and coverslipped while still wet with 5% n-propyl galate in glycerin. Animals that received bilateral

AAV-ΔFosB had serial coronal sections (40 μm) cut from frozen tissue, and then rinsed 3 times for 10 min in wash buffer. AAV-ΔFosB animals were only analyzed for ΔFosB expression and therefore were incubated in ΔFosB/FosB primary antibody (1:10000, sc-48 Santa Cruz Biotechnology Inc., Santa Cruz, CA) in wash buffer plus 0.3% Triton-X 100 at room temperature for 24 hr and then moved to 4 ºC for 24 hr. This concentration of primary antibody was chosen as it produces only minimal endogenous ΔFosB/FosB staining. Following incubation in primary antibody the sections were rinsed 3 times for 10 min in wash buffer, and then incubated in biotinylated-secondary antibody for 45 min at room temperature (1:200, Vector, Burlingame, CA). The sections were then washed 3 times for 10 min in wash buffer before being incubated in streptavidin Alexa Fluor 594 conjugate (1:500, Molecular Probes, Eugene, OR). Following this incubation, the sections were washed 3 times for 10 min in wash buffer then mounted on slides and coverslipped while still wet with 5% n-propyl galate in glycerin.

Microscopic Analysis. Slides were analyzed by a Leica DM4000B light microscope with fluorescent capability coupled to a Leica DFC500 digital camera. Digital images of both the right and left injection sites of each section were serially analyzed by fluorescence microscopy to locate the injection placement in the NAc. The sections from each animal were analyzed to find the

rostral to caudal spread of viral expression and also the anatomical location of the largest diameter of expression. Further, within these sections the numbers of FosB stained cells were counted by an experimenter that was blind to experimental conditions.

Results

Time course of viral mediated overexpression of ΔFosB in the NAc of female Syrian hamsters.

A separate group of animals were utilized initially to find a time course of viral-mediated ΔFosB overexpression in the female hamster. Analysis of ΔFosB expression at the 3 (n=5), 6 (n=6), and 9 (n=2) week time points revealed that 3 weeks post stereotaxic surgery produced a level of ΔFosB overexpression which was maintained through 6 and 9 weeks post stereotaxic surgery. Viral expression was mostly nuclear, but was also found in the cytoplasm and even the dendrites of some overexpressing cells. Of the thirteen animals that comprised the time course experiment, four animals had rostral NAc core viral injections, one of which spread into the bed nucleus of the stria terminalis (BNST). The remaining nine animals had caudal injection placements, seven in the caudal core, and two in the caudal shell of the NAc. Only one of the caudal shell injections crossed caudally into the BNST, while six of the injections in the caudal core crossed caudally into the BNST. The average largest diameters of viral expression for each time point

were found to be 0.9 mm, 1.2 mm, and 1.0 mm for 3, 6, and 9 weeks, respectively. These average diameters were subjected to an analysis of variance and were not found to be significantly different. Therefore, in the following behavioral experiments, behavioral testing began around 3 weeks post stereotaxic surgery, and animals were sacrificed around 9 weeks post stereotaxic surgery to ensure that viral expression was maintained at a consistent level.

Immunohistological analysis of AAV-ΔFosB and AAV-GFP viral injections.

Brain sections from each animal used in the behavioral experiments were serially analyzed in a coronal plane for the anatomical location of viral injection. A total of 12 animals were analyzed for their bilateral ΔFosB expression by cell count, and injection placement, which was determined by tracing the residual needle tracks. Although injection placement was analyzed in a coronal section (**Figure 4**), protein expression extended in a rostral-caudal ellipse from the injection site, and also spread in a dorsal-ventral ellipse from the injection site. Of the five animals analyzed from Group 2, 70.5% of the overexpression cells were in the Nucleus accumbens (median= 16,864 cells, lower quartile= 7,551 cells, upper quartile=20,002 cells, interquartile range=12,451). The seven animals analyzed from Group 4 showed 65.6% viral overexpression in the nucleus accumbens (median=9,972 cells, lower quartile=5,683 cells, upper quartile=11,213 cells, interquartile range= 5530.). These cell counts represent

viral overexpression rather than endogenous staining due to the purposeful dilution of the primary antibody.

Figure 4

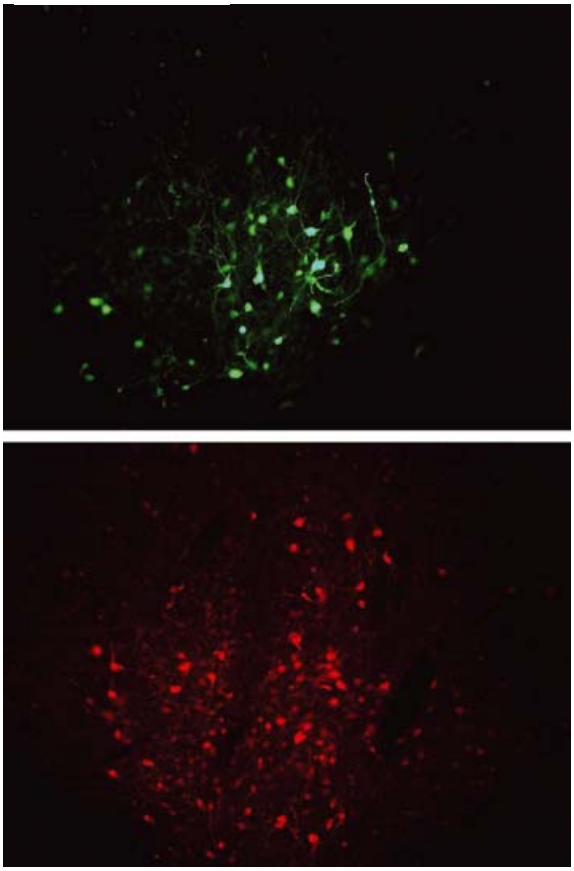


Figure 4. Protein expression levels mediated by AAV-GFP or AAV-ΔFosB 12 wks post-injection. **Top.** GFP overexpression was mostly nuclear but was also found to spread to the cytoplasm and dendrites of cells. **Bottom.** ΔFosB protein expression mimicked the expression pattern of the AAV-GFP overexpression cells (Hedges *et al*. 2009).

Of the 24 bilateral injection sites, twelve were in the rostral core of the NAc, six of which had viral expression that caudally spread into the BNST. The remaining twelve injection sites were in the caudal NAc. One of the twelve injections was in the caudal shell and spread caudally into the BNST. The last eleven injection sites were all in the caudal core of the NAc, eight of which spread caudally into the BNST. All injections were centered around the anterior

commissure except the one injection in the caudal shell of the NAc which was slightly more medial than the anterior commissure (**Figure 5**). All animals showed appropriate overexpression of either GFP or ΔFosB and were therefore used in subsequent behavioral analysis. No animals were excluded from the study because of poor anatomical injection placement. Further, because all injections were aimed at the accumbal core and only one injection included the shell no statistical analysis was done on the injection sites.

Figure 5

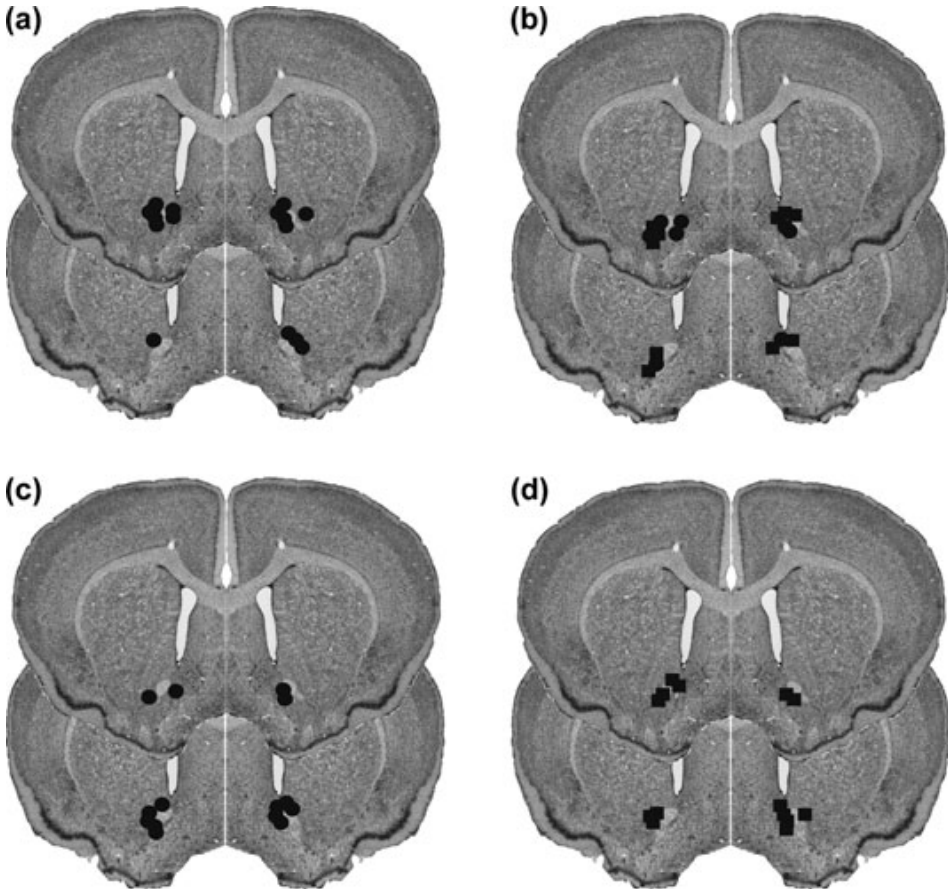


Figure 5. Anatomical localization of viral injection placements of experimental animals. Circles represent AAV-GFP placement and squares represent AAV-ΔFosB placement. **a.** AAV-GFP injection placements for animals with 5 wks sexual conditioning (Group 1). **b.** AAV-GFP and AAV-ΔFosB injection placements for animals with no sexual conditioning (Groups 2 and 3). **c.** AAV-GFP injection placements for animals with 2 wks sexual conditioning (Group 5). **d.** AAV-ΔFosB injection placements for animals with 2 wks sexual conditioning (Group 4) (Hedges *et al*. 2009).

AAV vector overexpression of ΔFosB in the NAc of female Syrian hamsters results in enhanced sexual reward.

To assess whether the overexpression of ΔFosB in the NAc had an effect on sexual reward we used the conditioned place preference paradigm. In this test, animals underwent either 0, 2, or 5 weeks of sexual conditioning. During sexual conditioning, lordosis latency and duration were recorded for each female hamster. Neither lordosis latency (Group 1: 553 sec ± 7 sec, Group 4: 552 sec ± 7 sec, Group 5: 561 sec ± 7 sec,) nor lordosis duration (Group 1: 485 sec ± 15 sec, Group 4: 522 sec ± 10 sec , Group 5: 522 sec ± 12 sec) during sexual conditioning differed significantly among groups throughout testing regardless of viral injection. Therefore neither the overexpression of GFP nor ΔFosB had any effect on the receptive behavior of the females.

Each group from the conditioned place preference procedure was analyzed individually with a repeated measure t-test between the amount of time spent in the conditioning compartment (gray compartment) during the pre-test and the post-test. The statistical analysis was not extended between groups. Previous

research has shown that five conditioning sexual experiences are sufficient to detect significant changes in place preference (Meisel & Joppa, 1994, Meisel *et al.*, 1996). Indeed, the positive control group consisting of female animals overexpressing GFP in the NAc that were given five conditioning sexual experiences spent significantly more time during the post test in the gray chamber paired with the sexual experience compared with the pre-conditioning performance, $t(8) = -3.13$, $P < 0.05$. As anticipated, animals that were not given any conditioning sexual experiences did not change significantly the amount of time in either chamber regardless of viral injection. Females overexpressing GFP that were given 2 conditioning sexual experiences did not demonstrate place conditioning, whereas females that were given two conditioning sexual experiences with overexpression of ΔFosB spent significantly more time in the chamber paired with sexual experience during this post test, $t(7) = -2.48$, $P < 0.05$ (**Figure 6**).

