The role of the nuclear receptor corepressor in brain development

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Psychology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2012

Date of final oral examination: 08/03/12

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Acknowledgements

First and foremost, I would like to offer my thanks and appreciation to my advisor, Tony Auger, and to Cathy Auger for their guidance, support and encouragement. I admire you both and I am grateful that you have been a part of my life.

I would also like to thank my committee; Tony Auger, Cathy Auger, Stephen Gammie, Brad Postle and Cathy Marler. They have encouraged me to think more critically about my research and have given their time and expertise to better my work.

I am also grateful to the members of the Auger lab, past and present, who have given me moral support and technical assistance. I would especially like to thank Michelle Edelmann, Mira Kolodkin, Meag Bychowski and Robin Forbes-Lorman for their friendship both in and out of the lab. Special thanks are given to Hannah White, an outstanding undergraduate student and now graduate student with whom it was my pleasure to work. Finally, I would like to thank members of the psychology department, especially the animal care staff. I would not have been able to complete this dissertation without them.

I would also like to thank my family. My dad, mom, sister and brothers are amazing people who inspire me and are there for a laugh or a hug. They have always been there for me and supported me without question. Finally, I am especially grateful for my husband, Joe, for his patience, support and love.

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The role of the nuclear receptor corepressor in brain development

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Abstract

Sexual differentiation of the neonatal rat brain is regulated by a dynamic processes occurring at the level of DNA, resulting in sexually dimorphic gene expression. Steroid hormone receptors act partly in the developing brain by recruiting coregulatory proteins and other transcription factors that lead to altered gene expression. Recent data indicate that epigenetic processes play an important role in sexual differentiation of the brain. The dynamic interaction between epigenetic factors and chromatin leads to functional plasticity within the brain that may explain the diversity of responses to endogenous and exogenous signals. Subtle variations in these epigenetic factors may also partially explain individual differences in the development of neurological and mental health disorders. In this dissertation, I will explore the role of particular epigenetic factors during early postnatal brain development and their lasting impact on programming juvenile and adult behavior. We now show that both coactivators and corepressors are sexually dimorphic in the developing amygdala. Specifically, males express higher levels of Steroid Receptor Coactivator 1 (SRC-1) and CREB Binding Protein (CBP) than do females and females express higher levels of Nuclear Receptor Corepressor (NCoR) mRNA than do males. In addition, we have now shown that a transient, targeted disruption of NCoR within the developing amygdala has lasting consequences on juvenile social play, anxiety-like behaviors and social recognition. We also report that variations in the somatosensory stimuli associated with maternal

grooming during the neonatal period alter expression levels of NCoR and Kaiso, but do not appear to affect expression levels of DISC1. Taken together, these data suggest a dynamic model of chromatin remodeling which may drive the organization of the developing rat brain.

Chapter 1: Introduction

Introduction

The study of epigenetic mechanisms is important for investigating how gene-by-environment interactions can have lasting impacts on brain function and behavior (Meaney, 2010). One of the interesting challenges is that a change in epigenetic processes may directly participate in the development of a disorder or it may increase the susceptibility of a disorder in response to further gene or environmental challenges. Epigenetic mechanisms appear to be important in regulating a variety of behaviors, including processes involved in learning and memory (Levenson and Sweatt, 2005). For our purposes, we will define epigenetics as a change to DNA that alters gene transcription without changing the genetic code. In general, epigenetic processes consist of methylation of DNA by DNA methyltransferases (DNMTs). While the methylation of DNA alone can interfere with protein binding and gene transcription, it is the binding of methyl-CpG binding proteins to methylated DNA, and subsequent recruitment of nuclear corepressors and histone deacetylases to form repressor complexes that may more efficiently repress gene expression. While proteins that place methylation marks, the writers, are important for understanding epigenetic alterations, it is the proteins that bind these marks, the readers, which ultimately dictate gene expression. In this dissertation, I will explore the role of particular epigenetic factors during early postnatal brain development and their lasting impact on programming juvenile and adult behavior. I demonstrate that a brief perturbation of an epigenetic reader within the developing amygdala can have lasting consequences on social interactions and anxiety-like behavior.

Sexual differentiation of rodent brain and behavior

One model that is well suited for investigating epigenetic mechanisms is sexual differentiation of the brain (McCarthy et al., 2009). The developing brain is elegantly sensitive to steroid hormone exposure, and the differential exposure to steroid hormones between neonatal males and females produces some lasting sex differences in brain physiology and behavior. This is a particularly useful model as an early, brief exposure to steroid hormones can have lasting changes on brain development and function. While non-steroidal mechanisms can contribute to the differentiation of male versus female brain (Olesen et al., 2005;De Vries et al., 2002), it is generally accepted that testosterone, and its metabolites estradiol and dihydrotestosterone, are crucial for organizing some of the most salient sex differences in brain and behavior (MacLusky and Naftolin, 1981;Morris et al., 2004). These differences are thought to result from two major surges of testosterone from the testes, one occurring around embryonic day 18 (Weisz and Ward, 1980), and another a few hours after birth (Rhoda et al., 1984), as castration before this surge disrupts masculinization, and neonatal testosterone treatment can masculinize females (Baum, 1979). Gonadal hormones influence sexual differentiation of the brain and behavior mostly by binding to intracellular nuclear receptors.

A variety of steroid receptors have been implicated in sexually differentiating the brain, such as estrogen receptor α (ERα), ERβ, androgen receptors (AR), and progestin receptors. In general, activation of nuclear receptors results in release of heat shock proteins and conformational change of the receptor. This conformational change is believed to enhance the ability of the steroid-receptor complex to bind to a response elements on DNA (Jensen et al., 1968;Walters, 1985). Once bound to DNA, the receptor complex interacts with various

combinations of co-regulatory proteins to influence a diverse array of cellular processes ranging from genomic transcription (Carson-Jurica et al., 1990;McKenna et al., 1999b), changes in second-messenger systems (Etgen et al., 1999), neurotransmitter synthesis and release (Etgen and Karkanias, 1994;McCarthy, 1994;Hull et al., 1999). These difference can elicit lasting changes in cell function, neurochemical phenotype, or neuroanatomical projections (De Vries and Simerly, 2002). Thus, steroid hormones act upon their respective steroid receptors in developing neurons to produce lasting differences in cell number, migration, phenotype and morphology, as well as behavior, between the sexes. Therefore, while the steroid hormone surge is transient, the outcomes of this exposure are lasting. It is possible that some of these sexually dimorphic outcomes result from epigenetic processes.

While steroid hormones can shape numerous behaviors, one of the first sexually dimorphic social behaviors to emerge during development is juvenile social play behavior. Specifically, juvenile male rats engage in social play behavior at a higher frequency than do juvenile female rats (Olioff and Stewart, 1978). While numerous factors, such as neurotransmitters, epigenetic factors and social experience, have been found to alter the development of juvenile social play behavior (Auger and Olesen, 2009), sex differences in juvenile play behavior are mainly organized by neonatal testosterone exposure (Meaney and Stewart, 1981;Beatty et al., 1981). More specifically, androgen receptors play a critical role in organizing sex difference in juvenile social play behavior (Casto et al., 2003;Meaney and Stewart, 1981), with some data suggesting a potential role for estrogen receptors (Olesen et al., 2005). Combined, these studies suggest that brief changes in steroid receptor activity can have lasting consequences on sexually dimorphic and non-sexually dimorphic behaviors. Therefore,

this model is particularly suited for the epigenetic investigation of gene by environmental interactions.

Epigenetic processes and brain sexual differentiation

In general, DNA methylation has been associated with the suppression of gene transcription. There are several mechanisms by which DNA methylation can lead to gene repression. While methylation of DNA alone can interfere with gene transcription, it is the binding of methyl-CpG binding proteins to methylated DNA that results in more efficient gene repression. Methyl-CpG-binding proteins increase the interactions of chromatin remodeling co-repressor complexes with DNA and histones, resulting in gene repression (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006). Interestingly, corepressor complexes bound to DNA may recruit DNA methyltransferases resulting in DNA methylation (Fuks et al., 2000;Fuks et al., 2001). This suggests a complex relationship between DNA methylation, transcriptional repressors, and chromatin modification. Finally, increased intragenic DNA methylation can lead to decreased transcriptional elongation (Lorincz et al., 2004).

DNA methyation and Methyl-binding proteins

Methylation of DNA occurs when a methyl group attaches to a cytosine within a 5'-CpN-3' dinucleotide site through an enzymatic reaction that is catalyzed by DNA cytosine-5-methyltransferases (DNMTs). The strong bond between the cytosine nucleotide and methyl group results in a stable, but reversible, modification in gene expression (Metivier et al., 2008). While most studies focus on methylation of DNA at CpG sites, there is evidence indicating that

methylation also occurs at CpA, CpC and CpT sites but at lower rates (Grafstrom et al., 1985) (Ramsahoye et al., 2000). The functional impact of methylation at these different CpN sites remains to be determined. Methylation at CpG sites usually occur within the promoter region upstream from the transcription start site and several CpG nucleotides within a promoter may be methylated. It is believed that the number or pattern of methylated CpG sites is important for functional differences. While the role of DNMTs in sexual differentiation of brain and behavior remains to be determined, recent data indicate that certain DNMTs are sexually dimorphic during certain parts of early development (Kolodkin and Auger, 2011).

A family of methyl-CpG-binding was first discovered after the characterization of the methyl-CpG-binding domain (MBD); this binding domain is responsible for binding to the methylated CpG dinucleotides (Hendrich and Bird, 1998). Members within the family of methyl-CpG-binding proteins include Kaiso, MBD1, MBD2, MBD3, MBD4, and Methyl-CpG-binding protein 2 (MeCP2). Upon binding to methylated DNA, methyl binding proteins (MBPs) recruit corepressor proteins and HDACs to modify chromatin and repress gene transcription. MBPs are thought to be critical for typical cell function, as disruptions of MBPs have been implicated in the etiology of several disorders.

In the rodent brain, the expression of MeCP2 is sexually dimorphic early in development. Females express higher levels of MeCP2 mRNA compared to males on postnatal day 1 (Kurian et al., 2007). Transient disruption of MeCP2 expression has been found to reduce the levels of juvenile social play behavior in males to female-typical levels. As testosterone action in the developing amygdala is critical for normal organization of male juvenile social play, it is possible that reducing MeCP2 expression interfered with testosterone-induced masculinization of

play. Interestingly, transient disruption of MeCP2 in the developing amygdala had no effect on juvenile social play behavior in females. Furthermore, reduced Mecp2 expression did not alter juvenile sociability or adult anxiety-like behavior, suggesting this disruption may be associated with subtle behavioral modification (Kurian et al., 2008). This transient disruption in MeCP2 expression has also been found to be important for the organization of the sex difference in vasopressin expression (Forbes-Lorman et al., 2012). These data support the concept that epigenetic factors, such as methyl-binding proteins, likely contribute to sexual differentiation of the brain and social behavior.

Nuclear Corepressors and methyl-binding proteins

Binding of methyl-CpG binding proteins to methylated DNA leads to the subsequent recruitment of nuclear corepressor and histone deacetylase repressor complexes. The HDACs within these complexes inhibit gene expression by removing acetyl groups from histones, which results in condensation of the chromatin and gene repression (Klose and Bird, 2006). These chromatin remodeling complexes may cause additional modifications to the chromatin including, methylation, ubiquitylation, phosphorylation and sumoylation (Wu et al., 1986) which result in gene repression (Klose and Bird, 2006). Numerous corepressor complexes are recruited by methyl-binding proteins, which include Sin3, NuRD, CoREST, and the NCoR/SMRT repressor complexes (Cunliffe, 2008). These multi-protein complexes share many of the same proteins with some notable differences (Cunliffe, 2008). For example, both Sin3 and NuRD complexes contain HDAC1, HDAC2, RbAp46, and RbAp48. However, the Sin3 complex contains Sin3a,

SAP18, and SAP30; whereas, the NuRD complex contains MBD3, MTA-2, and Mi-2. Greater dissimilarity is found within the CoRest and SMRT/NCoR repressor complexes. The CoREST complex contains HDAC1, HDAC2, CoREST, SHARP and Sin3; whereas, the SMRT/NCoR complex contains HDAC3, SMRT, and NCoR. It is likely that different protein combinations yield different functional consequences on gene transcription. As the recruitment of corepressor complexes to DNA can occur following interactions with nuclear receptors or methyl-binding proteins, there exists the potential for increased diversification of function as a result of which combinations are formed upon DNA. NCoR has been shown recently to interact directly and indirectly with methyl-binding proteins (Yoon et al., 2003;Cukier et al., 2008;Kokura et al., 2001;Alland et al., 1997). Specifically, NCoR has been shown to interact directly with Kaiso, MeCP2 and other methyl-binding proteins (Kokura et al., 2001;Yoon et al., 2003;Cukier et al., 2008) and may interact indirectly with methyl-binding proteins through the Sin3 corepressor complex (Alland et al., 1997).

Nuclear Corepressors and Nuclear Receptors

The most widely studied corepressors, NCoR (Nuclear Receptor CoRepressor) and SMRT (Silencing Mediator of Retinoid and Thyroid Receptors), were first discovered and identified through their interaction with thyroid and retinoid hormone receptors (Horlein et al., 1995;Chen and Evans, 1995). NCoR and/or SMRT also interact with androgen receptors (Cheng et al., 2002;Yoon and Wong, 2006), estrogen receptors (Lavinsky et al., 1998), and progestin receptors (Liu et al., 2002). It remains to be determined if some corepressors have preferential

interactions with certain nuclear receptors. While some corepressors can interact weakly, but directly, with nuclear receptors in the presence of the ligand, this interaction can be much stronger in the presence of the antagonists.

Through these interactions with nuclear receptors, corepressors are also known to function by decreasing nuclear receptor induced transcriptional activity. This is in contrast to the function of nuclear receptor coactivators, which is to increase transcriptional activity. Coactivators are thought to function by facilitating access of transcriptional factors to the DNA promoter site (McKenna et al., 1999a). Many coactivators appear to accomplish this through their own intrinsic histone acetylase transferase (HAT) activity or through their association with complexes having HAT activity. The hyperacetylation of histone tails, by HAT activity, decreases their positive charge which leads to the separation of the histone tails and the negatively charged DNA. This change in chromatin structure allows for the recruitment of additional transcriptional factors and subsequently leads to an increase in transcription (McKenna et al., 1999a;Rosenfeld and Glass, 2001). In contrast, corepressors recruit HDACs (Tsai and O'Malley, 1994), which restore a positive charge on histone tails and causes DNA to be less assessable to transcription factors (McKenna et al., 1999a), ultimately, leading to a decrease in transcription. Corepressors interact with a repressor domain on nuclear receptors that overlaps with the surface area that binds coactivators. Hormone binding to nuclear receptors causes a conformational change that favors the recruitment of coactivators over corepressors [for review see (Privalsky, 2004;Aranda and Pascual, 2001;Xu et al., 1999)]. Modifications of the coregulatory proteins, as well as the nuclear receptors, such as acetylation (Fu et al., 2002) and phosphorylation (Zhou et al., 2001), also can change the interacting affinities of these molecules. This sets up an interesting

competition for these sites between coactivators and corepressors, and results in histone and coregulatory protein modification that can take place in minutes to hours (Shang et al., 2000;Privalsky, 2004). These relatively rapid modifications may contribute to a cyclical patterning of nuclear receptor gene transcription.

The importance of the cellular expression of coreprossors is best illustrated by data indicating that these corepressors are partially responsible for the antagonist ability of some drugs. For example, tamoxifen acts as an estrogen receptor agonist in NCoR knockout mice cells and re-expressing NCoR within these cells restores the antagonistic ability of tamoxifen (Jepsen et al., 2000). RU-486 acts as a progestin receptor antagonist in cells that express a higher ratio of corepressors to coactivators and can switch to an agonist if the ratio is reversed (Liu et al., 2002). Therapeutically, it is important to understand the relative ratios of coregulatory protein expression within tissues as these ratios can determine if a drug will act as an agonist or antagonist when used in treatment for some forms of cancer, such as tamoxifen (Shang and Brown, 2002). Therefore, it is important to understand the function of corepressors, as well as the ratio of coregulatory protein expression, to better predict how a cell responds to environmental or endogenous signals.

Coactivator expression is known to be sexually dimorphic (Misiti et al., 1998;Auger et al., 2002a;Charlier et al., 2002;Duncan and Carruth, 2007) and regulated by steroid hormones (Mitev et al., 2003;Murphy and Segal, 1997;Charlier et al., 2006a). Coactivators are also important for the development and expression of adult sexually dimorphic behavior (Auger et al., 2000;Auger et al., 2002a;Charlier et al., 2006b;Molenda et al., 2002;Molenda-Figueira et al., 2006;Apostolakis et al., 2002). While more is known about the molecular mechanisms and

functions of coactivators, less is known about the functional role of corepressors and little is known about either coactivators or corepressors in the amygdala.

Environmental Influences

It is important to not only examine the function of these epigenetic factors, but to also examine what in the natural environment may cause changes in these factors and mechanisms. An exciting line of research has indicated that variations in the early social environment cause changes in epigenetic factors within the developing brain. For example, it appears that maternal care influences ERα expression by altering ERα promoter methylation (Champagne et al., 2006). As rat mothers typically groom the anogenital region of males more than females (Moore and Morelli, 1979;Moore, 1984), it is possible that variations in maternal care may be contributing to sex differences in ERα expression and DNA promoter methylation patterns. It has recently been reported that there is a sex difference in ERα promoter CpG methylation in both the rat preoptic area and amygdala (Kurian et al., 2010;Edelmann and Auger, 2011). To determine the contributions of maternal care to the sex difference in ERα promoter methylation and expression, simulated maternal grooming (SMG) was used to model a component of maternal care, the somatosensory stimulation of the anogenital region. This paradigm was chosen to control for other factors of maternal care and to focus on the tactile stimulation of licking and grooming. SMG provided to females was found to increase ERα promoter methylation and expression to male-like levels within the developing POA and amygdala (Kurian et al., 2010;Edelmann and Auger, 2011). As additional infant contact (e.g., SMG) places methylation marks upon DNA, we will examine if SMG alters the expression of epigenetic readers that interact with these marks.

In this dissertation, I examined the expression and function of the epigenetic reader, NCoR, on programming juvenile social behavior and anxiety-like behavior. I provide data that brief perturbations of NCoR within the developing amygdala can have lasting consequences on juvenile social interactions and anxiety-like behavior. Chapter two investigates potential sex differences in coactivator and corepressor mRNA expression levels in the amygdala of the developing rodent brain. Chapter three focuses on a specific corepressor, NCoR and examines its functional role in the developing amygdala. Chapter four further examines the functional role of NCoR in the developing rat amygdala on juvenile and adult social and anxiety-like behavior. Chapter five will examine the effects of SMG on other epigenetic factors within the developing amygdala, specifically NCoR and the methyl-CpG-binding protein, Kaiso.

Chapter 2: Sex differences in coactivator and corepressor expression within the developing rat amygdala

Abstract

Steroid hormones play an important role in the developing rat brain, causing many physiological and behavioral changes. Steroid hormone action is regulated at the level of DNA through a balance of recruitment of complexes which cause acetylation of histones and increases in gene expression and through the recruitment of complexes, such as coactivators, e.g. Steroid Receptor Coactivator-1 (SRC-1) and CREB binding protein (CBP), and corepressors, e.g. Nuclear Receptor CoRepressor (NCoR) and Silencing Mediator of Retinoid and Thyroid Receptors (SMRT), which cause deacetylation and decreases in gene expression. We used real-time RT-PCR to examine potential sex differences in coactivators and corepressors in the amygdala of the developing rat brain. On postnatal day 1 (PN1) we have found that within the amygdala males express higher levels of SRC-1 and CBP mRNA), but this differences is no longer present by postnatal day 10 (PN10). In contrast, females express a greater relative amount of NCoR mRNA as compared to males on PN1, interestingly this difference is still present on PN10. The specific function of these coregulatory proteins within in the developing amygdala is yet to be elucidated. Taken together, these data suggest that sex differences in some chromatin remodeling factors are transient, while others appear more lasting during early brain development. As males express higher levels of coactivators and lower levels of a corepressor, these data support the concept that there is a dynamic remodeling of chromatin which may drive sexual differentiation of the brain. The differential expression of these coregulatory proteins also likely contribute to how some areas of the brain are more or less sensitive to hormones and other environmental stimuli.

Introduction

Sexual differentiation of the brain is mediated, in part, by steroid hormones acting upon neurons within the developing brain during a critical time period of neonatal life. During the perinatal period, male rats are exposed to two surges of testosterone (Weisz and Ward, 1980;Rhoda et al., 1984). It is the difference in exposure to these surges that appear to drive many of the sex differences in brain and behavior. Testosterone produces these differences mainly via its metabolites estradiol and dihydrotestosterone. These hormones influence sexual differentiation of the brain and behavior mostly by binding to intracellular nuclear receptors. Hormone binding to these nuclear receptors results in release of heat shock proteins and conformational change of the receptor. This conformational change is believed to enhance the ability of the steroid-receptor complex to bind to response elements on DNA (Jensen et al., 1968;Walters, 1985). Once bound to DNA, the receptor complex interacts with combinations of co-regulatory proteins to influence cellular processes (Carson-Jurica et al., 1990;McKenna et al., 1999b;Etgen et al., 1999;Etgen and Karkanias, 1994;McCarthy, 1994;Hull et al., 1999), ultimately, leading to changes in cell function, neurochemical phenotype, or neuroanatomical projections (De Vries and Simerly, 2002). Nuclear receptor function on DNA is dependent on the availability of coregulatory proteins that form the transcriptional complex, some of which increase gene transcription, called coactivators, and others that decrease gene transcription, called corepressors.

Early evidence of the interaction of coactivators and nuclear receptors include studies in which the phenomenon of squelching was observed, where the transcription of one activated nuclear receptor was decreased in the presence of another unrelated activated nuclear receptor (Meyer et al., 1989). These initial studies lead to the cloning and characterization of SRC-1

(Steroid Receptor Coactivator-1) (Onate et al., 1995). Since then many coactivators have been discovered, including CBP (CREB binding protein) (Kwok et al., 1994). Coactivators are thought to function through histone acetylase transferase (HAT) activity. This leads to hyperacetylation of histone tails which decreases the positive charge of the histone tails. This leads to the repulsion of the histone tails from negatively charged DNA, ultimately leading to changes in chromatin structure which increases the accessibility for additional transcriptional factors and increases in transcription (McKenna et al., 1999a;Rosenfeld and Glass, 2001). Interestingly, coactivator expression has been found to be sexually dimorphic in various areas of the brain (Misiti et al., 1998;Auger et al., 2002a;Charlier et al., 2002;Duncan and Carruth, 2007) and regulated by steroid hormones (Mitev et al., 2003;Murphy and Segal, 1997;Charlier et al., 2006a). Coactivators have also been shown to be critical for both the organization and activation of adult sexual behavior (Auger et al., 2000;Auger et al., 2002a;Charlier et al., 2006b;Molenda et al., 2002;Molenda-Figueira et al., 2006;Apostolakis et al., 2002).

In contrast to the function of nuclear receptor coactivators, which is to increase gene transcription, corepressors are generally thought to decrease gene transcription. The most widely studied corepressors, NCoR (Nuclear Receptor CoRepressor) and SMRT (Silencing Mediator of Retinoid and Thyroid Receptors), were first discovered and identified through their interaction with thyroid and retinoid hormone receptors (Horlein et al., 1995;Chen and Evans, 1995). Corepressors are classically thought to decrease gene transcription through their association with histone deactylase complexes or HDACs (Tsai and O'Malley, 1994). The relative hypoacetylation restores a positive charge on the histone tails and causes the histones to more closely associate with negatively charged DNA, and subsequently less assessable to transcription factors (McKenna et al., 1999a). Interestingly, corepressors can either repress gene expression

through their interactions with nuclear receptors or independently of nuclear receptors via their direct or indirect interactions with methyl binding proteins, such as Kaiso (Yoon et al., 2003) and MeCP2 (Cukier et al., 2008;Kokura et al., 2001).

It is likely that differences in the expression of these coregulatory proteins contribute to how some areas of the brain are more or less sensitive to hormones and other environmental stimuli. As little is known about the expression of coactivators and corepressors within the developing amygdala, we investigated potential sex differences in coactivator and corepressor mRNA expression levels in the amygdala of the developing rodent brain. We now report that levels of the coactivators SRC-1 and CBP and the corepressor NCoR are sexually dimorphic within the developing amygdala. We show that males express higher levels of SRC-1 and CBP on postnatal day 1, but these differences are gone by postnatal day 10. In contrast, females express higher levels of NCoR on postnatal day 1 and this difference persists through postnatal day 10.

Methods

Animals

Adult female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were mated in our animal facility and allowed to deliver normally. Cages were checked regularly to determine the day of birth (postnatal day 0, PN0). Animals were maintained on a 12 hour light/12 dark light cycle and food and water were available ad lib. This research was approved by the University of Wisconsin Animal Care and Use Committee.

Tissue Collection

Animals were sacrificed by rapid decapitation at 24 hours after birth (12 males and 12 females), referred to as postnatal day 1 (PN1) or 10 days after birth (8 males and 12 female), referred to as postnatal day 10 (PN10). The brains were microdissected using razor blades and immediately frozen in isopentane on dry ice. For the dissections, the brain was placed ventral side up on a cold surface. Using a razor blade, we made two coronal cuts, one caudal to the optic chiasma and one caudal to the hypothalamus. The tissue was then placed rostral side up. The amygdala was dissected by making a cut was along the optic tract and another cut at approximately 60 degrees to form an approximate triangle. Both sides were collected, pooled, and frozen. The tissue was stored at -80°C until homogenization for real-time PCR.

Real-time PCR

Total RNA was isolated from snap-frozen tissue using the AllPrep DNA/RNA Mini Kit (Cat. #80004, Qiagen, Valencia, CA). RNA concentrations were determined using the Qubit Quantification Platform (Cat. #Q32857, Invitrogen, Carlsbad, CA) and cDNA was generated with ImProm-II Reverse Transcription System (Cat. #A3800, Promega) according to manufacturer recommendations in an Eppendorf MasterCycler Personal PCR machine. Samples were stored at −80 °C. Real-time RT-PCR was conducted with a Stratagene Mx3000P™ real-time PCR system. cDNA was amplified using Sybr® Green I (S7563, Invitrogen), GoTaq Colorless Master Mix (Cat. #M7132, Promega), and ROX (Cat. #12223012, Invitrogen) was used as a passive reference dye to control for baseline fluorescence in each sample. All primers were found to have efficiencies between 90% and 110%. The amplification protocol is as follows: an initial melting step at 95 °C for 2 min followed by 40 cycles of a 95 °C melting step for 30 s, a 60 °C annealing step for 30 s, and a 72 °C elongation step for 30 s. Following

amplification, a dissociation curve analysis was used to ensure purity of the products, a single peak was observed for all products. Relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Ywhaz was used as a housekeeping gene for all comparisons. All samples were run in duplicate in the same run. Data were analyzed with a two-tailed Student's t-test. The following are the primer sequences: NCoR: Forward AGGTGAGCTGGCAGGACTTA, Reverse AGATAAAGGGCCTCCTCCAA, SMRT: Forward AAGCCAACTCATGAGGGTGTGGTA, Reverse TACGTCGTGTTTCTTGGAGCCACT, SRC-1: Forward GCTCATTTGCATCTGGCCCATCAT, Reverse AAGAAGGACAGTGCGTCAGCTTCT, CBP: Forward TAATGGAGGCTGCCCAGTGTGTAA, Reverse CTGGCGGAGCTTGTGTTTGATGTT, Ywhaz: Forward TTGAGCAGAAGACGGAAGGT, Reverse GAAGCATTGGGGATCAAGAA.