Figure 6

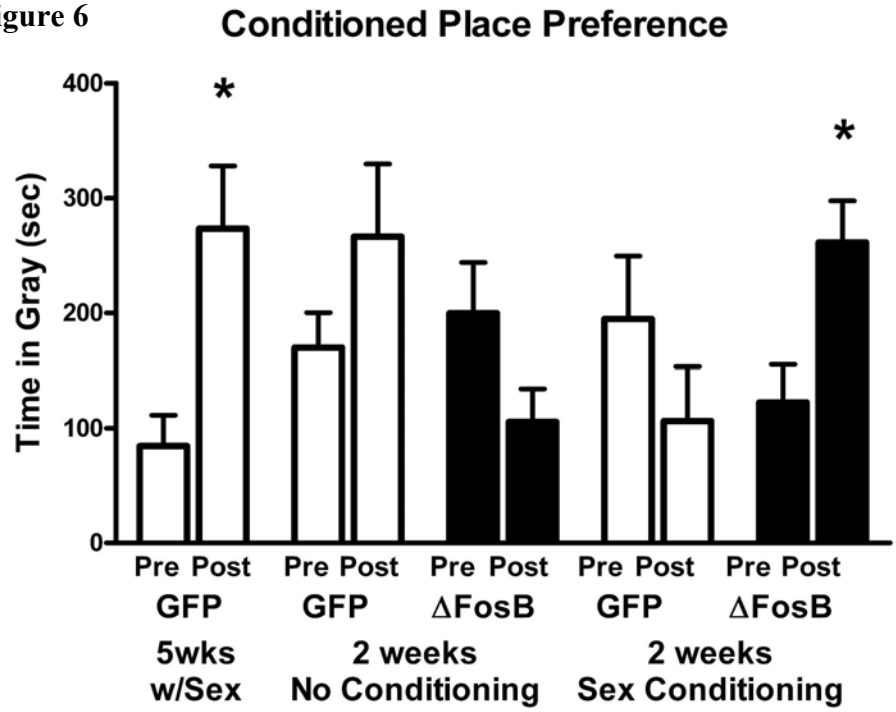


Figure 6. Conditioned place preference following viral injection. This graph shows the mean (±S.E.M) number of seconds during the pre-conditioning test (Pre) and the postconditioning test (Post) that each group of hamsters spent in the gray compartment. AAV-GFP animals received either 0 weeks (no conditioning), 2 weeks, or 5 weeks of conditioning with a male. AAV-ΔFosB animals received either 0 weeks (no conditioning) or 2 weeks of conditioning with a male.* $p<0.05$ vs. Pre-Test. (Hedges *et al*. 2009).

AAV vector overexpression of ΔFosB in the NAc of female Syrian hamsters improves their copulatory efficiency with naïve males.

One week following the conditioned place preference post-test, females with 2 weeks of sexual conditioning tests (Groups 4 and 5) were subjected to a

naïve male sexual behavior test. In this test, AAV-ΔFosB females with 2 prior sexual experience tests significantly improved their copulatory efficiency more than did AAV-GFP females with 2 prior sexual experiences (**Figure 7**). The hit rate (the proportion of total mounts that included intromission) of sexually naïve males that were paired with the AAV-ΔFosB females was significantly higher than the hit rate of naïve males paired with AAV-GFP females, $t(14)= 4.089$ $P<0.005$.

Figure 7

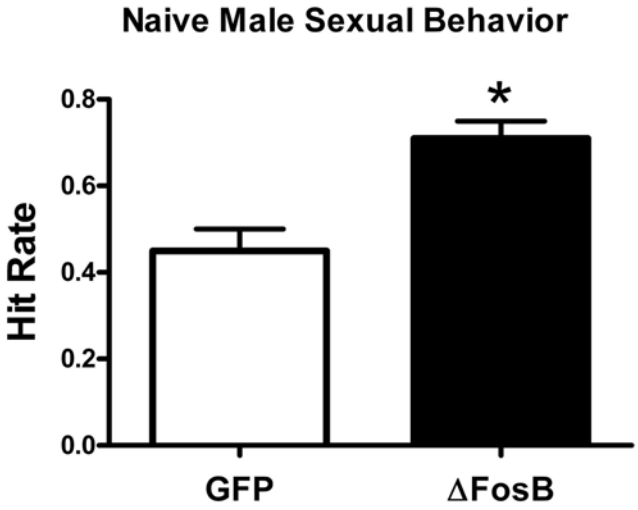


Figure 7. Copulatory efficiency of naïve male hamster partners. This graph shows the mean (± S.E.M) hit rate (the proportion of total mounts that included intromission) of the naïve male hamsters that were paired either with AAV-GFP females or AAV-ΔFosB females. The males paired with AAV-ΔFosB females had a significant increase in the hit rate compared to males paired with AAV-GFP females. * $p<0.05$ vs. GFP. (Hedges *et al*. 2009).

Discussion

Previous experiments that utilized AAV vectors for overexpression of ΔFosB were conducted in either rat or mouse model systems (Berton *et al.*, 2007, Olausson *et al.*, 2006, Winstanley *et al.*, 2007, Zachariou *et al.*, 2006). We validated the viral expression patterns in the hamster brain by immunohistochemical staining. This analysis demonstrated effective expression of ΔFosB that appeared as soon as 3 weeks after intracranial injection and remained elevated for 9 weeks in our time course analysis and up to 12 weeks in the behavioral experiments.

In our model of sexual experience, repeated copulatory interactions by the male leads to a sensitization of dopamine release in the NAc (Kohlert & Meisel, 1999, Kohlert *et al.*, 1997) which has reinforcing consequences in a conditioned place preference paradigm (Meisel & Joppa, 1994, Meisel *et al.*, 1996). This dopamine sensitization, as well as the ability of female hamsters to regulate successful intromission by the mounting male as a result of repeated sexual encounters, demonstrates an associative response (Bradley *et al.*, 2005b). We have shown that this reinforced sexual behavior can be enhanced by overexpression of ΔFosB in the NAc in the context of subthreshold sexual experience, analogous to the enhancement in instrumental responses to cocaine, morphine, or food consumption following similar overexpression of ΔFosB (Colby *et al.*, 2003, Olausson *et al.*, 2006, Zachariou *et al.*, 2006). This

enhancement in sexual interactions with the male following sexual experience was mirrored by the acquisition of a conditioned place preference. It is reasonable to consider ΔFosB as acting as a transcriptional nexus that is mediating both long-term modifications in behavior and the underlying neuronal plasticity consequent to the activation of the downstream targets of ΔFosB.

Given that elevation of ΔFosB produces these effects, the underlying mechanisms must then be questioned. There are very few molecular consequences that result from the accumulation of ΔFosB, therefore it is unclear how these molecular events fit into plasticity and dendritic spine formation. It has been proposed that accumulation of ΔFosB increases the expression and activity of the serine/threonine cyclin dependent kinase-5 (Cdk5) (Bibb, 2003, Kumar *et al.*, 2005). Key to our research is the role that Cdk5 has in synaptic plasticity by increasing dendritic spine formation (Bibb, 2003, Cheung *et al.*, 2006, Norrholm *et al.*, 2003). Accumulation of ΔFosB also increases the activity of additional transcription factors, such as nuclear factor kappa B (NF-κB), which have been implicated in synaptic plasticity, learning, and memory and may contribute to the long-term behavioral effects from drugs of abuse as well as other motivated behaviors (Ang *et al.*, 2001, Peakman *et al.*, 2003). Lastly, the prevalence of GluA2 subunits in AMPA receptors can be directly amplified by induction of ΔFosB (Nestler, 2001, Peakman *et al.*, 2003). In future studies we plan on concentrating on these and other potential downstream transcriptional targets of

ΔFosB to determine how their activity fluctuates with the accumulation of ΔFosB following repeated sexual behavior.

There is a vast literature postulating distinct roles that the shell and core of the NAc play in motivated behaviors (Brenhouse & Stellar, 2006, Cadoni & Di Chiara, 1999, Perrotti *et al.*, 2008, Pierce & Kalivas, 1995). Previous research in our laboratory has consistently identified cellular effects of sexual experience on the core of the accumbens (Bradley *et al.*, 2005a, Bradley *et al.*, 2005b, Bradley & Meisel, 2001, Bradley *et al.*, 2004, Kohlert & Meisel, 1999, Kohlert *et al.*, 1997, Meisel *et al.*, 1993), forming the basis for our targeting of the NAc core in this study. Our analysis of the anatomical extent of ΔFosB overexpression indicated that though the injections were indeed targeted to the caudal core of the NAc, ΔFosB expression often spread caudally into the rostral BNST. Although the caudal NAc and rostral BNST are certainly anatomically distinct nuclei, they are not necessarily functionally distinct as both regions modulate many of the mechanistic elements key to motivational processes (e.g., (Koob *et al.*, 2004). In our microdialysis studies of female hamsters (Kohlert *et al.*, 1997), we noted an inability to distinguish rostral BNST probe placements from those in the caudal NAc in terms of basal dopamine levels, dopamine responses to sexual interactions with males, or patterns of dopaminergic afferent innervation. Rather than viewing the spread of infection into the BNST as methodologically problematic, these results support the idea of a functional continuum between the NAc and BNST.

Although we have shown that overexpression of ΔFosB in female hamsters is *sufficient* to produce a conditioned place preference to sexual responding and to enhance copulatory interactions with males, it remains unknown whether ΔFosB expression is also *necessary* for these behavioral consequences of sexual experience. Recent studies have utilized an AAV-ΔJunD virus, which decreases ΔFosB mediated transcription by competitively heterodimerizing with ΔFosB before binding the AP-1 region on genes (Winstanley *et al.*, 2007). Pitchers et al. utilized this AAV-ΔJunD to knockdown ΔFosB mediated transcription, and determined that ΔFosB is required for sexual reward in male sexual behavior (Pitchers *et al.*, 2010b). Our goal is to complete a similar experiment to test whether ΔFosB is also necessary for the behavioral plasticity we have observed following female sexual experience, which will complement the results of the study presented here. If the accumulation of ΔFosB and its subsequent activation of downstream targets are causing both behavioral and cellular plasticity, then the knockdown of ΔFosB should abolish these effects.

CHAPTER 3

Long-term changes in gene expression following repeated sexual experience

Rationale

In the previous experiment, viral overexpression of ΔFosB resulted in increased sexual reward and copulatory efficiency in female hamsters. Accumulation of ΔFosB protein should result in the concurrent upregulation of its downstream target proteins. Although the viral injection was limited to the NAc in the previous study, one would assume that under normal sexual behavior conditions, there would be an activation of the entirety of the mesocorticolimbic dopamine circuit rather than the NAc alone. Because ΔFosB has been shown to accumulate in neurons in a variety of brain regions (Perrotti *et al.*, 2008), the purpose of the following experiment was to determine how sexual experience affects long-term gene expression of targets of ΔFosB regulated transcription in a limbic circuit.

Introduction

Activation of the mesocorticolimbic dopamine circuit underlies both the long-term cellular and behavioral changes associated with motivated behaviors and reward. For example, rewarding behaviors as diverse as wheel running (Greenwood *et al.,* Werme *et al.*, 2002), sucrose intake (Christiansen *et al.,* Smith & Berridge, 2005, Wallace *et al.*, 2008, Wyvell & Berridge, 2001), maternal behavior (Brown *et al.*, 1996, Hedges *et al.*, 2010, Numan, 2007, Stolzenberg & Numan, 2011), pair-bonding (Hedges *et al.*, 2010, Young *et al.*, 2005) and sexual

behavior (Carlezon & Thomas, 2009, Frohmader *et al.*, 2010, Hedges *et al.*, 2010, Meisel & Mullins, 2006, Pfaus *et al.*, 1995, Pitchers *et al.*, 2010a, Pitchers *et al.*, 2010b, Stolzenberg & Numan, 2011, Wallace *et al.*, 2008) all result in similar long-term behavioral and cellular consequences, suggesting a common set of neurobiological mechanisms that regulate these processes throughout an animal's life.