Results

Sex Differences at PN1

We report that on PN1, males express higher levels of SRC-1 mRNA than do females in the amygdala ($p<0.05$, *t*-test, Figure 1A). Males were also found to express higher levels of CBP mRNA within the amygdala on PN1 ($p<0.05$, *t*-test, Figure 1B). In contrast, females were found to express higher levels of NCoR mRNA within the amygdala on PN1 ($p<0.05$, *t*-test, Figure 1C), this confirms earlier results (Jessen et al., 2010a). SMRT expression levels were not found to be significantly different between males and females on PN1 ($p>0.05$, t-test, Figure 1D).

Sex Differences at PN10

We report that on PN10, SRC-1 (Figure 2A) and CBP (Figure 2B) expression levels were not significantly different between males and females on PN10 ($p>0.05$, t-test). However, females were found to express higher levels of NCoR mRNA within the amygdala on PN10 ($p<0.05$, *t*-test, Figure 2C). SMRT expression levels were not found to be significantly different between males and females on PN10 ($p>0.05$, t-test, Figure 2D).

Discussion

We find that the relative levels of coactivator and corepressor mRNA expression are differentially expressed between the sexes during the first week of postnatal life. Males express higher levels of SRC-1 and CBP mRNA within the amygdala on PN1, however this difference is no longer present on PN10. In contrast, females express higher levels of NCoR mRNA within the amygdala on PN1 and this difference persists through PN10. SMRT mRNA expression levels were not found to be significantly different between males and females on PN1 or PN10. These data support the idea that differences in coregulatory protein distribution may contribute to how some areas of the brain are more or less sensitive to hormones and other environmental stimuli. In addition, the relative higher levels of coactivators and lower levels of a corepressor expression are likely to enhance steroid mediated sexual differentiation the male developing amygdala.

We now report that males express greater levels of SRC-1 and CBP mRNA within the rat amygdala on PN1. These data are consistent with findings reporting sex differences in SRC-1 and CBP in various animal models. For example, males have been reported to express higher levels of SRC-1 in rat anterior pituitary cell lines (Misiti et al., 1998) and in the preoptic area of adult Japanese quail (Charlier et al., 2002). These data are also consistent with our previously

reported data indicating that males express higher levels of CBP within the rat ventromedial hypothalamus, medial preoptic area, and the arcuate nucleus on the day of birth (PN0) (Auger et al., 2002b). We also show that this sex difference in SRC-1 and CBP is eliminated by PN10. As coactivator expression is known to be regulated by steroid hormones (Mitev et al., 2003;Murphy and Segal, 1997;Charlier et al., 2006a), it is possible that the sex differences in these coactivators result from differences in steroid hormone exposure during early development between the sexes, that is the perinatal testosterone surges. It is interesting to consider that circulating hormone levels may both contribute to controlling expression levels of coactivators and that these coactivators help to control nuclear receptor activation by these steroid hormones. While coactivators have also been shown to be important for the organization and activation of adult sex behavior (Auger et al., 2000;Auger et al., 2002a;Charlier et al., 2006b;Molenda et al., 2002;Molenda-Figueira et al., 2006;Apostolakis et al., 2002), many of these studies have focused on determining the function of coactivators within hypothalamic regions; therefore, more research is needed to determine the function of coactivators within the developing amygdala.

The expression of NCoR and SMRT mRNA appear to be ubiquitously present throughout the adult rat brain; however, differences in the expression levels between NCoR and SMRT have been observed in some areas including the brain stem, thalamus, hypothalamus and hippocampus (van der Laan et al., 2005a). Subcellular distribution of corepressors also appears to be important for the function of the cell. Differences in NCoR cytoplasmic versus nuclear location has been indicated in abnormal function in colorectal cancer cells (Nagy et al., 1997a) as well as critical in determining cell fate (Hermanson et al., 2002). In early studies, estradiol treatment was not found to affect NCoR levels, however, estradiol treatment lead to a brief increase, followed by a rapid decrease in SMRT levels in the anterior pituitary (Misiti et al., 1998). In a more recent study,

estradiol was found to down-regulate NCoR mRNA, but not SMRT levels, in estrogen receptor-positive breast cancer cells (Frasor et al., 2005). Interestingly, we have recently shown that NCoR levels are sexually dimorphic in the developing amygdala and medial basal hypothalamus on PN1 and responsive to estradiol. Females were found to express higher levels of NCoR mRNA as compared to males and these levels were decreased by estradiol treatment in both of these areas (Jessen et al., 2010a). We have now confirmed that females express higher levels of NCoR mRNA within the amygdala on PN1 and shown that this sex difference persists until at least PN10. In contrast, SMRT levels were not found to be sexually dimorphic at either PN1 or PN10. The function of sex differences in coregulatory expression is still unclear, however, it is possible that females express higher levels of NCoR mRNA to serve as protection from brief exposures to hormones that may otherwise lead to masculinization of the brain. It is possible that SMRT expression levels are not sexually dimorphic within the developing amygdala because it is involved in processes that must develop similarly in males and females.

While NCoR and SMRT have been found to be very similar in structure and some function, differences do exist. For example, NCoR and SMRT appear to be affected by different growth factors and cytokines which appear to affect intracellular location of these corepressors and cell fate (Jonas and Privalsky, 2004). While the use of knockouts have been useful for investigating the function of coactivators, there are fewer models to examine the functional role of corepressors in the brain. Interestingly, the most widely studied corepressor knockout, NCoR, is embryonically lethal. Therefore, NCoR appears to be a required component during early development and SMRT, or other corepressors, cannot fully compensate for the loss of NCoR.

The function of corepressors within the brain has yet to be fully elucidated. As corepressors interfere with nuclear receptor action and are recruited by methyl-binding proteins to repress gene expression, it is possible that corepressors are involved in both processes that are sensitive to steroid hormones and epigenetic processes which may influence behavior. The amygdala has been found to be critical in the development of juvenile social play behavior (Meaney et al., 1981a), as well as, in regulating behaviors such as anxiety-like behavior (LeDoux, 1998;Graeff et al., 1993). Our lab has reported many sex differences in expression levels of epigenetic factors in the developing amygdala, including DNMT3a (Kolodkin and Auger, 2011) and in the methyl-binding protein, MeCP2 (Kurian et al., 2007). In addition, we have recently reported that disruption of a methyl-binding protein, MeCP2 (Kurian et al., 2008), and of NCoR (Jessen et al., 2010a) disrupts the organization of juvenile social play behavior. Specifically, transient and targeted disruption of MeCP2 or NCoR expression within the developing amygdala interfered with the organization of juvenile social play behavior in males only. This disruption in NCoR was also found to increase anxiety-like behavior in both males and females (Jessen et al., 2010a). As nuclear receptor action has been found critical for the development of sex differences in juvenile social play behavior, it is possible that NCoR affects the organization of juvenile social play behavior through its interaction with nuclear receptors. Conversely, as anxiety-like behavior was disrupted in both males and females, it is possible that NCoR affects the organization of anxiety-like behavior through interaction with methyl-binding proteins.

Disruptions in coactivator and corepressor complexes have been implicated in many human disorders, including many cancers and even Rett syndrome and Huntington's disease.

Mutations in the X-linked MECP2 gene are thought to be the direct cause of Rett syndrome, a progressive neurodevelopmental disorder (Amir et al., 1999). Recently, MeCP2 has been shown to directly interact with Sin3a, Rest, as well as NCoR, and it has been suggested that these proteins may be better therapeutic targets than MeCP2 (Cukier et al., 2008). MeCP2 has also been reported to interact with the SMRT corepressor complex. Interestingly, the truncated form of MeCP2, which occurs in Rett syndrome, does not interact with the SMRT complex in developing neurons (Stancheva et al., 2003). The failure of MeCP2's interaction with SMRT is suggested to cause abnormal neuronal differentiation. Furthermore, abnormal subcellular distribution NCoR is found in humans with Huntington's disease and in the mouse model of Huntington's disease (Boutell et al., 1999a).

In summary, these data show that both coactivators and corepressors are sexually dimorphic in the developing amygdala. The exact function of these sex differences are yet unknown, however, together these data suggest that appropriate coactivator and corepressor expression are necessary for the organization of typical brain physiology and behavior.

Figure Legends

Figure 1

Sex differences in coactivators and corepressors at PN1. A. Males express significantly higher levels of SRC-1 mRNA within the amygdala on PN1. * = $p<0.05$. B. Males express significantly higher levels of CBP mRNA within the amygdala on PN1. * = $p<0.05$. C. Females express significantly higher levels of NCoR mRNA on PN1. * = $p<0.05$. D. SMRT expression levels were not significantly different between males and females on PN1.

Figure 2

Sex differences in coactivators and corepressors at PN10. A. SRC-1 expression levels were not significantly different between males and females on PN10. B. CBP expression levels were not significantly different between males and females on PN10. C. Females express significantly higher levels of NCoR mRNA on PN10. * = $p<0.05$. D. SMRT expression levels were not significantly different between males and females on PN10.

Figures

Figure 1

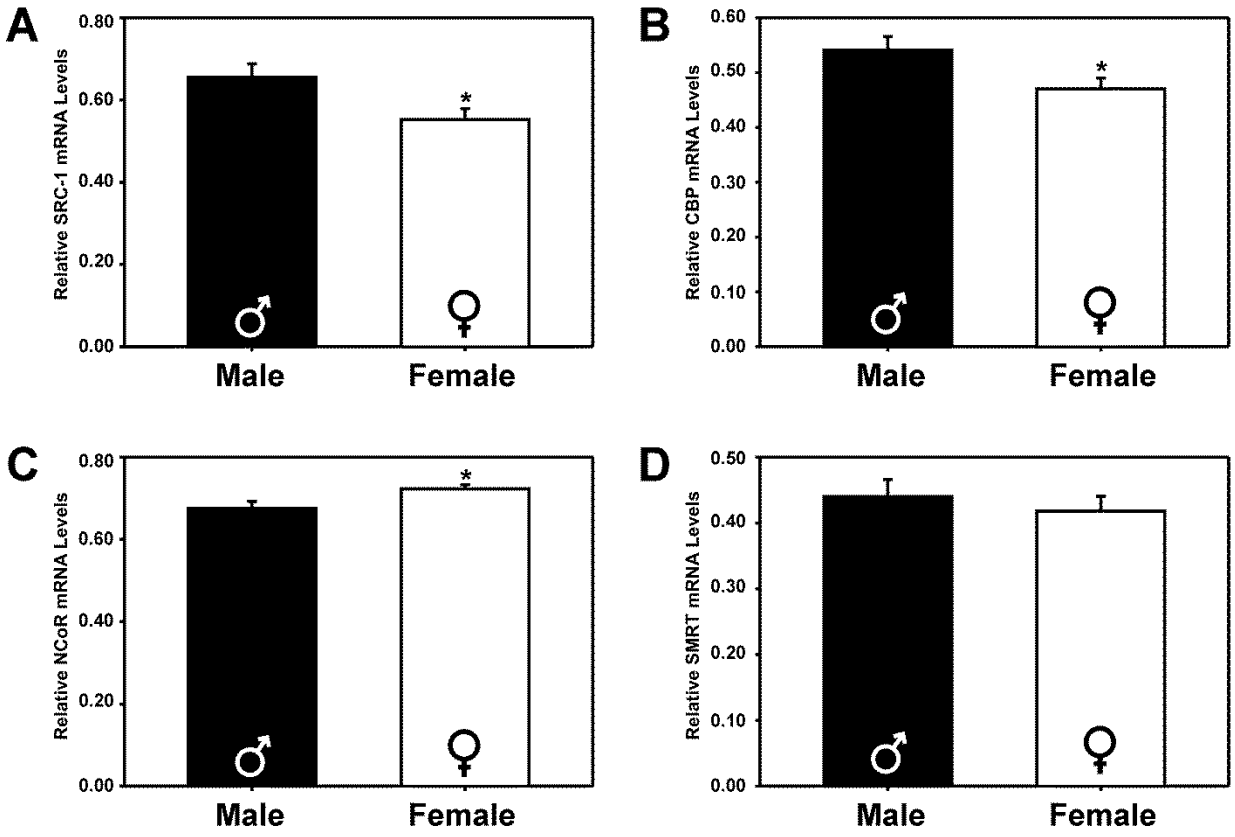
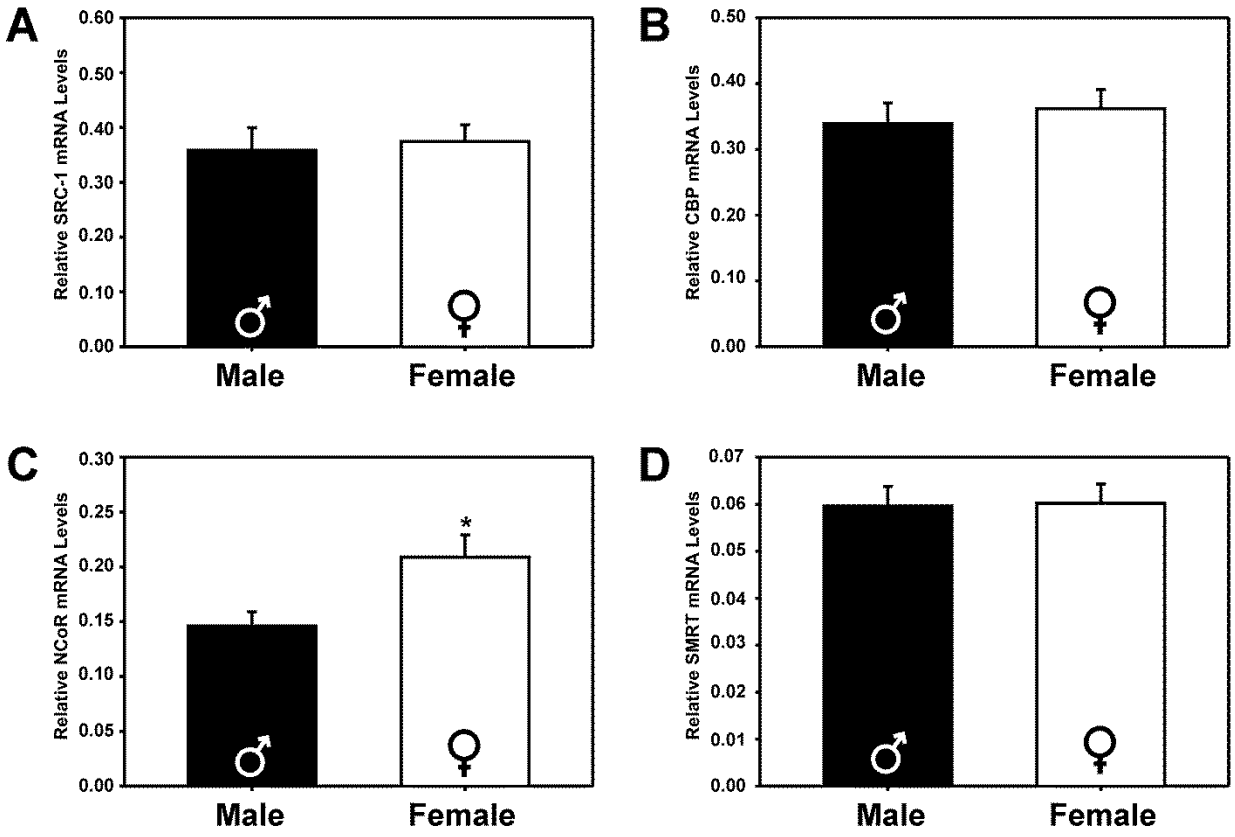