Our lab has focused on the transcription factor ΔFosB because of its unique properties that allow it to accumulate following repeated neuronal activity, possibly situating ΔFosB as an important component of both long-term behavioral and cellular plasticity (Chen *et al.*, 2009, Mcclung *et al.*, 2004, Nestler, 2004, Nestler *et al.*, 1999). To test whether this accumulation of ΔFosB plays a role in natural motivating behavior, we virally overexpressed ΔFosB in the NAc of female hamsters given minimal sexual experience. This manipulation increased reward as tested through conditioned place preference, and improved copulatory efficiency with a male, which in the absence of overexpression failed to produce similar results (Hedges *et al.*, 2009).

This study shows the importance of ΔFosB action in the NAc in sexual reward and behavioral plasticity, however, it remained unknown how our overexpression paradigm affected ΔFosB regulated transcription to ultimately augment behavior. There is a limited set of identified gene targets of ΔFosB mediated transcription, including the α-amino-3-hydroxyl-5-methyl-4-isoxazole-

propionate (AMPA) glutamate receptor 2 (GluA2) subunit, the serine/threonine kinase, cyclin-dependent kinase 5 (Cdk5), nuclear factor kappa B and the opioid peptide dynorphin (Ang *et al.*, 2001, Mcclung & Nestler, 2003, Russo *et al.*, 2009). Such gene expression occurs in the NAc and is the main focus of studies on motivated behaviors. Yet it remains unclear if these behavioral and cellular changes result simply from direct dopaminergic activation of the NAc, or if they include activity within the entirety of the circuit.

In this study our aim was to use female sexual behavior to better understand what molecular changes, as measured through quantitative real-time polymerase chain reaction (RT-PCR), result from repeated behavioral experience within various areas of this circuit. We have focused our findings here to include two targets that have been shown to be downstream of ΔFosB mediated transcription (i.e. GluA2 and Cdk5), as well as an additional two targets (i.e. Arc and MEF2A) that have been shown to play a role in structural plasticity. The immediate early gene Arc, and a downstream phosphorylation target of Cdk5, MEF2A, both play critical roles in experience-based structural plasticity (Bramham, 2008, Bramham *et al.*, 2010, Pulipparacharuvil *et al.*, 2008, Rodriguez *et al.*, 2005). In addition to the NAc, a further goal of this study was to determine if repeated sexual experience also produces molecular changes in other areas of the mesocorticolimbic dopamine circuit. Here, we examined another target of VTA dopaminergic projections, the prefrontal cortex (PFC), and a NAc output

pathway known to be an important component of the reward circuit, i.e. the ventral pallidum (VP). Understanding how this circuit changes as a result of a repeated natural rewarding experience will give a better understanding of how the motivational components of sexual behavior are regulated.

Materials and Methods

Animals. Male and female Syrian hamsters were delivered at approximately 60 days of age from Charles River Laboratories (Wilmington, MA). Females were individually housed in plastic cages (50.8 cm long x 40.6 cm wide x 20.3 cm high), whereas stimulus males were group housed in identical plastic cages in groups of two or three. The animal room was maintained at a controlled temperature of 22°C with a 14:10 light-dark schedule (lights off between 1330 and 2330 hrs). Food and water were available to all animals *ad libitum*. All procedures used in this experiment were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Surgery. Female hamsters were bilaterally ovariectomized under sodium pentobarbital anesthesia (Nembutal; 8.5 mg per 100 g body weight, i.p.) one week

following arrival to the laboratory and allowed to recover for one week prior to experimental testing.

Sexual Experience. All ovariectomized female hamsters were primed for sexual experience once a week by giving subcutaneous injections of estradiol benzoate (10 μg in 0.1 ml of cottonseed oil) approximately 48 and 24 hrs prior to sexual testing, followed by a subcutaneous injection of progesterone (500 μg in 0.1 ml of cottonseed oil) 4-6 hr prior to sexual testing on the third day. Females assigned to sexual experience groups were given weekly sexual behavior tests by being paired with a sexually experienced male hamster in the female's home cage for a 10 min session, whereas the naïve females were still hormone primed, but remained in the colony room. Each male and female were paired only once for the duration of the experiment. Throughout the experiment, measurements of female sexual behavior, i.e. lordosis latency and duration, were made weekly. There were no differences across weeks between groups in the sexual behavior parameters measured.

During the sixth week of sexual experience, one group of experienced females was sacrificed 3 hrs following their last sexual experience test (Experience/3 hr, n=9) to examine immediate changes in mRNA expression, while the other group of sexually experienced females was sacrificed 3 days following their last sexual experience test (Experience/3 day, n=9) to determine

long-lasting changes in mRNA expression. The sexually naïve females were sacrificed either 3 hrs or 3 days following their last progesterone injection and were combined into a single control group (No Experience, n=9). All females were sacrificed by rapid decapitation. The brain was quickly removed and placed in a brain matrix on ice and 2 mm slices at the levels of the prefrontal cortex, nucleus accumbens/caudate nucleus, and ventral pallidum were taken for each female. Bilateral punches (1 mm diameter x 2 mm deep) of the four aforementioned brain areas were collected on ice and placed in RNAlater (Qiagen). All punches were stored at 4°C for 48 hrs and then stored long-term at -20°C.

RNA extraction and cDNA synthesis. RNAlater was aspirated from the tissue and the punches were homogenized with a tissue shredder in a commercially available lysis buffer (RNeasy mini kit, Qiagen). RNA was extracted with a standard kit (RNeasy mini kit, Qiagen) by eluting with 30 µL of RNAse DNAse free PCR grade water according to the manufacturer's instructions and the extracts stored on ice. Sample RNA was immediately reverse transcribed into cDNA using a Roche Transcriptor First Strand cDNA synthesis kit by a combination of both anchored-oligo(dt) and random hexamer primers (Roche). The template-primer mixture was denatured by heating at 95°C for 10 min in a thermal block cycler with a heated lid. The remaining components of the reverse

transcription reaction were added to each PCR tube and the reaction was run for 10 min at 25°C, followed by 30 min at 55°C, and 5 min 85°C. The reaction was terminated by placing tubes immediately on ice. Sample cDNAs were diluted 1:5 with RNAse DNAse free PCR grade water, aliquotted to one-use microcentrifuge tubes and stored at -20°C until later analysis by RT-PCR.

*Primers***.** Real-time PCR assays were designed using the Roche ProbeFinder online software (www.Universalprobelibrary.com) which designs optimal PCR primers by using the Primer3a primer design algorithm. Primers generated by the software are checked by a Roche developed *in silico* PCR algorithm that searches the relevant genome and transcriptome for possible mis-priming sites for both of the generated PCR primers. If sites are identified, the assay rank is down-graded and flagged. The program pairs the designed primers with a Universal Probe Library (UPL) probe to create a complete optimal RT-PCR assay. The Universal Probe Library is comprised of 165 presynthesized dual-labeled fluorogenic probes. The UPL probes themselves are short hydrolysis probes comprised of chimeras of DNA and locked nucleic acids (LNA). A LNA is a derivative of RNA in which the ribose ring is constrained by a methylene linkage between the 2' oxygen and the 4' carbon resulting in increased thermal stability and binding strength of the duplexes. Combining sequence specific primers with UPL probes ultimately increases the specificity of results, because

unlike SYBR green which binds to all double stranded DNA, fluorescence is only detected when both the probe and primer are optimally bound to the template cDNA. Assays were designed for a reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and four genes of interest, GluA2 and Cdk5 using the respective available Syrian hamster gene sequences, and Arc and MEF2A, using the available known rat sequences. Forward and reverse primer sequences, UPL probe, product length, and GenBank accession code can be found in **Table 2**. Primer sequences were checked using a BLAST search to verify specificity. PCR products of each primer set were run out on 1.5% agarose gels and the detection of a single band on the gel further verified product specificity. Additionally the efficiency of each primer set was calculated by using standard curves of serially diluted template cDNA. As expected, all efficiencies were found to be approximately two.

Table 2. **PCR primer sequences, UPL probe #, product length, and GenBank accession code.**

Gene	Primer Sequences	UPL Probe #	Product Length (b.p.)	GenBank Accession Code
GAPDH	Fwd GTCTACTGGCGTCTTCACCAC	57	69	DQ403055.1
	Rev ATGACCCTCTTGGCTCCAC			
GLUA2	Fwd GAATCAACTAACGAATTTGGGATT	126	61	GU586153.1
	Rev GCATAAAGGCACCCAAGGA			
CDK5	Fwd TGAGGAAGAAGACACGAGCAT	41	76	CB884593.1
	Rev TCCTCCAAACGCCTTAATTTT			
ARC *	Fwd GCTGAAGCAGCAGACCTGA	3	74	NM019361.1
	Rev TCTGCTTTTCTTCACTGGTATGA			
MEF2A *	Fwd AAGGCTTTACTTCCCCTGGA	64	74	NM001014035.1
	Rev CGCTTGTCCATAGATGGTGCT			

Table 2. PCR primer sequences, UPL probe #, product length, and GenBank accession code. Quantitative real-time PCR information for GAPDH, GluA2, Cdk5, Arc, and MEF2A. * Represents the use of rat sequences as hamster sequences are currently unknown.

Real-time PCR. Real-time PCR reactions were carried out in a 20 µL reaction consisting of 4 µL RNAse DNAse free PCR grade water, 100 nM UPL probe, 200 nM of appropriate forward and reverse primers, 1X LightCycler 480 Probes Master Mix and 4 µL diluted (1:5) cDNA template. Real-time PCR was measured using a LightCycler 480 machine (Roche). Reactions were pre-incubated at 95°C for 10 min with a ramp rate of 4.4°C/sec. The reactions were amplified for 45 cycles (GAPDH/Cdk5, GAPDH/GluA2) or 55 cycles

(GAPDH/Arc, GAPDH/MEF2A) with the following cycle parameters: 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s, for each amplification cycle followed by a cooling cycle at 40°C for 30 s.

Analysis. Crossing point (Cp) values were averaged across triplicate samples for the gene of interest (GluA2, Cdk5, Arc or MEF2A) and normalized to the reference gene (GAPDH) (ΔCt) and then averaged Cp values were analyzed with the ΔΔCt method to determine relative changes in gene expression between sexually experienced females sacrificed at two different time points and sexually naïve females. Data were analyzed with a one-way ANOVA and Newman-Keuls posthoc analysis (Statistica 9.0) and considered to be statistically significant at $P<0.05$.

Results

To analyze the immediate, as well as long lasting effects of repeated sexual experience on gene expression in the limbic circuit, female hamsters were given six weeks of repeated sexual experience and sacrificed either 3 hr or 3 days following their last sexual test to determine changes in both short-term and long-term gene expression. Four brain regions within the mesocorticolimbic dopamine circuit were analyzed with quantitative real-time PCR for relative expression of two targets of ΔFosB mediated transcription, specifically the GluA2 subunit of

the AMPA receptor and Cdk5. Further, two additional gene targets were analyzed due to their known involvement in structural plasticity, specifically Arc (Bramham *et al.*, 2010) and MEF2A (Pulipparacharuvil *et al.*, 2008). All genes of interest were normalized to GAPDH as an internal reference gene for analysis due to its stability and constitutively high expression in cells.

First concentrating on the NAc, one of the primary outputs of VTA dopaminergic projection neurons, we observed that repeated sexual experience resulted in differential gene expression. A one-way ANOVA with a Students Newman-Keuls multiple comparison test revealed that there was a significant increase in GluA2 mRNA expression in the NAc following repeated sexual experience at both the short (3 hrs) and long (3 days) time points compared to sexually inexperienced females, ($P<0.05$) (**Figure 8**). No effects of sexual experience on Cdk5, Arc, or MEF2A were observed. There was however a trend for an increase in MEF2A in the NAc at both the 3 hr ($p=0.10$) and 3 day ($p=0.10$) time points.

Figure 8

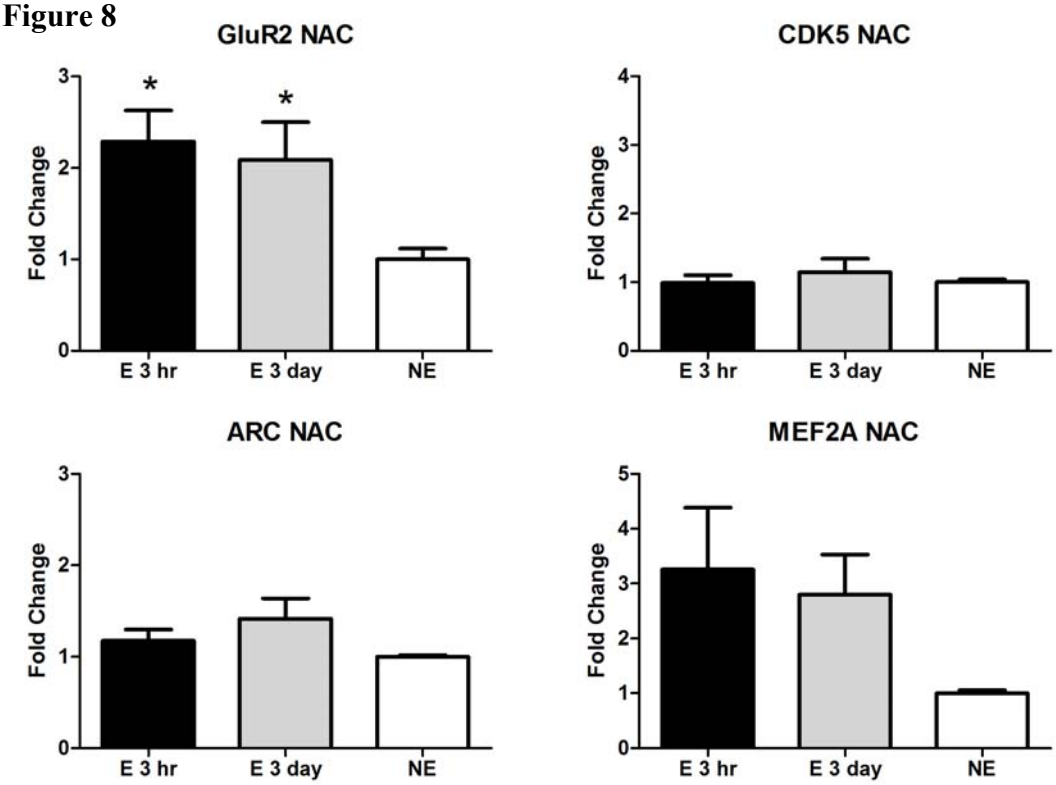


Figure 8. Changes in gene expression following repeated sexual experience in the NAc of female hamsters. Female hamsters sacrificed either 3 hrs (E 3 hr) or 3 days (E 3 day) following their last sexual experience test had significantly increased mRNA expression of the GluA2 AMPA receptor subunit in the NAc relative to sexually inexperienced (NE) females (* $p<0.05$). Repeated sexual experience was not found to significantly change gene expression of Cdk5, Arc, and MEF2A within the NAc. There is a trend present towards increased MEF2A expression at both tested time points.

We also analyzed the prefrontal cortex (PFC), another brain area that receives direct dopaminergic innervation, but also has glutamatergic projections that feed back to the NAc (Humphries & Prescott, 2010). Although there was a trend towards an increase in Cdk5 in the PFC at both measured time points (3 hr

p=0.09, 3 day p=0.29), one-way ANOVA did not detect any significant changes in expression of GluA2, Cdk5, Arc or MEF2A in this brain area at 3 hr or 3 day following the last sexual experience (**Figure 9**).

Figure 9

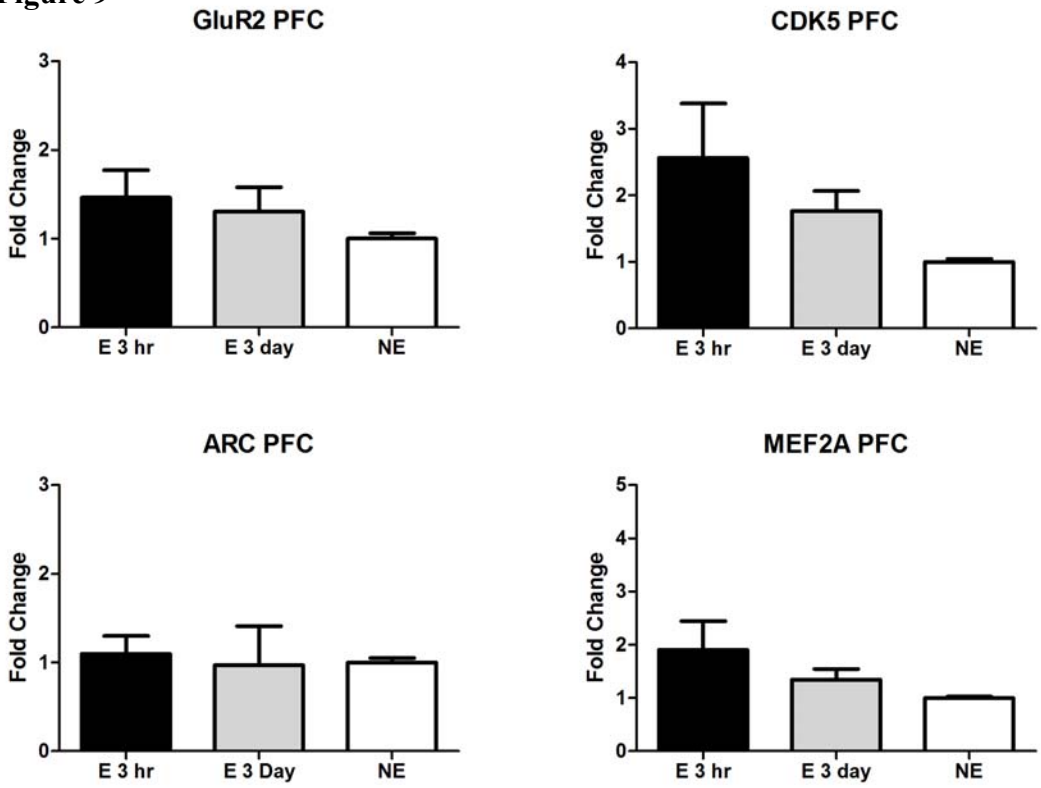


Figure 9. Changes in gene expression following repeated sexual experience in the PFC of female hamsters. Repeated sexual experience was not found to significantly change gene expression of GluA2, Cdk5, Arc, or MEF2A within the PFC at either measured time point.

We next analyzed the dorsal striatum, specifically the caudate nucleus (CU), as an anatomical control region which receives dopaminergic input primarily from the substantia nigra (Humphries & Prescott, 2010, Pierce & Kalivas, 1997a, Pierce & Kumaresan, 2006). Interestingly, although this brain area has been shown to be activated following stimulation through administration of drugs of abuse, we have never observed changes in the CU as a result of our sexual experience paradigm. We did not observe any significant changes in gene expression of GluA2, Cdk5, Arc or MEF2A in the CU at either the 3 hr or 3 day time points (**Figure 10**). However, there was a trend for an increase in MEF2A within this brain region as a result of repeated sexual experience at both time points tested compared to naïve control females (3 hr $p=0.13$, 3 day $p=0.12$).

Figure 10

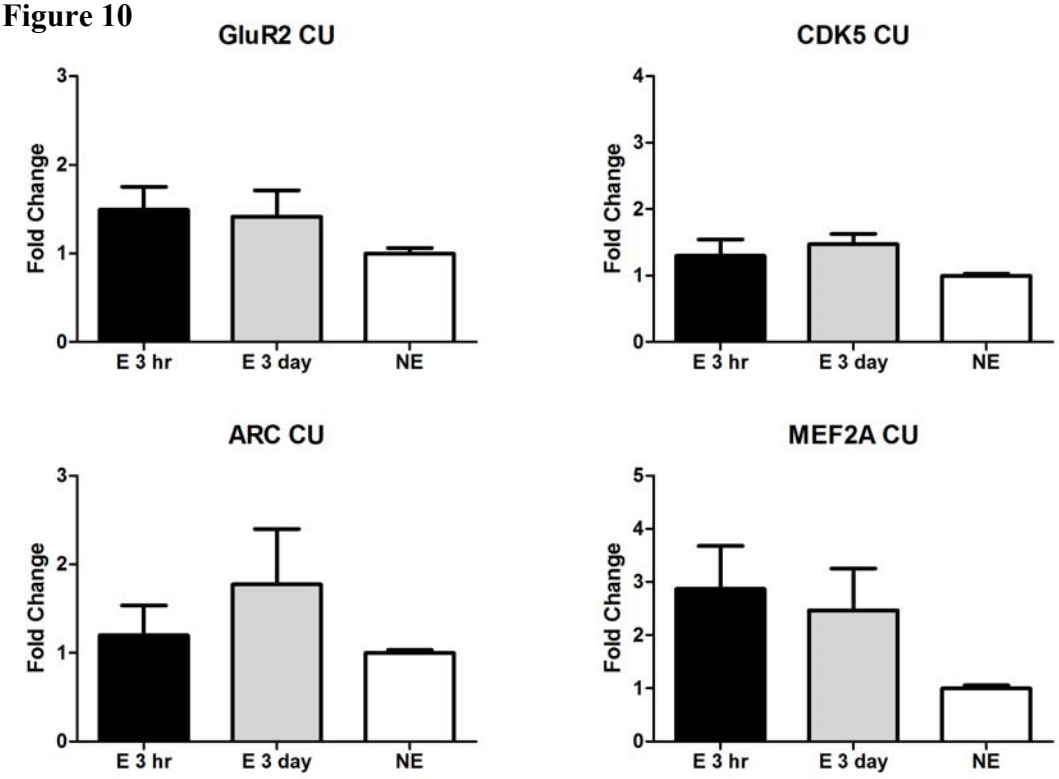


Figure 10. Changes in gene expression following repeated sexual experience in the CU of female hamsters. No significant changes in GluA2, Cdk5, Arc, or MEF2A mRNA expression were observed following repeated sexual experience at either of the time points analyzed.

Lastly, we analyzed the ventral pallidum (VP) which is an output of NAc medium spiny neurons primarily possessing D2 receptors (Humphries & Prescott, 2010). In this way, the VP also served as a control brain area, as the postulate of our research has been that the long-term plasticity observed following repeated sexual experience is mediated through D1 receptor activation (Bradley & Meisel, 2001, Bradley *et al.*, 2004, Hedges *et al.*, 2010, Meisel & Mullins, 2006). There

were no observed changes in gene expression in any of the gene targets examined (**Figure 11**).