Figure 2


A
Relative SRC-1 mRNA Levels
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Male
Female
B
Relative CBP mRNA Levels
0.50
0.40
0.30
0.20
0.10
0.00
Male
Female
C
Relative NCoR mRNA Levels
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
Male
Female
D
Relative SMRT mRNA Levels
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Male
Female

Chapter 3: The nuclear receptor corepressor has organizational effects within the developing amygdala on juvenile social play and anxiety-like behavior

Published in Endocrinology, 2010 Mar;151(3):1212-20.

Abstract

Nuclear receptor function on DNA is regulated by the balanced recruitment of coregulatory complexes. Recruited proteins that increase gene expression are called coactivators, and those that decrease gene expression are called corepressors. Little is known about the role of corepressors, such as Nuclear receptor Corepressor (NCoR), on the organization of behavior. We used real-time PCR to show that NCoR mRNA levels are sexually dimorphic, females express higher levels of NCoR mRNA within the developing amygdala and hypothalamus, and that NCoR mRNA levels are reduced by estradiol treatment. To investigate the functional role of NCoR on juvenile social behavior, we infused siRNA targeted against NCoR within the developing rat amygdala and assessed the enduring impact on juvenile social play behavior, sociability, and anxiety-like behavior. As expected, control males exhibited higher levels of juvenile social play than control females. Reducing NCoR expression during development further increased juvenile play in males only. Interestingly, decreased NCoR expression within the developing amygdala had lasting effects on increasing juvenile anxiety-like behavior in males and females. These data suggest that the corepressor, NCoR, functions to blunt sex differences in juvenile play behavior, a sexually dimorphic and hormone-dependent behavior, and appears critical for appropriate anxiety-like behavior in juvenile males and females.

Introduction

Differential nuclear receptor activity during development has been shown to result in many of the structural and behavioral differences between males and females. These lasting changes are mainly produced by steroid hormones acting upon intracellular nuclear receptors (Blaustein and Olster, 1989;Cooke et al., 1998). During development, male rodents are exposed to higher levels of testosterone (Weisz and Ward, 1980), leading to differences in neurogenesis, cell death, and cell migration within the brain (Cooke et al., 1998), as well as lasting sex differences in behavior. One of the first sexually dimorphic social behaviors to emerge during development is juvenile social play behavior. Juvenile males engage in social play behavior at a higher frequency than do juvenile females (Auger and Olesen, 2009). This difference is known to be influenced by neonatal estradiol and testosterone exposure, as manipulating these hormone levels can sex reverse differences in juvenile social play behavior in rats (Olesen et al., 2005) and non-human primates (Goy and Deputte, 1996;Wallen, 2005).

Generally, steroid hormones act by binding to nuclear receptors, dimerizing with another ligand bound nuclear receptor, which then interact with hormone response elements within DNA leading to the formation of a transcriptional complex (Tsai and O'Malley, 1994). Nuclear receptor function on DNA is critically dependent upon available coregulatory proteins that form within the transcriptional complex, which can either increase gene transcription, called coactivators, or decrease gene transcription, called corepressors. Coactivators increase nuclear receptor function by increasing the acetylation of histone tails, which decreases their positive charge and allows the DNA to be more assessable to transcription factors (McKenna et al., 1999a). Various coactivators, such as SRC-1 (Steroid Receptor Coactivator-1) and CBP (CREB-

Binding Protein), have been found to be sexually dimorphic, (Misiti et al., 1998;Auger et al., 2002a), regulated by hormones (Mitev et al., 2003;Murphy and Segal, 1997;Charlier et al., 2006a) and necessary for the development of adult sexually dimorphic behavior (Auger et al., 2000;Auger et al., 2002a). Research on corepressor function in the developing brain has been much more limited.

Corepressors are thought to decrease gene transcription through their association with histone deactylase complexes, or HDACs (Tsai and O'Malley, 1994), which restores a positive charge on the histone tails and causes DNA to be less assessable to transcription factors (McKenna et al., 1999a). The corepressor, Nuclear receptor Corepressor or NCoR, was first discovered and identified through its interaction with thyroid hormone receptors (Horlein et al., 1995). Since then, it has been shown to be a corepressor molecule for androgen and estrogen receptors (Cheng et al., 2002;Yoon and Wong, 2006;Lavinsky et al., 1998). More recently, it has been found that NCoR can also repress gene expression independently of nuclear receptors via its direct or indirect interactions with methyl binding proteins, such as Kaiso (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006) and possibly MeCP2 (Cukier et al., 2008;Kokura et al., 2001). This is intriguing as disruption of normal methyl-binding protein function during brain development is associated with several neural developmental disorders; therefore, it is possible that abnormal NCoR expression and function might underlie some of the atypical behavioral patterns observed in individuals with epigenetic neural developmental disorders, such as Rett syndrome (Amir et al., 1999) and Huntington’s disease (Boutell et al., 1999b). While the distribution of NCoR within the rat brain (van der Laan et al., 2005b) has been reported, little is known about the functional role of NCoR in the developing brain, and whether

NCoR function has lasting consequences on the formation of typical behavior. We now report that the levels of NCoR mRNA are sexually dimorphic within the developing brain, females express higher levels of NCoR mRNA within the developing amygdala and hypothalamus, and that NCoR mRNA levels are reduced by estradiol treatment in females. We then targeted the amygdala with siRNA as it is critical in the development of social and anxiety behavior. We show that decreases in NCoR mRNA within the neonatal amygdala influences the organization of typical juvenile social play behavior and anxiety-like behavior.

Methods

Animals

Adult female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were mated in our animal facility and allowed to deliver normally. Cages were checked regularly to determine the day of birth (postnatal day 0, PN0). Animals were maintained on a 12 hour light/12 dark light cycle and food and water were available ad lib. This research was approved by the University of Wisconsin Animal Care and Use Committee.

Tissue Collection for NCoR expression studies

Animals were sacrificed by rapid decapitation at 24 hours after birth, referred to as postnatal day 1 (PN1). Two separate groups of animals were used to assess NCoR expression. One group was used for the detection of NCoR mRNA in the medialbasal hypothalamus (MBH; n = 4 males and n = 5 females) and a second to detect NCoR mRNA in the amygdala (n = 8

males and n = 11 females). The brains were microdissected using razor blades and immediately frozen in isopentane on dry ice. For the dissections, the brain was placed ventral side up on a cold surface. Using a razor blade, we made two coronal cuts, one caudal to the optic chiasma and one caudal to the hypothalamus. The tissue was then placed rostral side up and both MBH and amygdala were collected from this slab. For the amygdala, a cut was made along the optic tract and another cut at approximately 60 degrees to form an approximate triangle. Both sides were collected, pooled, and frozen. For the MBH, we then made two cuts adjacent and medial to the optic tract, followed by a perpendicular cut dorsal of the third ventricle. The tissue was stored at -80°C until homogenization for real-time PCR.

Hormone Treatment

Two additional sets of animals were used to assess the impact of hormone treatment. One set was injected subcutaneously with 100µg estradiol benzoate dissolved in 0.1ml sesame oil (6 females) or 0.1ml control sesame oil (7 males and 6 females) on the day of birth (PN0). The brains were collected and the MBH microdissected on PN1 as previously described. This dose of estradiol was chosen as it has been shown to result in male typical estradiol levels within the developing hypothalamus (Amateau et al., 2004). A separate set of animals were used to assess the impact of hormone treatment in the amygdala. Animals were injected subcutaneously with 100ug estradiol benzoate dissolved in 0.1ml sesame oil (6 females) or 0.1ml control sesame oil (6 females and 8 males) on the day of birth (PN0). The brains were collected and the amygdala microdissected on PN1 as previously described. India ink injected into the paw was used to mark groups at PN0.

NCoR Disruption with siRNA

To examine the functional role of NCoR during brain development on the organization of juvenile behavior, we infused small interfering RNA (siRNA) to reduce NCoR expression within the developing amygdala. Lyophilized NCoR (Santa Cruz Biotechnology, Cat# sc-36002) and nonsense control (Santa Cruz Biotechnology, Cat# sc-37007) siRNA were resuspended in RNAse-free water and oligofectamine reagent (Invitrogen, Cat# 12252-011) to make a 100µM solution. Litters used in this experiment were combined and the pups were randomly assigned treatment groups. Animals were cryoanesthetized and infused bilaterally with 1µl of siRNA, either NCoR or control, using a modified stereotaxic device and a 2-µl Hamilton syringe. Infusion occurred 12 hours after birth and again 28 hours after birth. As previously described, infusions were aimed at the amygdala, 1 mm lateral, 2 mm caudal and 5.5 mm ventral from the center and bregma suture lines (Kurian et al., 2008a). India ink injected into the paw was used to mark individuals within treatment groups. Following treatment, pups recovered under a warm lamp and were placed back with dams so that each new litter contained animals from both sexes and both treatment groups. Animals used to determine if NCoR siRNA decreased expression of NCoR mRNA within the amygdala were sacrificed 8 hours after the second infusion, approximately 36 hours after birth (4 males and 6 females with control siRNA and 8 males and 7 females with NCoR siRNA) or on PN10 (8 males with control siRNA and 6 males with NCoR siRNA). Animals used for behavioral testing were allowed to develop typically with the dam until weaning at PN21 (8 males and 7 females treated with control siRNA, 13 males and 8 females treated with NCoR siRNA).

Real-time PCR

Total RNA was isolated from the snap frozen tissue using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Cat# RTN-70). A StrataScript First Strand Synthesis system kit (Stratagene, Cat# 200420) was used to reverse transcribe RNA to cDNA in an Eppendorf MasterCycler Personal PCR machine. The cDNA was amplified in a Stratagene Mx3000P™ real-time PCR system using Platinum® qPCR SuperMix‑UDG (Invitrogen, Cat#11730-017). NCoR and SMRT primers were designed using Invitrogen D-LUX Designer (NCoR: accession number AF124821.1, Forward GGACCCGAGGGAAGACTACCA, Reverse CGGACTTCCCTTGCATCCTTGTC[FAM]G, product size-96 bases, Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT): accession number AF113001.1, Forward GGGCAAGCCCGACATAGAAT, Reverse cgccGTATCGGGTAGTAGCTCCAGG[FAM]G, product size-67 bases). All primers were found to have efficiencies between 93% and 100%. Primers for the gene of interest were multiplexed with 18s primers (Invitrogen, Cat#115HM-02) to allow for comparison within the same tube. ROX was used as a passive reference dye to control for baseline fluorescence in each sample. The amplification protocol was as follows – 50°C for 2 min, an initial denaturing step at 95°C for 2 min followed by 40 cycles of a 95°C denaturing step for 15 sec, a 55°C annealing step for 30 sec, and a 72°C elongation step for 30 sec. Following amplification, a dissociation curve analysis was used to ensure purity of the products, a single peak was observed for all products. Samples were run in duplicate in the same run. Relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Data were analyzed with a two-tailed Student's t-test or a one-way ANOVA and Tukey's *post hoc* comparison tests where appropriate.

Behavioral Testing and Statistical Analysis

All behavioral tests were performed and analyzed as previously reported (Kurian et al., 2008a) with the following exceptions. Briefly, behavioral tests were performed under dim red light approximately one to two hours following the beginning of the dark phase of the light cycle. Each behavior was recorded and then analyzed by a trained technician blind to all treatments using The Observer (Noldus Information Technologies) or Stopwatch+ (Center for Behavioral Neuroscience) with the exception of the light/dark chamber which was scored in real time. Behavioral data from each test, with the exception of the sociability test, were analyzed using a two-way ANOVA (sex by siRNA treatment) and Tukey's *post hoc* comparison tests were used to follow up any significant main effect or interaction. The sociability test was analyzed with a two-way repeated measures ANOVA and one-way ANOVAs for follow up comparisons to assess normal sociability.

Juvenile Social Play Behavior

Animals were weaned on postnatal day 21 and housed in groups of 5-6. All groups contained at least one animal from each treatment group (male/NCoR, male/control, female/NCoR and female/control). Animals were observed on postnatal days 25 through 29 for four minutes twice a day (one hour and three hours after the start of the dark cycle), as described previously (Olesen et al., 2005). Play behavior was scored using methods adapted from Casto et al. and Meaney and McEwen (Casto et al., 2003;Meaney and McEwen, 1986). Reported play behavior scores resulted from adding all instances that animals engaged in wrestling/boxing, biting, pinning or pouncing during the observation period. These behaviors were defined as wrestling/boxing: two rats engaged in rolling and tumbling over each other or making jabbing movements at each other with their forepaw; biting: one rat biting another; pouncing: one rat

pounces or lunges at another; and pinning: one rat standing over another, with its forepaws on the ventral surface of the opposing rat.

Sociability

The sociability test was adapted from Crawley et al. (Crawley, 2004;Crawley et al., 2007). An apparatus was split into three chambers with removable doors; the two end chambers each contained one empty perforated plastic container (7 X 7 X 16 cm). First, test animals (PN32) were placed into the center chamber and allowed to acclimate to all three chambers for ten minutes. The removable doors were then replaced containing the animal in the center chamber. A stimulus animal (an age and sex matched novel rat) was placed into one of the perforated plastic containers (social chamber), while the other container remained empty (non-social chamber). Doors were then removed and the test animal was allowed to explore all three chambers. Time in each chamber was recorded.

Elevated Plus Maze

On postnatal day 34, rats were tested for anxiety-like behavior within the elevated plus maze. The elevated plus maze consists of 2 opposing runways, one open and one closed, each measuring 100 cm in length and constructed of black Plexiglas. Each arm of the closed runway is fitted with 39 cm high Plexiglas walls on either side of the runways. The maze stands 50 cm off the floor. Rats were placed into the center of the maze facing an open arm and their behavior was video recorded for five minutes as they explored the maze. Time spent in open and closed arms was later recorded by a trained technician blind to the treatment groups. An entry was scored when all four paws crossed into the new portion of the maze.

Light/Dark Chamber

Following testing in the elevated plus maze, rats were tested for anxiety-like behavior in the Light/Dark chamber task. The light/dark chamber is one large Plexiglas chamber which is divided into two chambers by an opaque Plexiglas insert: one large lit side (35 X 38 X 39 cm) constructed of clear Plexiglas and one smaller dark side (25 X 38 X 39 cm) constructed entirely of opaque material with a lid. An opening in the lower corner on the insert (6 X 10 cm) allows the animal to move freely between the two sides. A white incandescent light is situated above the light side. The animals were placed into the middle of the light side of the box facing the opening and observed for five minutes. Total time in the light was recorded. A cross was scored when all four paws were on the new side.

Results

Sex difference in NCoR mRNA levels

We report that on PN1, females express higher levels of NCoR mRNA than do males in the MBH ($p<0.05$, *t*-test, Figure 1A). Females were also found to express higher levels of NCoR mRNA within the amygdala on PN1 ($p<0.05$, *t*-test, Figure 1B).

Estrogen regulation of NCoR mRNA levels

We found that females expressed significantly higher levels of NCoR mRNA within the MBH contrasted to males, a confirmation of the sex difference, and that estradiol treatment in females reduced the levels of NCoR mRNA to male-like levels ($p<0.01$, ANOVA, $p<0.05$, Tukey's *post hoc* comparison test, Figure 1C). We also report that estradiol treatment in females

reduced the levels of NCoR mRNA within the amygdala to male-like levels ($p<0.01$, ANOVA, $p<0.05$, Tukey's *post hoc* comparison test, Figure 1D). These data suggest that the sex difference in NCoR expression between males and females may be partially due to differences in steroid hormone exposure.

siRNA Treatment

NCoR siRNA infused into the developing amygdala was confirmed to decrease the levels of NCoR mRNA in males and females ($p<0.05$, *t*-test, Figures 2A and 2B). We also performed controls to demonstrate that NCoR infusion into the amygdala was both targeted and transient. NCoR expression levels were not significantly different between control and NCoR siRNA treated animals within the MBH ($p>0.05$, t-test, Figure 3A), unlike within the targeted amygdala in the same animals. NCoR expression levels were also not significantly different between control and NCoR siRNA treated animals within the amygdala when examined at PN10 ($p>0.05$, t-test, Figure 3B), indicating that the effect of the NCoR siRNA infusion is transient. We next investigated if the effect of the siRNA treatment was limited to the gene of interest, NCoR. Indeed, mRNA expression levels of another corepressor, SMRT, were not significantly different between control and NCoR siRNA treated animals within the amygdala ($p>0.05$, t-test, Figure 3C).

Juvenile Social Play Behavior

We assessed the enduring impact of a reduction in NCoR expression on the development of juvenile social play behavior. A typical sex difference in juvenile play behavior was observed with control males exhibiting higher levels of play than control females ($p<0.05$, Tukey's *post*

hoc comparison test, Figure 4), as well as a general main effect of sex ($p<0.001$, two-way ANOVA, Figure 4). A general main effect of treatment was also observed ($p<0.05$, two-way ANOVA). Interestingly, males with a decrease in NCoR mRNA expression within the developing amygdala exhibited significantly higher levels of juvenile social play when compared to control males ($p<0.05$, Tukey's *post hoc* comparison test, Figure 4). The effect of reduced NCoR expression increasing the masculinization of juvenile social play was observed in males only, as no effect on juvenile social play behavior was observed in females.

Sociability

Normal sociability is defined as a significant difference between the time spent in the social chamber versus the non-social chamber (Crawley, 2004). In the current experiment, all animals showed normal levels of sociability. That is, animals in all treatment groups spent more time in the social chamber than in the non-social chamber ($p<0.05$, ANOVA, Figure 5). This suggests that while juvenile social play behavior is altered in males by targeted reduction of NCoR expression within the developing amygdala, there was no impact on typical sociability exhibited by these juveniles.

Anxiety-like Behavior

A targeted decrease in NCoR expression within the developing amygdala was found to significantly increase anxiety-like behavior during the juvenile period in males and females. Animals infused with NCoR siRNA showed an increase in anxiety-like behavior as compared to controls in both the elevated plus maze ($p< 0.05$, two-way ANOVA, Figure 6A) and the light/dark box ($p<0.01$, two-way ANOVA, Figure 6B). These data suggest that transient

disruptions in NCoR expression within the developing amygdala can lead to lasting increases in anxiety-like behavior in juvenile males and females.

Discussion

NCoR was one of the first corepressors to be identified (Horlein et al., 1995) and is thought to function through its association with the Sin3 complex and histone deacetylase complexes (HDACs) (Heinzel et al., 1997;Alland et al., 1997;Nagy et al., 1997b). While NCoR mRNA has been found to be ubiquitously distributed (van der Laan et al., 2005b) in the adult rat brain, we now show that females express higher levels of the corepressor, NCoR, within the MBH and amygdala during development. This is in contrast to the higher levels of coactivators, i.e. SRC-1 and CBP, found in males (Misiti et al., 1998;Auger et al., 2002a). The sex differences in NCoR expression are found in areas of the rat brain known to be rich in nuclear receptors, and therefore it is likely that differences in steroid hormone exposure contribute to the differential expression of NCoR between the sexes. Indeed, we report that treatment of females with estradiol decreases the levels of NCoR mRNA to male-like levels with the amygdala and hypothalamus. These data are consistent with previously published *in vitro* data showing that estradiol can down-regulate NCoR expression (Frasor et al., 2005), and again is in contrast to the upregulation of coactivator expression following estradiol treatment, as reported with SRC-1 (Mitev et al., 2003;Murphy and Segal, 1997;Charlier et al., 2006a) and CBP (Mitev et al., 2003;Murphy and Segal, 1997;Charlier et al., 2006a). The function of these sex differences in coregulatory expression remains unclear; however, as male rats have two surges of hormones, one prenatal and one postnatal, it is possible that the first prenatal surge partially masculinizes some systems and reduces corepressors so that a second neonatal surge a few days later can

further masculinize the brain. Likewise, it is possible that the higher levels of NCoR observed in females may serve to partially protect their brains from masculinization that could result from a brief developmental exposure to hormones. Regardless, these data do suggest that steroid hormone exposure alters the available expression of coregulatory complexes found within a cell, and may change the way a cell responds to future hormonal or environmental challenges.

Given the amygdala's role in regulating social play (Meaney et al., 1981a), we chose to investigate the functional role of NCoR on the organization of juvenile social behavior. To accomplish this, we used siRNA to induce a targeted reduction of NCoR expression within the developing amygdala and assessed the functional consequence of reduced expression on the formation of typical juvenile social play behavior and sociability. We chose to disrupt NCoR expression within the first 36 hours of neonatal life, as this is a time point in which NCoR expression is sexually dimorphic and this period of time is known to be critical for sexual differentiation by steroid hormones. Our data suggest that NCoR plays an important organizational role in the masculinization of juvenile social play behavior. The typical sex difference in juvenile social play behavior (Meaney and Stewart, 1981) was observed with control males showing higher levels of social play contrasted to control females. Interestingly, males experiencing a targeted disruption of NCoR expression within the developing amygdala displayed higher levels of juvenile social play behavior when compared to control males; no differences in juvenile play behavior was observed in females. These findings are consistent with NCoR functioning as a corepessor of nuclear receptors. That is, reducing the expression of the nuclear corepressor, NCoR, allowed for further masculinization of juvenile social play behavior in males. Reducing NCoR expression in females did not alter juvenile social play behavior.

While it is possible that NCoR levels remain high in females due to the sex difference in NCoR exression, it more likely that increased masculinization of social play did not occur in NCoR siRNA treated females because they are not exposed to the masculinizing actions of the testosterone surge that is occurring in males. Interestingly, while juvenile social play behavior was altered by NCoR disruption, no differences in sociability were detected. This suggests that not all social behaviors were altered by NCoR disruption within the developing amygdala.

Juvenile social play behavior is classically thought to be due to activation of androgen receptors (Meaney and Stewart, 1981;Meaney and McEwen, 1986), with some data suggesting a possible involvement of estrogen receptors (Olesen et al., 2005). As NCoR appears to interact with both androgen (Cheng et al., 2002;Yoon and Wong, 2006) and estrogen receptors (Lavinsky et al., 1998), it is possible that reducing NCoR expression enhances the function of both nuclear receptors to further masculinize juvenile social play. These results are also interesting when considered with findings suggesting that coactivators are critical in mediating sexual differentiation of the brain (Auger et al., 2000;Auger et al., 2002a). Together, they suggest that appropriate coactivator and corepressor expression are necessary for the organization of typical sex differences in social behavior. That is, coactivators may act to enhance sex differences, and corepressors may act to restrict or lessen the magnitude of some sex differences in males. Compensatory mechanisms to reduce overt sex differences in some behaviors have been proposed previously (De Vries, 2004;McCarthy and Konkle, 2005) and it is possible that NCoR may be a molecule involved in reducing some sex differences in social play behavior.

As the amygdala has also been found to be critical in regulating anxiety-like behavior (LeDoux, 1998;Graeff et al., 1993), we investigated the effects of NCoR disruption during

development on juvenile anxiety-like behavior. We have found that NCoR siRNA treatment had a lasting effect of increasing anxiety-like behavior during the juvenile period. This increase in juvenile anxiety-like behavior due to targeted disruption of NCoR occurred independent of sex. While sex differences in anxiety-like behavior are known to occur in adults (Johnston and File, 1991), we found no sex differences in anxiety-like behavior during the juvenile period. Given that NCoR disruption increases juvenile anxiety-like behavior in both males and females, it is important to consider that NCoR can also repress gene expression via its interactions with methyl-binding proteins, such as Kaiso (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006) and MeCP2 (Kokura et al., 2001;Cukier et al., 2008). Therefore, it is possible that the long term changes in anxiety-like behavior may result from decreased interaction of NCoR with methyl-binding proteins during the neonatal period.