Figure 11

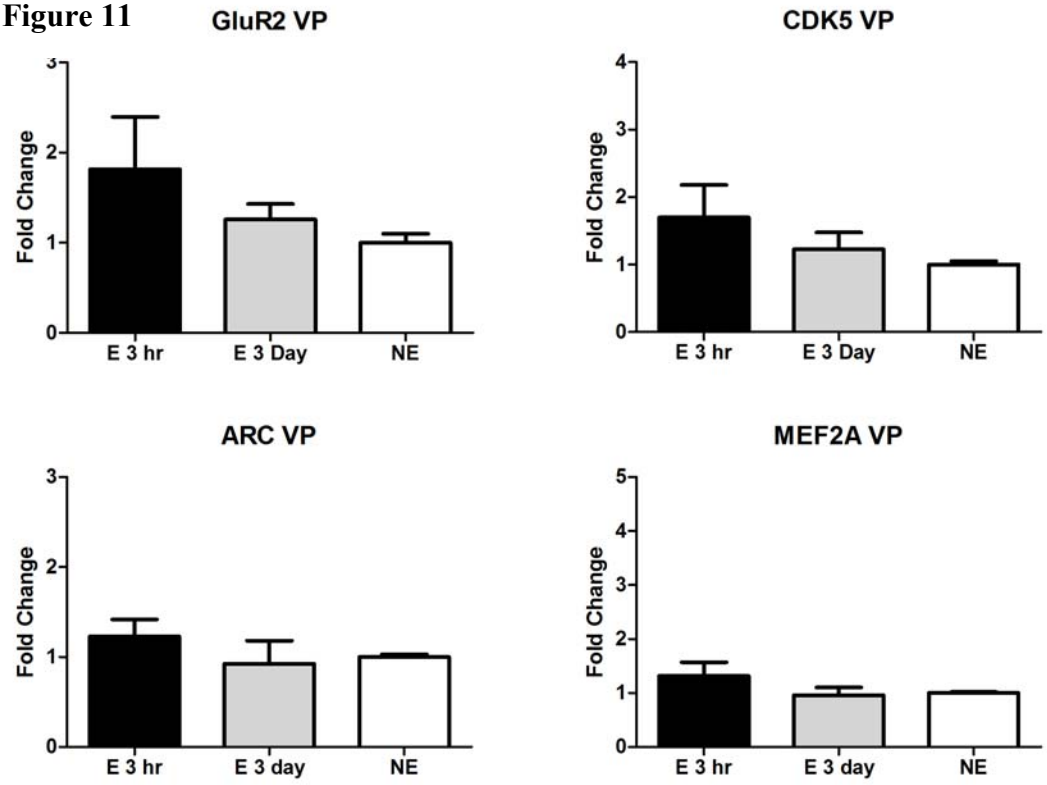


Figure 11. Changes in gene expression following repeated sexual experience in the VP of female hamsters. No significant changes in GluA2, Cdk5, arc, or MEF2A mRNA expression were observed following repeated sexual experience at either of the time points analyzed however there was a trend of an increase in MEF2A gene expression at both time points.

Discussion

We hypothesized that activation of the mesocorticolimbic dopamine system by a naturally rewarding behavior, specifically female sexual behavior, would result in a pattern of gene expression that could explain the long-term

behavioral, cellular, and structural plasticity observed following repeated sexual experience. We previously conducted a large gene expression microarray study that analyzed differences between sexually experienced and sexually naïve females (Bradley *et al.*, 2005a). In the aforementioned study, we used a rat Affymetrix chip to hybridize hamster brain cDNA as the Syrian hamster genome has not been sequenced. Although there is a large homology between the rat and hamster sequences, it is possible that differences between the genomes could attribute to type II error, or false negatives, in gene detection. A false negative occurs when we fail to observe differences that are occurring as a result of poor sensitivity, or in this case non-optimal hybridization between rat probes and hamster cDNA sequences. To take a more focused approach in determining changes in gene expression, we used the more sensitive quantitative real-time PCR assay. In this study we chose to focus on four gene targets, the GluA2 subunit of the AMPA receptor, Cdk5 (cyclin-dependent kinase 5), Arc (activity-regulated cytoskeleton-associated protein), and MEF2A (myocyte enhancer factor 2A). Both GluA2 and Cdk5 are downstream targets of ΔFosB, a highly stable transcription factor that has been shown to play an important role in addiction-related behavior and plasticity. For example, overexpression of ΔFosB in bitransgenic mice resulted in increased levels of GluA2 (Kelz *et al.*, 1999), and viral overexpression of GluA2 enhanced the rewarding effects of cocaine in a conditioned place preference paradigm (Kelz *et al.*, 1999). Cdk5 activity and

protein levels also have been shown to increase following either chronic cocaine administration or overexpression of ΔFosB (Bibb, 2003, Mcclung & Nestler, 2003). Interestingly, inhibition of Cdk5 blocks the cocaine-induced increase in dendritic spine formation in the NAc (Norrholm *et al.*, 2003).

Our goal in this study was to determine whether gene expression for these proteins also occurs following experience with natural rewards. We also analyzed two additional gene targets, Arc and MEF2A, which may be important in mediating experience-based structural plasticity. Arc is an immediate early gene whose mRNA is rapidly induced by synaptic activity, and is then locally translated within dendritic spines to ultimately mediate long-term potentiation and long-term depression (Link *et al.*, 1995, Lyford *et al.*, 1995, Moga *et al.*, 2004, Rodriguez *et al.*, 2005, Steward *et al.*, 1998). Lastly, we analyzed MEF2A, a phosphorylation target of Cdk5, due to its identified role in cocaine regulated behavioral and synaptic plasticity (Pulipparacharuvil *et al.*, 2008). Specifically, this study found that suppression of MEF2A through Cdk5 phosphorylation via dopamine D1 receptor signaling was linked to increased dendritic spine formation (Pulipparacharuvil *et al.*, 2008)

Our experiment showed that repeated sexual experience increased the mRNA expression of the GluA2 subunit of the AMPA receptor. We analyzed expression through RT-PCR at two sacrifice time points to discern differences in short-term and long-term gene expression. GluA2 mRNA expression was

increased in only the NAc of experienced females sacrificed at both the short and long-term time points compared with naïve control females. This result indicates that increased mRNA expression of GluA2 may be an inherent outcome of activating the NAc through naturally motivated behaviors, and may be playing a critical role in sexual reward and plasticity.

When we examined Cdk5 expression in various areas of the mesocorticolimbic dopamine pathway following repeated sexual experience, there were no significant changes at either time point analyzed. Further, we also did not detect any significant differences in expression of two genes involved in structural plasticity, Arc and MEF2A, following repeated sexual experience in any brain area analyzed either 3 hrs or 3 days following the female's last sexual experience. However, we did observe trends for increased MEF2A expression in both striatal regions, the NAc and CU, as a result of repeated sexual experience. In prior unpublished studies we were unable to detect changes in GluA2 or Cdk5 protein levels following repeated sexual experience in the NAc, CU, PFC, or VP. Reasoning that our failure to perceive differences may be due to the inability of the assay to detect very small changes in protein, we chose to test our hypothesis again using a gene expression PCR approach. We believed that the use of quantitative real-time PCR would be a much more sensitive assay to measure what may be very small changes in expression occurring as a result of repeated sexual experience.

The endpoints that we chose to analyze in this study were based off of reported changes in these brain areas following administration of addictive drugs. Although we did not directly measure differences in gene expression between sexually experienced and drug experienced animals, we made the assumption that these brain areas would be similarly activated through natural motivated behaviors. There are two possible explanations for our inability to detect differences in gene expression of targets that are either downstream of ΔFosB mediated transcription or are related to structural plasticity. First, it might be that sexual experience produces a pattern of gene expression in the endpoints that we measured that does not mirror the pattern of expression found following administration of drugs of abuse. Another possible explanation for these seemingly negative results is that the regulation of our gene targets in our measured endpoints is not at the level of mRNA, or even protein, as our previous unpublished Western blotting experiment was unable to detect differences in protein expression. Instead, it may be that the regulation of these proteins is through other post-translational mechanisms, such as activity through phosphorylation, which is of particular interest due to the ability of Cdk5 to affect MEF2A activity through phosphorylation.

Our study serves as an interesting demonstration of changes in gene expression resulting from a natural motivated behavior, specifically female sexual behavior. Understanding how natural motivated behaviors affect the rewarding

neurocircuitry in the brain allows researchers to better understand how this system functions as a result of normal rewarding behaviors. Our observed increase in gene expression of the GluA2 subunit of the AMPA receptor points to the possibility that repeated sexual experience may act to alter cellular excitability. Therefore, determining the relative distribution of AMPA receptors containing GluA2 subunits between the cell surface and intracellular stores could give insight into the possible ramifications of the upregulation of this specific AMPA receptor subunit on synaptic plasticity.

CHAPTER 4

Trafficking of GluA1 and GluA2 AMPA receptor subunits following repeated sexual experience

Rationale

Results from the previous experiment demonstrated that when female hamsters are given repeated stimulation from a natural motivated behavior, specifically sexual behavior, we observed a long-lasting increase in mRNA expression of the GluA2 subunit of the AMPA receptor within the NAc. To look further into this potentially important increase in a specific AMPA receptor subunit, with ramifications for cellular excitability, I used a cell-surface protein cross-linking method to examine the trafficking of this AMPA subunit, as well as the GluA1 AMPA subunit, to measure receptor distribution between the cell surface and intracellular stores following repeated sexual experience.

Introduction

The nucleus accumbens (NAc) is critical for the expression of motivated behaviors and reward (Chen *et al.*, 2009, Everitt & Robbins, 2005, Kalivas & Volkow, 2005, Kelz *et al.*, 1999, Koob *et al.*, 2004, Martinez & Paredes, 2001, Mcclung & Nestler, 2003, Mcclung & Nestler, 2008, Meisel & Mullins, 2006, Nestler, 2004, Pitchers *et al.,* Wolf *et al.*, 2004). Although dopamine neurotransmission within the NAc has been the focus of the majority of research involving mechanisms of reward, glutamate neurotransmission through AMPA receptor activation mediates the majority of excitatory transmission in the brain, and has also been shown to be critical in psychostimulant addiction (Hu & White,

1996, Kalivas *et al.*, 2005, Kalivas & Volkow, 2005, Kessels & Malinow, 2009, Kourrich *et al.*, 2007, Kourrich & Thomas, 2009, Pennartz *et al.*, 1990, Sun *et al.*, 2008, Thomas *et al.*, 2008, Wolf *et al.*, 2004). Research has identified that glutamate AMPA receptor trafficking between intracellular stores and the synapse is an important component of plasticity by acting to change synaptic strength, specifically through long-term potentiation (LTP), long-term depression (LTD), and synaptic scaling (Bredt & Nicoll, 2003, Carroll *et al.*, 2001, Keifer & Zheng, Malinow & Malenka, 2002, Song & Huganir, 2002, Suzuki *et al.*, 2003, Thomas & Malenka, 2003, Turrigiano & Nelson, 2004, Wolf & Ferrario, 2010). AMPA receptors are assembled as tetramers on medium spiny neurons within the NAc consisting of GluA1, GluA2, and GluA3 subunits (GluA4 is not found on medium spiny neurons) (Cull-Candy *et al.*, 2006, Greger & Esteban, 2007)**.** The tetrameric composition of an AMPA receptor directly contributes to its physiological properties as well as its trafficking (Wolf & Ferrario, 2010). The hypothesis is that AMPA receptors composed of GluA2/3 subunits cycle constitutively in an out of synapses, whereas AMPA receptors containing GluA1 subunits are inserted into synapses in an activity-dependent manner (Malinow, 2003, Malinow & Malenka, 2002, Takahashi *et al.*, 2003). Further, the insertion of GluA2 subunits into AMPA receptors results in the receptor having decreased Ca^{2+} permeability, as well as decreased cellular excitability (Thiagarajan *et al.*, 2007). There is a small subpopulation of AMPA receptors that lack the GluA2 subunit, therefore

the insertion or removal of a small number of these GluA2 lacking AMPAR could result in strong changes in synaptic strength (Cull-Candy *et al.*, 2006, Guire *et al.*, 2008, Isaac *et al.*, 2007, Liu & Zukin, 2007, Liu & Cull-Candy, 2000, Plant *et al.*, 2006)**.**

Although a great deal of research has focused on AMPA receptor mediated plasticity and trafficking following administration of drugs of abuse, their actions in naturally rewarding behaviors are far less known. Our laboratory has successfully used sexual behavior in female Syrian hamsters as a model of motivational pathways, with repeated sexual experience ultimately causing long-term behavioral, cellular, and structural plasticity (Bradley *et al.*, 2005a, Bradley *et al.*, 2005b, Bradley & Meisel, 2001, Bradley *et al.*, 2004, Hedges *et al.*, 2009, Meisel & Mullins, 2006). We have shown that repeated sexual experience results in an accumulation of the uniquely stable transcription factor ΔFosB within the NAc (Meisel & Mullins, 2006), which also has been demonstrated following numerous other naturally rewarding behaviors (Christiansen *et al.,* Greenwood *et al.*, 2010, Hedges *et al.*, 2009, Olausson *et al.*, 2006, Pitchers *et al.,* Vialou *et al.,* Vialou *et al.,* Wallace *et al.*, 2008, Werme *et al.*, 2002). ΔFosB has been a transcription factor of interest in the drug abuse literature due to its unique stability and accumulation following chronic stimulation. Due to these properties, ΔFosB is optimally situated to play an important role in mediating reward and experience-dependent long-term plasticity. To determine if ΔFosB played a role

in sexual reward, we virally overexpressed ΔFosB in the NAc of female Syrian hamsters and tested them in a conditioned place preference paradigm for changes in reward. Overexpression of ΔFosB resulted in increasing the rewarding consequences of sexual behavior and also increasing copulatory efficiency with a male (Hedges *et al.*, 2009).

To determine what molecular changes were occurring as a result of ΔFosB overexpression, we chose to focus on one target of ΔFosB mediated transcription, the AMPA receptor subunit GluA2 (Kelz *et al.*, 1999). Female hamsters given repeated sexual experience showed a significant increase in mRNA expression of GluA2 in the NAc, but not in the caudate putamen (Chapter 3). The goal of the present study was not only to determine if the increased mRNA expression of GluA2 following repeated sexual experience was mirrored by increases in protein expression, but also to determine if sex experience differentially altered AMPA receptor subunit trafficking to the cell surface. We chose to include an additional AMPA receptor subunit, GluA1, due to its abundant expression in AMPA receptors and also because GluA1 surface expression has been shown to increase following 21 days of withdrawal from cocaine administration (Boudreau & Wolf, 2005). To analyze GluA1 and GluA2 trafficking we utilized a protein cross-linking technique developed by Marina E. Wolf's laboratory. This protocol employs bis(sulfosuccinimidyl)suberate (BS^3), a membrane-impermeable protein cross-linking agent, to distinguish between cell surface and intracellular receptor

pools in brain samples (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Gao & Wolf, 2007, Sun *et al.*, 2008). This chemical agent anchors proteins on the surface of the plasma membrane (Archibald *et al.*, 1998, Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Broutman & Baudry, 2001, Clayton *et al.*, 2002, Conrad *et al.*, 2010, Ferrario *et al.*, 2011, Gerges *et al.*, 2004, Grosshans *et al.*, 2002a, Grosshans *et al.*, 2002b, Hall *et al.*, 1997, Hall & Soderling, 1997a, Hall & Soderling, 1997b, Nelson *et al.*, 2009, Sun *et al.*, 2008)**.** Western blots can then identify proteins in high molecular weight (surface localized), and intracellular receptor pools (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Ferrario *et al.*, 2011, Nelson *et al.*, 2009, Wolf & Ferrario, 2010). Consequently, this study examined if there is differential long-term AMPA receptor trafficking observed following sexual experience in female hamsters.

Materials and Methods

Animals. Male and female Syrian hamsters were delivered at approximately 60 days of age from Charles River Laboratories (Wilmington, MA). All females were housed individually in clear plastic cages (50.8 cm long x 40.6 cm wide x 20.3 cm high), whereas stimulus males were group housed in identical plastic cages in groups of two or three. The animal room was maintained at a controlled temperature of 22°C with a 14:10 light-dark schedule (lights off between 1330 and 2330 hrs). Food and water were available to all animals *ad*

libitum. All procedures used in this experiment were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals are were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Surgery. Female hamsters were bilaterally ovariectomized under sodium pentobarbital anesthesia (Nembutal; 8.5 mg per 100 g body weight, i.p.) one week following arrival in the laboratory and allowed to recover for two weeks prior to behavioral testing.

Sexual Experience. All ovariectomized female hamsters were primed for sexual experience once a week by giving subcutaneous injections of estradiol benzoate (10 μg in 0.1 ml of cottonseed oil) approximately 48 and 24 hrs prior to sexual testing, followed by a subcutaneous injection of progesterone (500 μg in 0.1 ml of cottonseed oil) on the third day, 4-6 hrs prior to sexual testing. Females receiving sexual experience were paired weekly with a sexually experienced male hamster in the female's home cage for a 10 min sexual experience session. The sexually naïve females were still hormone primed, yet remained in the colony room. Males and females were paired only once for the duration of the experiment. Throughout the experiment, measurements of female sexual behavior were made weekly. Neither lordosis latency (E 7 days 38 sec ± 5 sec, E 21 days

46 sec ± 7 sec) nor lordosis duration (E 7 days 482 sec ± 10 sec, E 21 days 454 sec ± 15 sec) were ultimately found to differ between the two tested time points. Following the six weeks of sexual experience, all females (experienced and naïve) continued to be hormonally primed as described previously for either 1 or 3 additional weeks. Half of the experienced and naïve females were sacrificed 7 days following their last sexual experience, while the remaining females were sacrificed 21 days following their last sexual experience. Sacrifice times are modeled from previous experiments conducted by the Wolf laboratory (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005).

BS^3 Crosslinking. Our cross-linking assay is modeled from the protocol successfully used by Marina E. Wolf's laboratory (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Ferrario *et al.*, 2011, Nelson *et al.*, 2009, Sun *et al.*, 2008, Wolf & Ferrario, 2010). Either 7 or 21 days following their last sexual test, animals were rapidly decapitated without anesthesia and brains were quickly removed, placed in a brain block and sliced into a 2 mm coronal section at the level of the NAc on ice. Bilateral punches were taken of the NAc and CU (1 mm x 2 mm) on an ice cold platform and chopped roughly in half with a razor blade. Punches were aimed at the NAc core, though because of the diameter of the punch, the tissue sample contained both NAc core and shell. The pieces of the punch from one side of the brain (cross-linked) were placed in a microcentrifuge

tube of ice cold artificial cerebral spinal fluid (ACSF) spiked with 2 mM BS^3 (Pierce, Rockford IL). The contralateral punch pieces (non-cross-linked) were placed in a tube of just ACSF. Punches were incubated in either solution for 30 min at 4°C with gentle agitation. Cross-linking was then quenched by the addition of 100 mM glycine to each microcentrifuge tube for 10 min at 4°C with gentle agitation. Samples were then centrifuged and the supernatant discarded. Punches were resuspended in 50 μL Western blotting processing buffer (1% SDS with 50 mM NaF and 3.3 mM EGTA with protein and phosphatase inhibitors (Pierce, Rockford, IL)), rapidly homogenized with a motorized micro hand homogenizer, aliquotted, and stored at -80°C for later protein assay and Western blotting analysis.

Western Blotting. Individual aliquots of each sample were analyzed for total protein concentration using the DC Protein Assay (BioRad Laboratories). Samples were divided so that each gel contained the total protein sample and cross-linked sample from each animal, as well as equal representation of each experimental group, plus a pre-stained protein standard (precision plus all blue protein standard, BioRad Laboratories, 161-0373). Each sample (35 μg of protein) was run out on two gels to blot for either GluA1 or GluA2. Protein samples were electrophoretically resolved on a 4-15% Tris-HCl gel (BioRad Laboratories) under reducing conditions for approximately 90 min at 100 V. Resolved proteins

were then transferred to a nitrocellulose membrane overnight at 25 V for immunoblotting. Nonspecific binding sites were blocked in Li-Cor blocking buffer (1:1 in 25 mM PBS) for 1 hr at room temperature with shaking. Blots were then incubated in mixed primary antibody for GluA1 (rabbit, 1:1000, Millipore AB1504) and GAPDH (mouse, 1:1000, Millipore MAB374) or GluA2 (rabbit, 1:1000, Millipore AB1768) and GAPDH (mouse, 1:1000, Millipore MAB374) in Li-Cor blocking buffer 1:1 with wash buffer (Tris-buffered saline with 0.1% Tween-20, Sigma) overnight at 4°C. Membranes were rinsed in wash buffer, and the blots were incubated with a mixture of infared labeled secondary antibodies, goat anti-rabbit IRDye 680 (1:10,000, Li-Cor Biotechnology) and goat anti-mouse IRDye 800 (1:10,000, Li-Cor Biotechnology). The use of species specific IRDyes allows for both a protein of interest (either GluA1 or GluA2) as well as a loading control (GAPDH) to be imaged on the same blot simultaneously, as long as each primary antibody is raised in a different species (Mathews *et al.*, 2009, Picariello *et al.*, 2006). Thus, two different antigens can be differentially detected through the use primary antibodies raised in different species in conjunction with appropriate IRDyes that are excited at two distinct wave lengths. This removes the need to repeatedly strip blots for re-probing. The blots were extensively rinsed in wash buffer prior to imaging and were scanned using a Li-Cor Odyssey Infared Imaging System scanner (Li-Cor, Lincoln, NE).

Analysis. Images were analyzed using the Odyssey Application software (Li-Cor) to determine integrated intensities of cell surface (S), intracellular (I), and total protein levels of GluA1 and GluA2. The surface and intracellular band densities were determined for each lane of cross-linked sample, whereas total protein levels were determined using the non-cross-linked tissue from the contralateral side of the brain. For all Western blots, density measurements of S, I and total protein (from the contralateral side of the brain) were normalized to an internal loading control, GAPDH. Because the S/I ratio is independent of protein loading these values were not normalized to a loading control. The resultant values of S, I, total and S/I for the experienced females were normalized to measurements from naïve, non-experienced control females. Therefore all data are presented as a percent of control females. Groups were statistically analyzed with a Student's t-test between experienced and naïve groups of females for each protein, at each time point, with significance set at $p<0.05$.

Results.

We were able to duplicate findings from other laboratories that BS^3 cross-linking is able to successfully distinguish cell surface and intracellular pools of GluA1 and GluA2 AMPA receptor subunits (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Sun *et al.*, 2008, Wolf & Ferrario, 2010, Wolf *et al.*, 2004). In this study bilateral brain samples from each animal were either cross-linked or left

unaltered. When cross-linked samples were electrophoretically separated by SDS-PAGE and immunoblotted with antibodies directed against GluA1 and GluA2, we observed a high molecular weight smear, representing the surface cross-linked protein, as well as a monomeric intracellular protein band, whereas non-cross-linked samples from the contralateral side of the brain produced only the monomeric intracellular protein band (**Figure 12**).

Figure 12

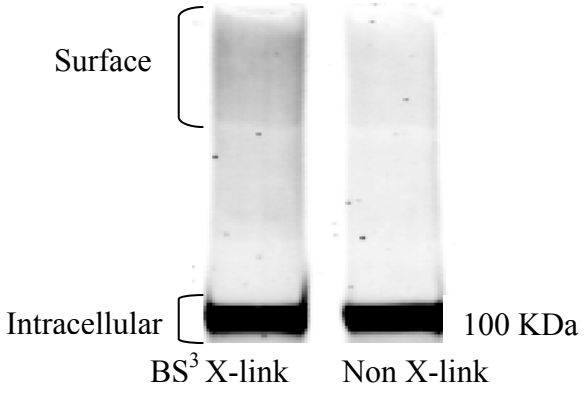


Figure 12. Example of BS^3 cross-linking by Western blotting. This Western blot represents a typical cross-linked sample vs. the contralateral non-cross-linked sample immunoblotted with an antibody against GluA1. There is a dark "smear" in the cross-linked sample corresponding to the surface protein expression, as well as a monomeric protein band, whereas the non-cross-linked sample only has the monomeric protein band present. Membranes immunoblotted with antibodies against GluA2 produced similar cross-linking results.

GluA1 and GluA2 distribution in the NAc and CU is not altered 7 days after the female's last sexual experience

Female hamsters that were given 6 weekly sexual experience pairings with a male hamster and sacrificed 7 days following their last sexual test showed no

significant changes in their S, I, or S/I ratio of either GluA1 or GluA2 protein expression (**Figure 13**). When non-cross-linked samples were analyzed for total protein levels of GluA1 and GluA2 it was found that total protein was not affected by repeated sexual experience at this time point. There was, however, a strong trend toward an increase in GluA1 total protein ($p=0.08$, $t=1.864$, $df=11$). Bilateral CU samples from the same sexually experienced and sexually naïve female hamsters were similarly treated with BS^3 cross-linking agent to analyze changes in trafficking of both GluA1 and GluA2. Repeated sexual experience was not found to produce changes in protein expression of S, I, or the S/I ratio of GluA1 or GluA2. There were also no changes in total protein levels of either protein as a result of repeated sexual experience.