While DNA methylation can cause gene inhibition directly by interfering with binding of transcriptional proteins to DNA, it can also cause gene repression by allowing binding of methyl-binding proteins to the DNA. Methyl-binding proteins then recruit corepressor complexes, some of which contain NCoR. Corepressor complexes cause gene repression through the additional recruitment of histone deactylase complexes (HDACs), which deacytalate histones leading to condensation of the chromatin and gene silencing (Klose and Bird, 2006a). While numerous corepressors have been identified that interact with methyl-binding proteins, there is evidence that NCoR can interact directly with methyl-binding proteins (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006;Kokura et al., 2001;Cukier et al., 2008) or indirectly via its interaction with the Sin3 corepressor complex (Alland et al., 1997). Interactions of NCoR with epigenetic proteins are intriguing as mutations in some of these proteins are implicated in several

neurodevelopmental disorders. For example, mutations in the X-linked MECP2 gene, which lead to disruption in MeCP2 protein function, are thought to be the direct cause of Rett syndrome, a progressive neurodevelopmental disorder (Amir et al., 1999). Recently, MeCP2 has been shown to directly interact with Sin3a, Rest, as well as NCoR, and it has been suggested that these proteins may be better therapeutic targets than MeCP2 (Cukier et al., 2008). In addition, abnormal cellular distribution of NCoR has been directly linked to Huntington's disease (Boutell et al., 1999b). While it is not clear if abnormal developmental expression of NCoR directly impacts these disorders, it is interesting to note that individuals with these disorders report an increase in anxiety (Sansom et al., 1993;Caine and Shoulson, 1983;Paulsen et al., 2001). As our findings suggest that NCoR plays an important role in the organization of anxiety-like behavior, it is possible that the increase in anxiety observed in these disorders may be partially due to abnormal NCoR function during brain development.

In summary, we report that levels of NCoR are sexually dimorphic during brain development, responsive to hormones and, more importantly, seem to have organizational effects on juvenile social and anxiety behavior. We find that a transient decrease in NCoR expression within the developing amygdala leads to an increase in juvenile social play behavior in males, and an increase in anxiety-like behavior in both males and females. It remains to be determined if these effects occur via NCoR's association with nuclear receptor complexes, methyl-binding proteins, or both. Regardless, these data illustrate the importance of understanding the balance of coactivator and corepressor expression, and adds an interesting layer of complexity to how we understand sexual differentiation of the brain, nuclear receptor function, and the organization of behavior.

Figure Legends

Figure 1

Sex differences and estradiol regulation of NCoR expression within neonatal brain. A. Females express significantly higher levels of NCoR mRNA within the MBH on PN1. * = $p<0.05$ (4 males, 5 females). B. Females express significantly higher levels of NCoR mRNA within the amygdala on PN1. * = $p<0.05$ (8 males, 11 females). C. Oil treated females (n=6) expressed significantly higher levels of NCoR mRNA within the developing MBH when compared to oil treated males (n=7) and estradiol treated females (n=6). Different letters represent a statistically significant difference, $p<0.05$. D. Oil treated females (n=6) expressed significantly higher levels of NCoR mRNA within the developing amygdala when compared to oil treated males (n=8) and estradiol treated females (n=6). Different letters represent a statistically significant difference, $p<0.05$.

Figure 2

Confirmation of siRNA treatment within the amygdala on PN0/1. A. NCoR siRNA infused into the developing amygdala decreased the expression of NCoR mRNA in males. * = $p<0.05$ (control siRNA n=4, NCoR siRNA n=8). B. NCoR siRNA infused into the developing amygdala also decreased the expression of NCoR mRNA in females. * = $p<0.05$ (control siRNA n=5, NCoR siRNA n=5).

Figure 3

Controls for the siRNA treatment within the amygdala on PN0/1. A. NCoR expression levels were not significantly different between control and NCoR siRNA treated females within the MBH, indicating that the siRNA treatment did not spread from the amygdala (control siRNA n=6, NCoR siRNA n=7). B. NCoR expression levels were not significantly different between control and NCoR siRNA treated males within the amygdala on PN10, indicating that the effect of the NCoR siRNA infusion is transient (control siRNA n=8, NCoR siRNA n=6). C. mRNA expression levels of SMRT were not significantly different between control and NCoR siRNA treated females within the amygdala (control siRNA n=6, NCoR siRNA n=7).

Figure 4

Reduced NCoR expression within the developing amygdala enhances masculinization of juvenile social play behavior in males only. A typical sex difference in juvenile play behavior was observed, males exhibited higher levels of play than did females. * = $p<0.001$. Interestingly,

males with a transient decrease in NCoR mRNA expression during development exhibited significantly higher levels of juvenile social play when compared to males infused with a control siRNA. # = $p<0.05$. This effect was not seen in females. (8 males and 7 females treated with control siRNA, 12 males and 8 females treated with NCoR siRNA)

Figure 5

All animals showed normal juvenile sociability, spending more time in the social chamber than in the non-social chamber. * = $p < 0.05$ (8 males and 7 females treated with control siRNA, 13 males and 8 females treated with NCoR siRNA)

Figure 6

Animals that experienced a transient decrease in NCoR mRNA in the developing amygdala showed increased levels of anxiety-like behavior during the juvenile period. A. NCoR siRNA treatment decreased the ratio of time in the open vs closed arms on the elevated plus maze. * = $p< 0.05$. B. NCoR siRNA treatment decreased the time spent in the light side of the light/dark box. * = $p<0.01$ (8 males and 7 females treated with control siRNA, 13 males and 8 females treated with NCoR siRNA)

Figures

Figure 1

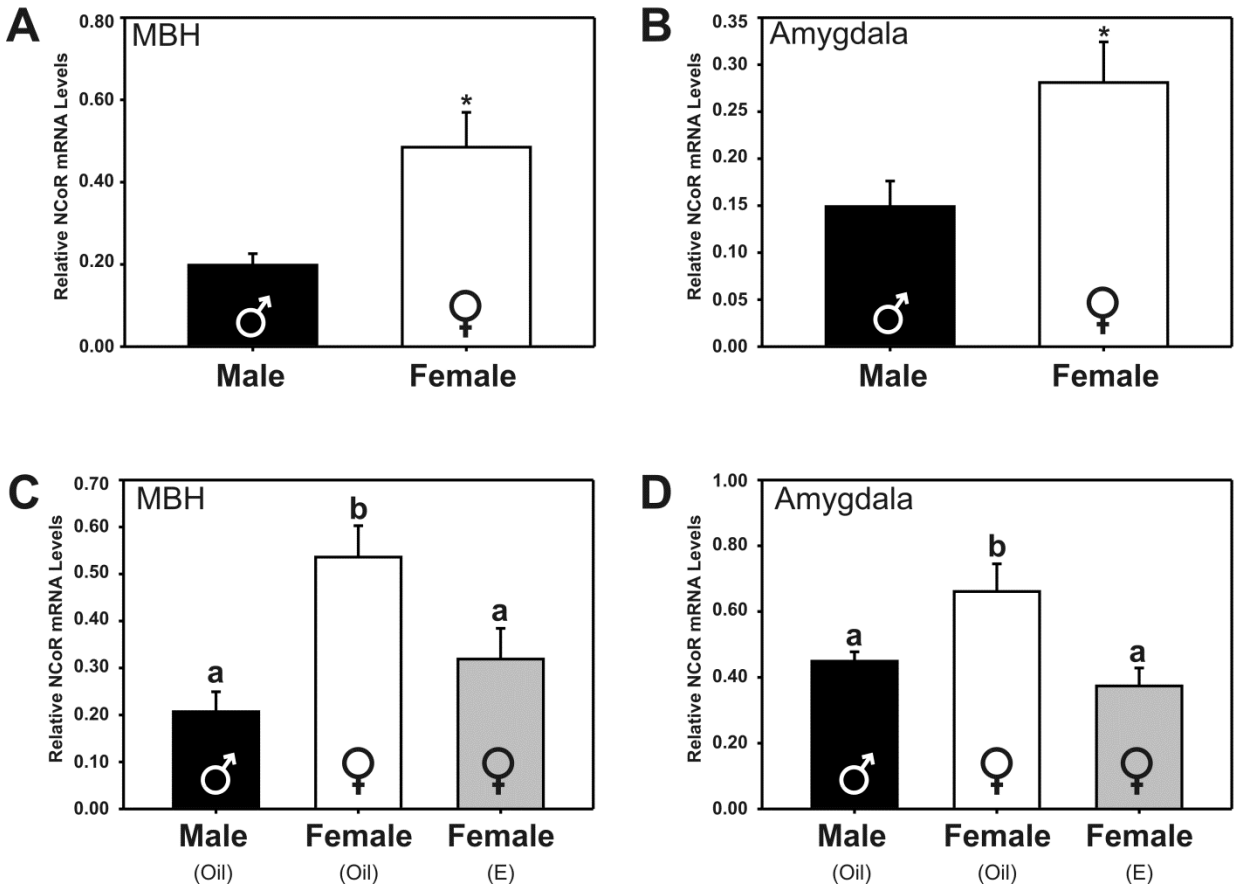


Figure 2

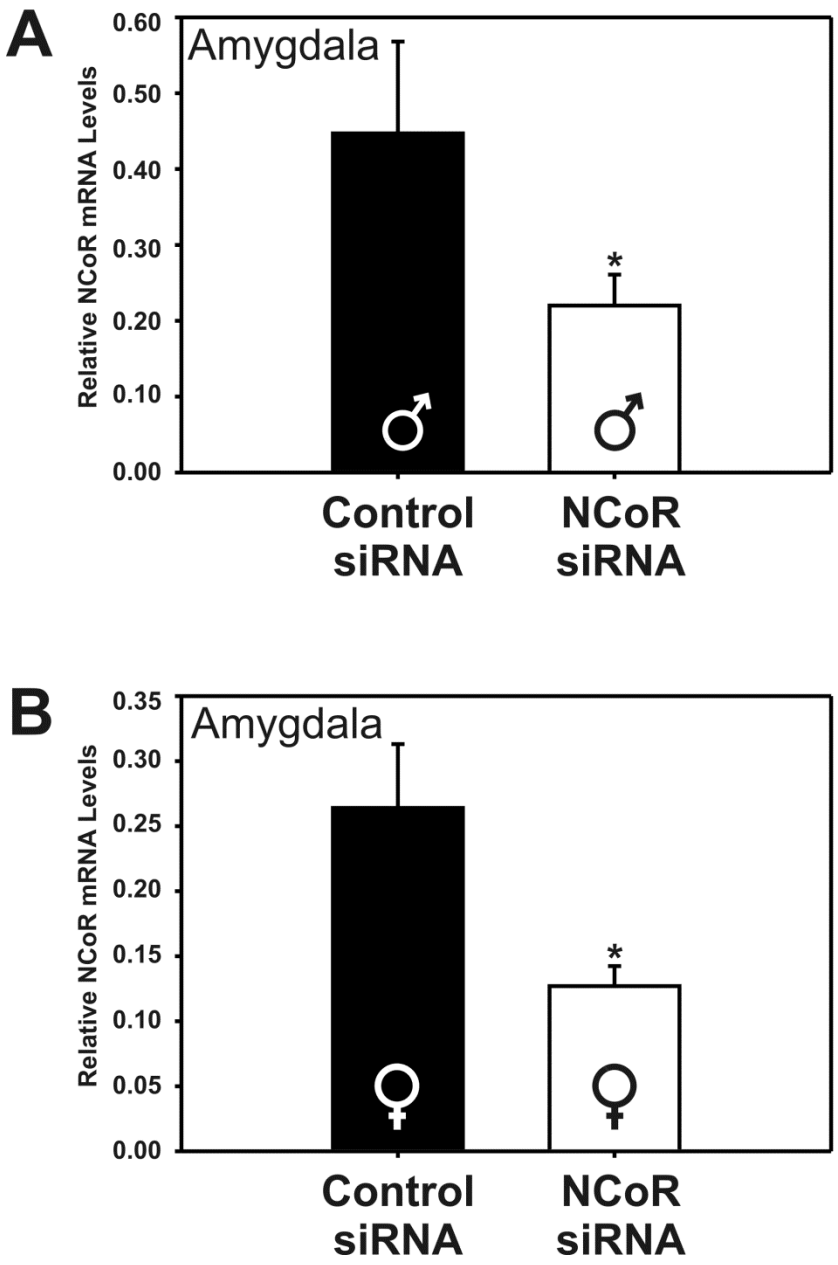
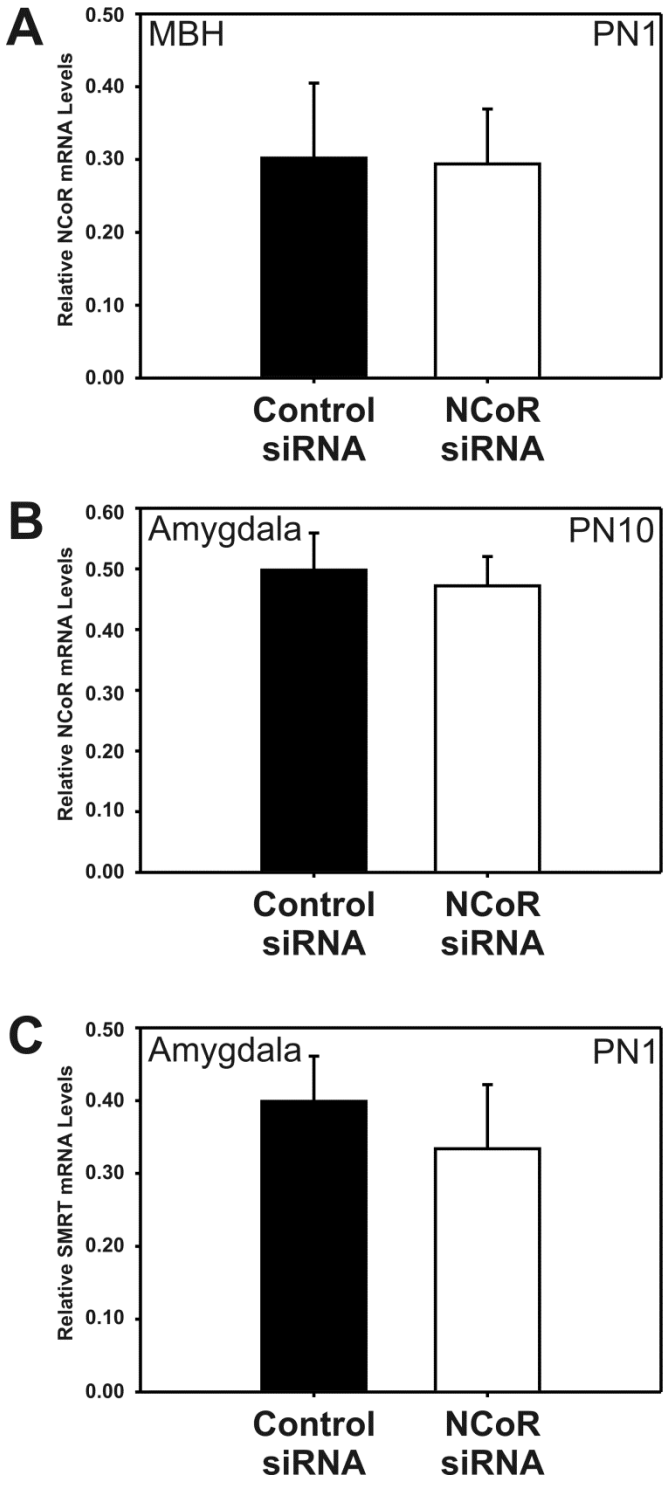