Figure 13

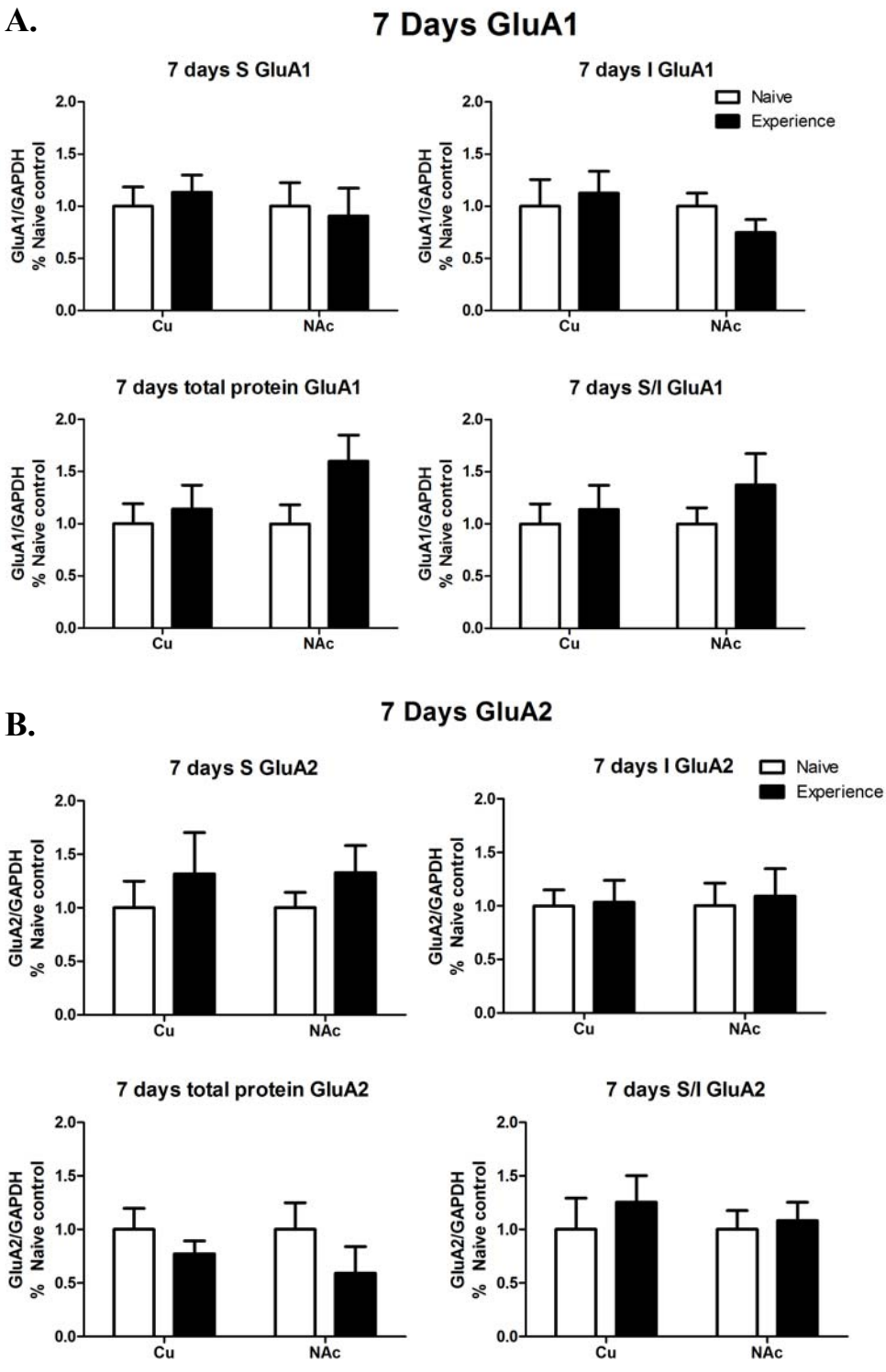

A.
7 Days GluA1
7 days S GluA1
7 days I GluA1
Naive
Experience
GluA1/GAPDH
% Naive control
2.0
1.5
1.0
0.5
0.0
Cu
NAc
7 days total protein GluA1
7 days S/I GluA1
B.
7 Days GluA2
7 days S GluA2
7 days I GluA2
GluA2/GAPDH
% Naive control
7 days total protein GluA2
7 days S/I GluA2

Figure 13. Surface (S), Intracellular (I), S/I ratio, and total protein levels of GluA1 (A) and GluA2 (B) 7 days following the last sexual experience. (A). Surface (S), Intracellular (I), total protein, and S/I ratio for GluA1 in the NAc and CU of experienced and naïve females represented as a percent of the naïve control females. (B). Surface (S), Intracellular (I), total protein, and S/I ratio for GluA2 in the NAc and CU of experienced and naïve females represented as a percent of the naïve control females.

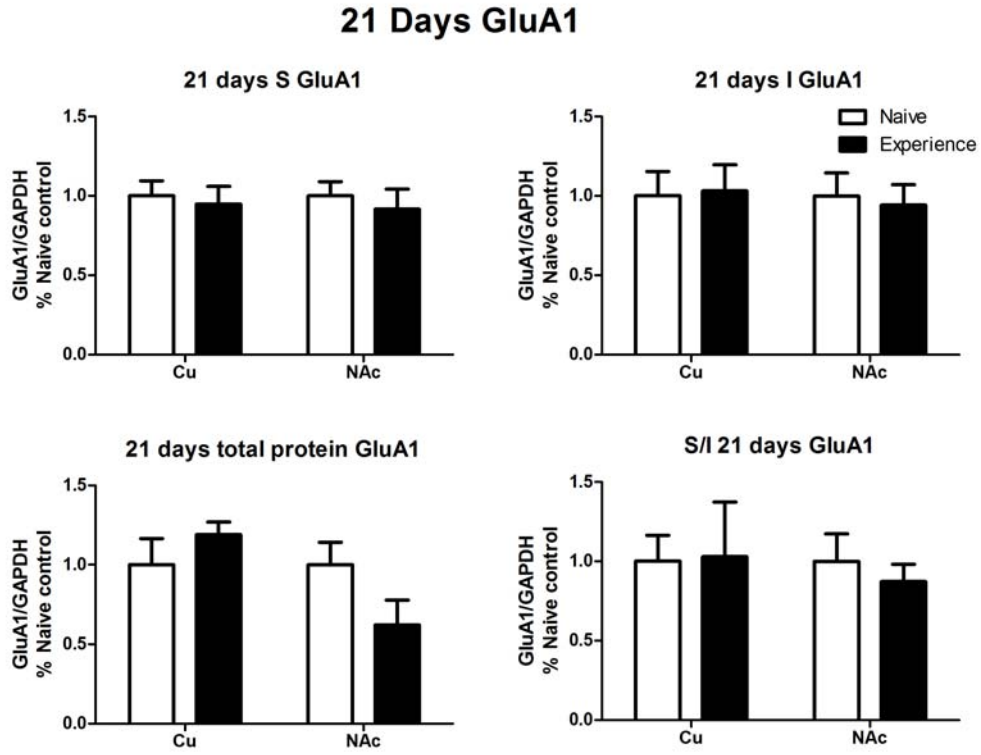
GluA1 and GluA2 distribution in the NAc and CU is not altered 21 days after the female's last sexual experience

Sexually experienced female hamsters and sexually naïve female hamsters did not show any significant changes in S, I, or S/I ratio of either GluA1 or GluA2 in the NAc when sacrificed 21 days following their last sexual experience (Figure 14). Total protein levels of GluA1 and GluA2 that were determined from non-cross-linked samples were also unchanged as a result of repeated sexual experience, however there was a trend for decreased total GluA1 ($p=0.09$, $t=1.782$, $df=14$), interestingly the opposite pattern of results observed at 7 days following the last sexual experience. Bilateral CU samples from the same sexually experienced and sexually naïve female hamsters were similarly treated with BS^3 cross-linking agent to analyze changes in localization of both GluA1 and GluA2, with a similar pattern of results. There was however a trend for a decrease in intracellular protein of GluA2 in the CU ($p=0.13$, $t=1.576$, $df=13$), with a corresponding trend of an increase in the S/I ratio ($p=0.09$, $t=1.825$, $df=12$).

Repeated sexual experience was not found to produce changes in S, I, S/I ratio or total protein expression of GluA1 or GluA2 protein in the CU.

Figure 14

A.



B.

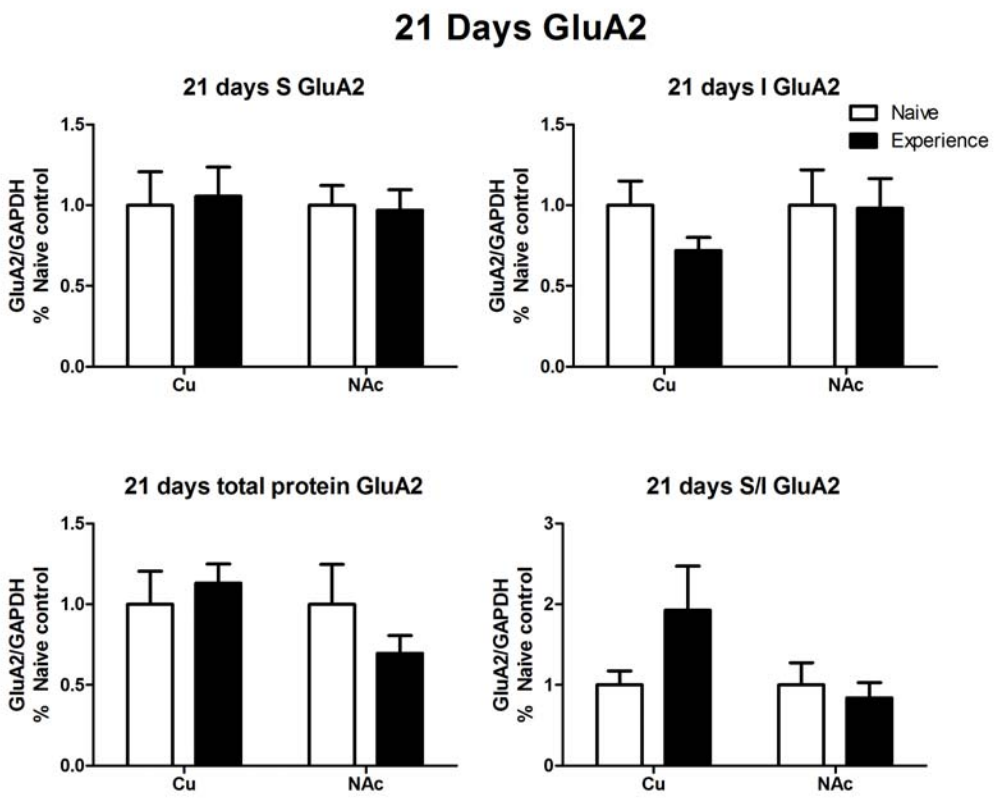


Figure 14. Surface, Intracellular, S/I, and total protein levels of GluA1 (A) and GluA2 (B) 21 days following the last sexual experience. (A). Surface (S), Intracellular (I), total protein, and S/I ratio for GluA1 in the NAc and CU of experienced and naïve females represented as a percent of the naïve control females. (B). Surface (S), Intracellular (I), total protein, and S/I ratio for GluA2 in the NAc and CU of experienced and naïve females represented as a percent of the naïve control females.

Discussion

Neurotransmission through AMPA glutamate receptors mediates the majority of excitatory transmission within medium spiny neurons of the NAc. The trafficking of these AMPA receptors into and out of the synapse has been

demonstrated to be an important component of behavior and plasticity (Kessels & Malinow, 2009, Malinow & Malenka, 2002). The implication of the insertion and removal of AMPA receptors into the synapse is that this trafficking results in changes in cellular excitability. Although we have not measured changes in cellular excitability following repeated sexual experience, the assumption was that our observed increase in gene expression of the GluA2 subunit of the AMPA receptor (Chapter 3) could potentially be affecting the excitability of NAc medium spiny neurons.

To test our hypothesis, in this experiment we studied different levels of regulation of AMPA receptor trafficking. We looked first at the regulation of individual subunits of the AMPA receptor tetramer, the composition of which is postulated to ultimately determine the physiology of the assembled receptor (Bredt & Nicoll, 2003, Conrad *et al.*, 2008, Cull-Candy *et al.*, 2006, Liu & Zukin, 2007, Liu & Cull-Candy, 2000, Malinow, 2003, Malinow & Malenka, 2002, Plant *et al.*, 2006, Song & Huganir, 2002, Takahashi *et al.*, 2003). Here, we chose to focus on two of the subunits found within medium spiny neurons in the NAc, the GluA1 and GluA2 subunits, which play an important role in mediating the physiology of AMPA receptors. Our unpublished results demonstrated that repeated sexual experience increases the mRNA expression of the GluA2 subunit in the NAc. In this study we continued to investigate the regulation of AMPA receptor trafficking through measuring total protein expression of both the GluA1

and GluA2 subunits. We did not find that repeated sexual experience altered the total protein expression of either the GluA1 or GluA2 subunits, despite the change we observed in GluA2 mRNA expression.

This experiment also investigated another level of AMPA receptor regulation by measuring the trafficking of the GluA1 and GluA2 subunits between intracellular stores and the cell surface. By utilizing the cross-linking agent BS^3 we were able to distinguish cell surface and intracellular receptor pools. In this study we tested the hypothesis that repeated sexual experience would alter trafficking of AMPA receptor subunits to the synapse as well as affecting the total protein expression of these subunits. Based off our results, it remains unclear if sexual experience affects glutamatergic AMPA receptor signaling. A large body of research supports that sexual experience, as well as other naturally motivated behaviors, affects dopaminergic plasticity. However, whether sexual experience has the functional consequence of affecting glutamatergic signaling had never been tested. We were unable to detect any changes in GluA1 or GluA2 trafficking between intracellular stores and the cell surface through the measure of the surface protein expression, intracellular protein expression, or the surface/intracellular ratio. Our results suggest that AMPA receptor signaling and the regulation of AMPA receptor trafficking may not be important in mediating cellular excitability in medium spiny neurons of the NAc for the behavioral output of sexual experience.

Nonetheless, two features of our Western blots may suggest that our surface cross-linking was not as successful as has been previously reported in the literature (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Conrad *et al.*, 2010, Ferrario *et al.*, 2011, Gao *et al.*, 2006, Nelson *et al.*, 2009, Wolf & Ferrario, 2010). First, the high molecular weight component in our Western blots did not seem as distinct as in published images. In line with this first observation is the high intensity of the intracellular receptor, which was comparable to that of the non-cross-linked punches. Unfortunately, our study did not have a positive control for BS^3 cross-linking, such as treatment with amphetamine, to verify that our cross-linking protocol was indeed successful. Further, our inability to detect changes in total protein could be due to sensitivity issues inherent to Western blotting, a technique that can easily detect large changes in protein levels, but may not be capable of detecting very small differences in protein. This confound, however, does not necessarily mean that the receptor trafficking results from the cross-linking experiment are flawed, as the S/I ratio should be independent of our ability to detect total protein differences.

Still, glutamatergic signaling though AMPA receptors has been demonstrated to be important in mediating the plasticity observed following administration of drugs of abuse (Boudreau *et al.*, 2007, Boudreau & Wolf, 2005, Bredt & Nicoll, 2003, Conrad *et al.*, 2010, Conrad *et al.*, 2008, Ferrario *et al.*, 2011, Humphries & Prescott, 2010, Kourrich *et al.*, 2007, Malinow, 2003,

Malinow & Malenka, 2002, Nelson *et al.*, 2009, Song & Huganir, 2002, Wolf & Ferrario, 2010, Wolf *et al.*, 2004). Because drugs of abuse are believed to usurp the neurocircuitry that mediates naturally rewarding behaviors, we hypothesized that the increased surface/intracellular ratio observed following cocaine administration would be mimicked by repeated sexual experience. However, this pattern of changes was not observed following activation of the system by sexual experience. Notably, the cellular plasticity resulting from natural motivated behaviors does not necessarily need to completely mirror the plasticity from drugs of abuse. In fact, this difference may partially explain why sexual behavior and other naturally rewarding behaviors are not normally addictive. Overall, this study has just begun to develop an understanding of the potential importance of AMPA receptor mediated glutamatergic signaling in the plasticity consequent of naturally motivated behaviors. By further determining whether repeated sexual experience changes the cellular excitability of NAc medium spiny neurons we will gain a better understanding of the mechanisms underlying long-term behavioral, cellular, and structural plasticity.

CHAPTER 5

Overall Discussion and Conclusions

The studies of this dissertation have illustrated that ΔFosB plays an important role in mediating the long-term cellular and behavioral plasticity following repeated sexual experience in female hamsters. I have demonstrated that ΔFosB mediates behavioral plasticity through sexual reward and that it may also mediate cellular plasticity by increasing expression of a target of ΔFosB regulated transcription, the GluA2 subunit of the AMPA receptor. Further, this increase in gene expression does not translate into changes in AMPA receptor trafficking. These studies now join a larger body of work that has demonstrated that ΔFosB does not only mediate the long-term plasticity observed following administration of potent pharmacological agents, but that it serves the more universal role of underlying the plasticity consequent of chronic stimulation of the system from natural motivated behaviors.

ΔFosB Mediates Sexual Reward

Viral overexpression of ΔFosB in the NAc of female hamsters, which mimics the accumulation of ΔFosB that occurs with chronic stimulation, resulted in an enhancement of sexual reward as well as increased copulatory efficiency with a male. ΔFosB has also been shown to play an important role in the rewarding consequences of additional natural motivated behaviors such as wheel running (Greenwood *et al.*, 2010), as well as male sexual behavior (Pitchers *et al.*) (see **Table 1** for review of known effects of ΔFosB and natural motivated

behaviors). These experiments suggest that ΔFosB accumulates following chronic stimulation from a variety of natural behaviors and that it serves an important role in mediating the rewarding aspects of these motivated behaviors, rather than simply being a consequence of administration of drugs of abuse.

Repeated Sexual Experience Increases Expression of a Target of ΔFosB Regulated Transcription

When changes in ΔFosB mediated gene transcription were measured following a 6 week regimen of repeated sexual experience, there was increased expression of the GluA2 subunit of the AMPA receptor in the NAc. Although GluA2 expression was heightened, repeated sexual experience did not alter trafficking of either GluA2 or GluA1 AMPA receptors to the cell surface. Excitatory glutamatergic neurotransmission through AMPA receptors on the cell surface of neurons has an important role in forms of synaptic plasticity such as long-term potentiation, long-term depression, and synaptic scaling. Importantly, the make-up of AMPA receptor tetramers has significant implications for the physiological properties of the receptor. Therefore, changes in the expression of certain AMPA receptor subunits, or changes in the cellular localization of these subunits, are believed to mediate changes in experience-dependent synaptic plasticity. Specifically, the presence of GluA2 containing AMPA receptors is of particular interest. GluA2 containing AMPA receptors are impermeable to Ca^{2+}

influx, whereas GluA2 lacking AMPA receptors are more permeable to Ca^{2+} influx. Therefore, even small changes in the expression of GluA2 containing AMPA receptors could have potent effects on cellular excitability.

Although I did not detect changes in either GluA1 or GluA2 surface expression following repeated sexual experience, this does not necessarily mean that cellular plasticity in unaffected by this behavior. Total protein changes in both GluA1 and GluA2 protein were found to be unaffected by repeated sexual experience. However, as a technique, Western blotting may not be sensitive enough to detect very small differences in protein levels. Considering this, my detection of increased mRNA expression using a more sensitive quantitative real-time PCR approach, that is not mirrored by increased protein through Western blot analysis may simply be a result of sensitivity issues. In any case, even very small changes in GluA2 expression on the cell surface can still have large effects on synaptic plasticity. To attain a better understanding of the potential changes in cellular excitability consequent of repeated sexual experience, additional methods such as electrophysiology, are needed.

Proposed mechanism of ΔFosB action in female sexual behavior

Repeated sexual experience results in persistent changes in behavioral, cellular, and structural plasticity. The studies of this dissertation support that chronic stimulation of the mesocorticolimbic dopamine pathway through repeated

sexual experience acts to increase ΔFosB, as well as its transcriptional targets to ultimately affect behavior, and possibly synaptic plasticity. Specifically, prior sexual experience in female hamsters changes the female's behavior in such a way that she learns to move her perineum in the direction of a male's thrusts to facilitate vaginal intromission. This persistent learned behavior allows female hamsters to improve the hit-rate (proportion of male mounts that result in intromission) of their naïve male sexual partners. Additionally, repeated sexual experience is rewarding as measured through a conditioned place preference paradigm. Underlying these changes in behavior are alterations in dopaminergic signaling in the mesocorticolimbic pathway in the brain. Ultimately, repeated stimulation of this pathway by sexual experience results in a sensitized release of dopamine within the NAc, which will give rise to increased dopaminergic signaling through D1 and D2 dopamine receptor mediated pathways, which act to alter synaptic plasticity through the formation of dendritic spines. Through previous studies, we believe that repeated sexual experience preferentially activates D1 mediated cell signaling pathways, which then leads to an accumulation of ΔFosB. I hypothesize that this increase in ΔFosB, and its consequent transcriptional activity, underlies the observed long-term behavioral, cellular, and structural plasticity that occurs with repeated sexual experience. From the studies of this dissertation, we know that ΔFosB definitely has an important role in mediating sexual reward and that sexual experience results in

increased gene expression of at least one target of ΔFosB mediated transcription. There undoubtedly numerous other consequences of ΔFosB accumulation and transcription that still require further exploration.

ΔFosB action in motivated behaviors

The experiments of this dissertation represent only a small piece of the puzzle concerning the action of ΔFosB in motivated behaviors. Research has identified that numerous motivated behaviors, including wheel running, instrumental food responding, sucrose drinking, male sexual behavior, and maternal behavior, are mediated at least in part through ΔFosB action (see Table 1 for summary). So, although accumulation of ΔFosB was originally described following chronic treatment with drugs of abuse and is still largely studied with respect to its actions following administration of numerous addictive drugs, it is clear that ΔFosB also mediates naturally rewarding behaviors. My work, as well as that of others, asserts the obvious contention that ΔFosB action evolved to mediate naturally rewarding behavior by acting as a molecular switch for plasticity that subserves adaptive motivational processes.

Health Relevance

The work of our laboratory implicates an animal's previous life history on their potential predisposition for addictive behaviors. Specifically, female

reproductive life experiences affect the mesocorticolimbic dopamine system making females uniquely susceptible to the addictive properties of drugs of abuse (Hedges *et al.*, 2010). As mentioned previously, this pathway is activated through sexual experience causing persistent behavioral, cellular, and structural plasticity. Experience with drugs of abuse also activates this natural reward pathway to cause long-term neuronal changes based on the inherent synaptic plasticity. We postulate that natural motivated behaviors, such as sexual behavior in female Syrian hamsters, act to sensitize components of the mesocorticolimbic dopamine pathway to increase copulatory efficiency that can facilitate reproductive success. The subsequent administration of addictive drugs accentuates this process, which in turn facilitates motivation for drugs instead of adaptive behavioral processes. This maladaptive transition to compulsive drug use is thought to underlie addiction.

By this logic, it follows that natural motivated behaviors could also become addictive, which does occur for extremes in sexual behavior, eating, shopping, internet use, and gambling (Albrecht *et al.*, 2007, Brezing *et al.*, 2010, Davis & Carter, 2009, Gearhardt & Corbin, 2011, Roller, 2007, Zack & Poulos, 2009). Just like the case of drug use, if the mesocorticolimbic dopamine system becomes biased toward expression of a particular motivated behavior, compulsive responses will develop. Studying the molecular events that give rise to these neuroadaptive changes following repeated sexual behavior are not only important

in their own right, but may also have implications in the etiology and development of therapeutic treatments for a spectrum of addictive disorders.

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