A
Amygdala
Relative NCoR mRNA Levels
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
♂
♂
Control siRNA
NCoR siRNA
B
Amygdala
Relative NCoR mRNA Levels
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
♀
♀
Control siRNA
NCoR siRNA

Figure 3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MBH
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0.30
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0.10
0.00
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Amygdala
PN10
Relative NCoR mRNA Levels
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0.50
0.40
0.30
0.20
0.10
0.00
Control siRNA
NCoR siRNA
C
Amygdala
PN1
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0.10
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Control siRNA
NCoR siRNA

Figure 4

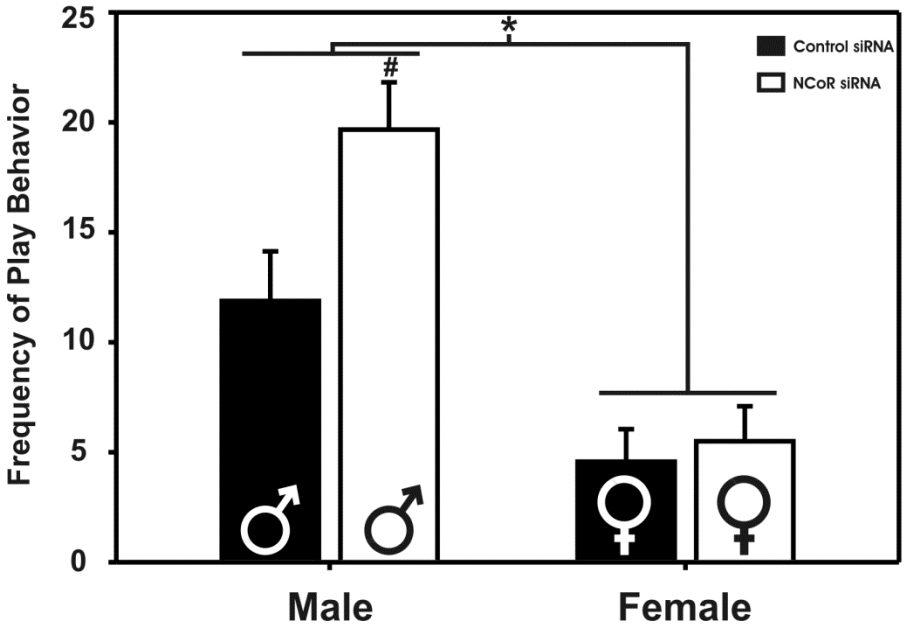


Figure 5

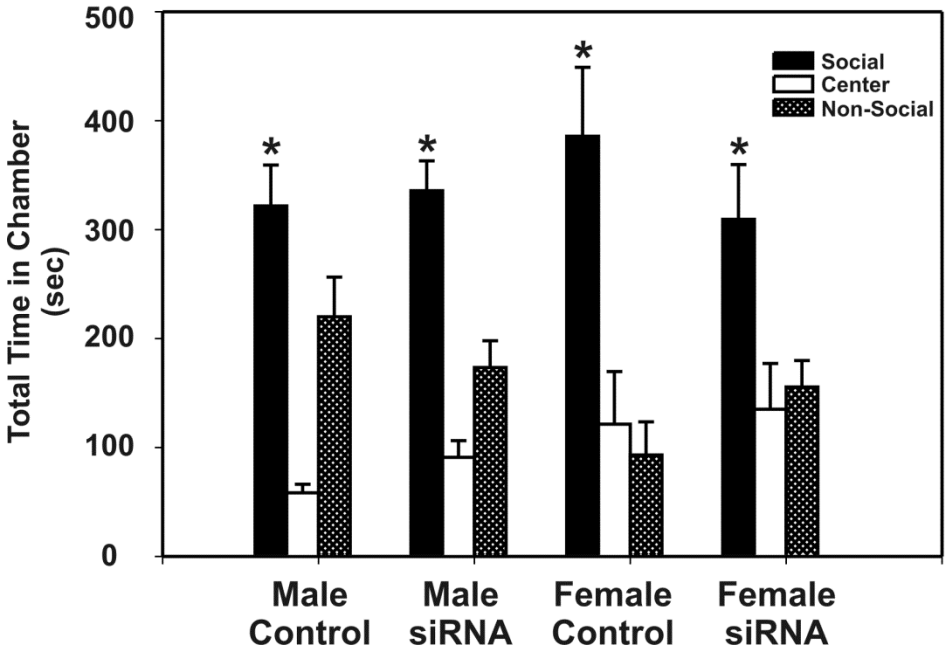
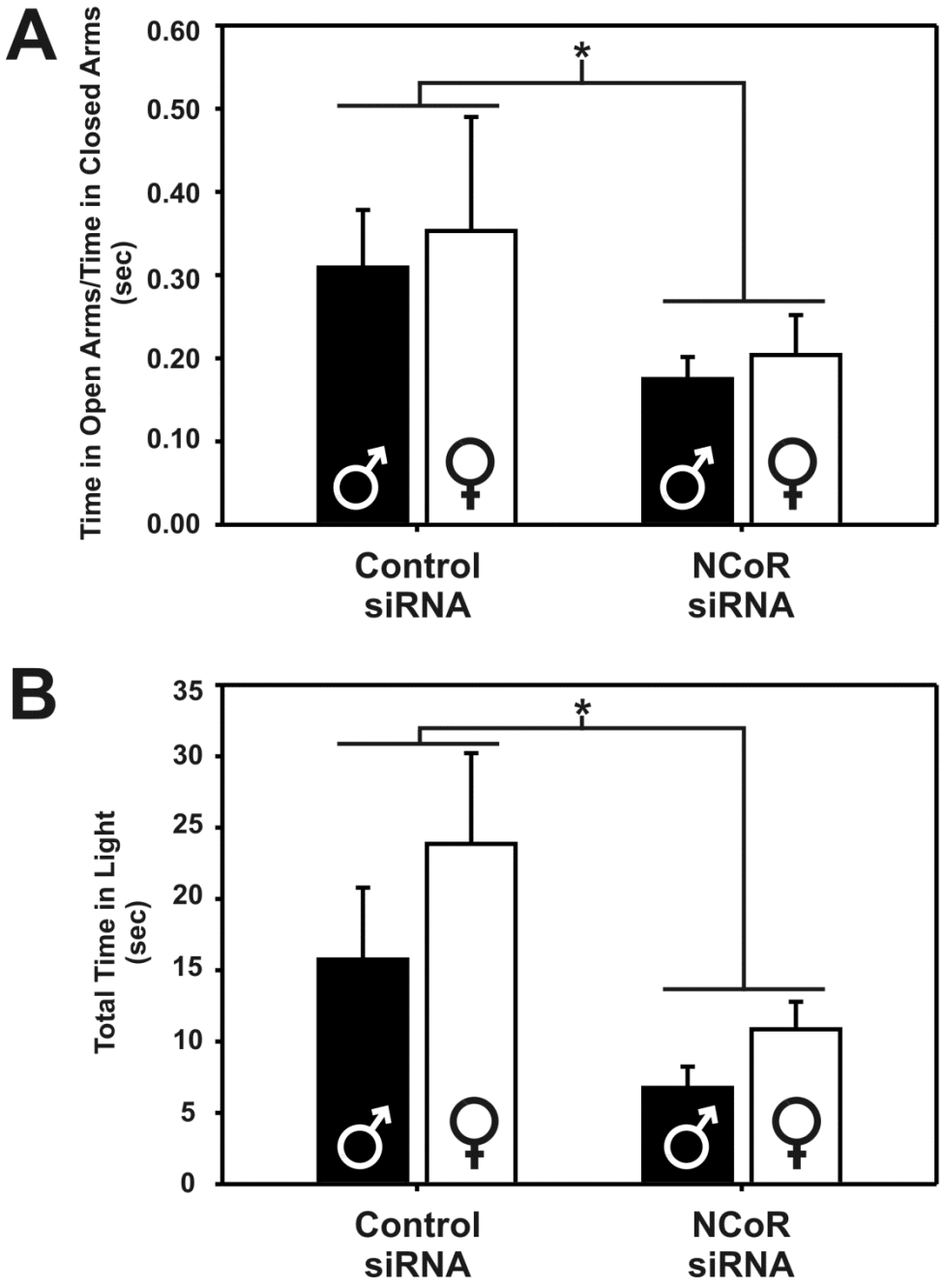


Figure 6


A
Time in Open Arms/Time in Closed Arms (sec)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
♂
♀
♂
♀
Control siRNA
NCoR siRNA
B
Total Time in Light (sec)
35
30
25
20
15
10
5
0
*
♂
♀
♂
♀
Control siRNA
NCoR siRNA

Chapter 4: The nuclear receptor corepressor has organizational effects within the developing amygdala on adult anxiety-like behavior and social discrimination

Abstract

Nuclear receptor function on DNA is regulated by the balanced recruitment of coregulatory complexes. Coactivators are recruited proteins that increase the acetylation of histones and subsequent gene expression; while corepressors lead to the deacetylation of histones and subsequently decrease gene expression. In contrast to coactivators, little is known about the role of corepresssors in brain development and behavior. We have previously reported a sex difference in the expression of Nuclear receptor Corepressor (NCoR) within the developing amygdala and shown that reduced NCoR expression during development increased juvenile play in males and had lasting effects on anxiety-like behavior in males and females. We now show that these effects on anxiety-like behavior persist until adulthood in males. To further examine the functional role of NCoR on social behavior, we examined the effects of decreased NCoR expression within the developing amygdala on juvenile social anxiety-like behavior and adult social discrimination behavior. We now show that decreased NCoR expression during development results in an increase in juvenile social anxiety-like behavior and disrupts social discrimination behavior in adults. These data suggest that NCoR is critical for the development of appropriate anxiety-like and social behavior.

Introduction

Epigenetics can be defined as a change to DNA that alters gene transcription without altering the underlying code. In general, DNA methylation has been associated with the epigenetic suppression of gene transcription. While methylation of DNA alone may lead to a decrease in gene transcription, it is the binding of methyl-CpG binding proteins to methylated DNA and subsequent increase in the interactions of chromatin remodeling corepressor complexes with DNA and histones that results in more efficient gene repression (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006). Nuclear receptor Corepressor, NCoR, has been found to be a component of some of these corepressor complexes (Cunliffe, 2008). Recently, NCoR has been found to interact directly or indirectly with methyl binding proteins, such as Kaiso (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006) and possibly MeCP2 (Cukier et al., 2008;Kokura et al., 2001).

NCoR was first discovered and identified through its interaction with thyroid hormone receptors (Horlein et al., 1995) and has since also been shown to be a corepressor molecule for androgen and estrogen receptors (Cheng et al., 2002;Yoon and Wong, 2006;Lavinsky et al., 1998). Through these interactions with nuclear receptors, NCoR is also known to function, independent of methyl binding proteins, by decreasing nuclear receptor induced transcriptional activity. This is in contrast to the function of nuclear receptor coactivators, which is to increase transcriptional activity. Nuclear receptor activation by steroid hormones plays an important role in the developing rat brain, causing many physiological and behavioral changes.

Corepressors are thought to decrease gene transcription through their association with histone deactylase complexes, or HDACs (Tsai and O'Malley, 1994), which restore a positive

charge on histone tails and causes negatively charged DNA to more tightly associate to the histones and thus less assessable to transcription factors (McKenna et al., 1999a). These chromatin remodeling complexes may cause additional modifications to the chromatin including, methylation, ubiquitylation, phosphorylation and sumoylation (Wu et al., 1986) which result in gene repression (Klose and Bird, 2006). As the recruitment of corepressor complexes to DNA can occur following interactions with nuclear receptors or methyl-binding proteins, there exists the potential for a dynamic model of chromatin remodeling which may allow for complex responses of different cells and tissues to diverse stimuli.

NCoR has been shown to be ubiquitously distributed in the rat brain (van der Laan et al., 2005b) and sexually dimorphic within the developing amygdala, females express higher levels of NCoR mRNA (Jessen et al., 2010a). Interestingly, NCoR was also found to be critical in the development of juvenile social and anxiety-like behavior, specifically that decreases in NCoR mRNA within the neonatal amygdala influences the organization of typical juvenile social play behavior and anxiety-like behavior, but not sociability (Jessen et al., 2010a). However, we still know relatively little about the functional role of NCoR in the developing brain, and whether disrupted NCoR function has lasting consequences on the formation of typical behavior into the adult period. We now show that male animals experiencing a transient decrease in NCoR expression within the developing amygdala showed a significant increase in social anxiety-like behavior during the juvenile period. We also show that male animals treated with NCoR siRNA showed increased anxiety-like into the adult period. In addition, social recognition is showed to be impaired in adult males that experienced NCoR disruption during development while animals infused with the control siRNA displayed normal social recognition behavior.

Methods

Animals

Adult female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were mated in our animal facility and allowed to deliver normally. Cages were checked regularly to determine the day of birth (postnatal day 0, PN0). Animals were maintained on a 12 hour light/12 dark light cycle and food and water were available ad lib. This research was approved by the University of Wisconsin Animal Care and Use Committee. Male animals from a study previously published were used in these experiments (Jessen et al., 2010a).

NCoR Disruption with siRNA

As previously reported (Jessen et al., 2010a), to examine the functional role of NCoR during brain development on the organization of juvenile and adult behavior, we infused small interfering RNA (siRNA) to reduce NCoR expression within the developing amygdala. Lyophilized NCoR (Santa Cruz Biotechnology, Cat# sc-36002) and nonsense control (Santa Cruz Biotechnology, Cat# sc-37007) siRNA were resuspended in RNAse-free water and oligofectamine reagent (Invitrogen, Cat# 12252-011) to make a 100µM solution. Litters used in this experiment were combined and the pups were randomly assigned treatment groups. Animals were cryoanesthetized and infused bilaterally with 1µl of siRNA, either NCoR or control, using a modified stereotaxic device and a 2-µl Hamilton syringe. Infusion occurred 12 hours after birth and again 28 hours after birth. As previously described, infusions were aimed at the amygdala, 1

mm lateral, 2 mm caudal and 5.5 mm ventral from the center and bregma suture lines (Kurian et al., 2008). India ink injected into the paw was used to mark individuals within treatment groups. Following treatment, pups recovered under a warm lamp and were placed back with dams so that each new litter contained animals from both sexes and both treatment groups. Animals were allowed to develop typically with the dam until weaning at PN21 (8 males were treated with control siRNA, 13 males were treated with NCoR siRNA). Male animals were separated at PN35 or PN36 and were randomly pair housed with a litter mate.

We have previously reported that NCoR siRNA infused into the developing amygdala was confirmed to decrease the levels of NCoR mRNA ($p<0.05$, *t*-test) and was both targeted and transient (Jessen et al., 2010a).

Behavioral Testing and Statistical Analysis

All behavioral tests were performed and analyzed as previously reported (Jessen et al., 2010a) and (Kurian et al., 2008) with the following exceptions. Briefly, behavioral tests were performed under dim red light approximately one to two hours following the beginning of the dark phase of the light cycle. Each behavior was recorded and then analyzed by a trained technician blind to all treatments using The Observer (Noldus Information Technologies) or Stopwatch+ (Center for Behavioral Neuroscience) with the exception of both light/dark chamber experiments which were scored in real time. Behavioral data from each test were analyzed with a two-tailed Student's t-test, except the social discrimination experiment which was analyzed using a paired t-test.

Social Light/Dark Chamber

Rats were tested for social anxiety-like behavior in the social Light/Dark chamber task on PN35 or 36. The light/dark chamber is one large Plexiglas chamber which is divided into two chambers by an opaque Plexiglas insert: one large lit side (35 X 38 X 39 cm) constructed of clear Plexiglas and one smaller dark side (25 X 38 X 39 cm) constructed entirely of opaque material with a lid. An opening in the lower corner on the insert (6 X 10 cm) allows the animal to move freely between the two sides. A white incandescent light is situated above the light side. Shavings from an unrelated, adult male rat cage were placed into the dark chamber. The animals were placed into the middle of the light side of the box facing the opening and observed for five minutes. The latency to enter the dark side of the box was measure in seconds using a stopwatch.

Elevated Plus Maze

Intact adult male rats (approximately two and half months after birth) were tested for anxiety-like behavior within the elevated plus maze. The elevated plus maze consists of 2 opposing runways, one open and one closed, each measuring 100 cm in length and constructed of black Plexiglas. Each arm of the closed runway is fitted with 39 cm high Plexiglas walls on either side of the runways. The maze stands 50 cm off the floor. Rats were placed into the center of the maze facing an open arm and their behavior was video recorded for five minutes as they explored the maze. Time spent in open and closed arms was later recorded by a trained technician blind to the treatment groups. An entry was scored when all four paws crossed into the new portion of the maze.

Light/Dark Chamber

Following testing in the elevated plus maze, rats were tested for anxiety-like behavior in the Light/Dark chamber task. The light/dark chamber is one large Plexiglas chamber which is divided into two chambers by an opaque Plexiglas insert: one large lit side (35 X 38 X 39 cm) constructed of clear Plexiglas and one smaller dark side (25 X 38 X 39 cm) constructed entirely of opaque material with a lid. An opening in the lower corner on the insert (6 X 10 cm) allows the animal to move freely between the two sides. A white incandescent light is situated above the light side. The animals were placed into the middle of the light side of the box facing the opening and observed for five minutes. Total time in the light was recorded. A cross was scored when all four paws were on the new side.

Social Discrimination

Intact adult male rats (approximately seven months after birth) were tested for social discrimination. The social discrimination paradigm was adapted from (Engelmann et al., 1995) and preformed as previously reported in (Bychowski and Auger, 2012) with the following exceptions. Briefly, animals were separated from their cage mates and singly housed 48 hours before testing. Testing occurred in the home cages of the adult male rats. In trial 1, a male juvenile rat was placed in the home cage of the adult rat and the adult was allowed to freely investigate for 5 minutes. After 5 minutes, the juvenile was removed and the adult was alone in its cage for 30 minutes. After the 30 minute intertrial interval, the juvenile from trial 1 plus a novel juvenile were placed in the adult's cage, and the adult was again free to investigate for 5 minutes. The juvenile rats were distinguishable to the researcher scoring the video by unique tail marks drawn with permanent marker. Adult investigation of the juvenile(s) was scored to include

direct contact between the nose of the adult and the analgenital region of the juvenile and close following behavior.

Results

Juvenile social anxiety-like behavior

A transient decrease in NCoR expression within the developing amygdala was found to significantly increase social anxiety-like behavior in juvenile males. That is, animals infused with NCoR siRNA during early development showed an increased latency to enter the dark side of the light/dark chamber as compared to control animals when adult male shaving were present in the dark side ($p<0.05$, t-test, Figure 1).

Adult anxiety-like Behavior

A transient decrease in NCoR expression within the developing amygdala was found to significantly increase anxiety-like behavior in adult males. Animals infused with NCoR siRNA showed an increase in anxiety-like behavior as compared to controls in both the elevated plus maze ($p<0.05$, t-test, Figure 2A) and the light/dark box ($p<0.05$, t-test, Figure 2B). These data suggest that transient disruptions in NCoR expression within the developing amygdala can lead to lasting increases in anxiety-like behavior in adult males.

Social Discrimination

We examined the enduring impact of a reduction in NCoR expression on the development of adult social discrimination. A typical pattern of social discrimination was observed with the control males, specifically, control males spent significantly more time investigation the anogenital region of the novel juvenile rat than the familiar juvenile rat ($p<0.05$, paired t-test, Figure 3A). Interestingly, males with a decrease in NCoR mRNA expression within the developing amygdala failed to discriminate between the novel and familiar juvenile rat ($p>0.05$, paired t-test, Figure 3B).

Discussion

Our data support the idea that NCoR plays a role in the organization of both social and anxiety-like behavior. We report that a transient decrease in NCoR expression within the developing amygdala during the first 36 hours of life, a time at which NCoR expression levels are sexually dimorphic and a period of time known to be critical for the organization of sex differences by steroid hormones, significantly increases social anxiety-like behavior in juvenile males, increases anxiety-like behavior in adult males and disrupts adult social discrimination in males.

We have previously shown that a targeted and transient reduction in NCoR expression within the developing amygdala leads to a disruption in juvenile social play behavior and juvenile anxiety-like behavior, but not sociability (Jessen et al., 2010a). The amygdala plays a critical role in regulating juvenile social play behavior (Meaney et al., 1981a), one of the first sexually dimorphic social behaviors to emerge during development. Juvenile males engage in social play behavior at a higher frequency than do juvenile females (Auger and Olesen, 2009). This difference is known to be influenced by neonatal steroid hormone exposure, specifically

estradiol and testosterone exposure (Olesen et al., 2005;Goy and Deputte, 1996;Wallen, 2005). Disruption in NCoR expression was shown to increase juvenile social play behavior in males, but had no effect in females. Anxiety-like behavior was also found to be higher in animals that experienced a decrease in NCoR expression during early development (Jessen et al., 2010a) and we now show that this increase persists into the adult period in males. Interestingly, we also now show that juvenile animals experiencing this disruption show an increase in the latency to enter the dark side of a light/dark box when shavings from adult male rats were placed into the dark side (there was no significant difference in latency to enter the dark side between control and NCoR siRNA animals when the shavings were not present, unreported data). This indicates that not only does NCoR influence the organization of a more generalized anxiety-like behavior, but also a more social component of anxiety-like behavior. Juvenile males rats should be wary of an adult male and disruption of NCoR during development appears to heighten this awareness. The mechanism by which NCoR has these effects are unknown and it is possible that NCoR has these effects through its interaction with nuclear receptors or methyl-binding proteins. Taken together, these data indicate that NCoR has a complex role in the organization of juvenile social behavior.

The amygdala has been found to be critical in regulating anxiety-like behavior (LeDoux, 1998;Graeff et al., 1993) and we have found that NCoR siRNA treatment had a lasting effect of increasing anxiety-like behavior during the juvenile and adult period. This increase in juvenile anxiety-like behavior due to targeted disruption of NCoR occurred independent of sex. Given that NCoR disruption increases juvenile anxiety-like behavior in both males and females, it is important to consider that NCoR can also repress gene expression via its interactions with methyl-binding proteins, such as Kaiso (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006) and MeCP2 (Kokura et al., 2001;Cukier et al., 2008). Therefore, it is possible that

the long term changes in anxiety-like behavior may result from decreased interaction of NCoR with methyl-binding proteins during the neonatal period. It is possible that NCoR disruption may play a role in the development of anxiety disorders in humans. This possible disruption may have been overlooked in the past because it may occur very early in development.

The ability to recognize a familiar conspecific is important across species living in social systems. In rodents, olfactory or pheromonal signals are used to encode social information and this typically involves investigation of the anogenital region. Laboratory tests of social recognition in rats are designed to take advantage of the animals tendency to intensely investigate unknown, or novel, individuals and the amygdala appears to play a role in ability to socially recognize a conspecific (Ferguson et al., 2002). Interestingly, there appears to be a sex differences in rat social discrimination and these differences appear to be due to circulating hormones. Females rats are found to have a lower levels of baseline investigatory behavior, but seem to show social recognition responses for a longer period of time. Castration of male rats leads to male rats performing like females in recognition tests and testosterone replacement restores male-like behavior. These differences appear to be due to the use of slightly different systems between the sexes (Bluthe and Dantzer, 1990;Bluthe et al., 1990;Dantzer, 1998). We have used the social discrimination paradigm to investigate social recognition. In this paradigm social recognition is assessed by comparing the difference in time spent investigating a previously encountered and a novel juvenile male. An animal is considered to have show social recognition if they investigate the novel juvenile male more than the familiar juvenile male. We have shown that animals that experienced a disruption in NCoR expression in the amygdala during development failed to show social recognition, animals that received a control infusion

showed typical social recognition. It is possible that reducing the expression of NCoR during development, allowed for further masculinization of the systems responsible for social recognition and the males receiving this disruption showed an even greater reduction in social recognition.

Many different factors appear to be important for the regulation of social recognition memory, such as vasopressin, oxytocin and dopamine (Ferguson et al., 2002;Keverne and Curley, 2004). The vasopressin system is highly sexually dimorphic in the rat brain and sensitive to steroid hormones (De Vries et al., 1994). Specifically, adult male rats express higher levels of vasopressin as compared to females. Castration has been found to result in reduced vasopressin expression and testosterone replacement restores this expression (Brot et al., 1993;De Vries et al., 1994;Miller et al., 1989). Progesterone appears to be another steroid hormone that regulates the vasopressin system. That is, progestin receptors have been found in nearly every vasopressin cell within certain areas of the brain, including parts of the amygdala (Auger and De Vries, 2002) and progesterone treatment appears to decrease vasopressin within these cells (Auger and Vanzo, 2006). Therefore, progesterone may also play an important role in regulating the vasopressin system and in vasopressin dependent behaviors. Recently, treatment with progesterone was shown to impair social recognition and this effect appears to be due to progestin receptor activation as it is blocked by a progestin receptor antagonist (Bychowski and Auger, 2012). Therefore, it is possible that NCoR influences the organization of social recognition by affecting androgen receptors or progestin receptor regulation of the vasopressin system. These effects are very interesting because while most effects on the social recognition system are activational, these effects are organizational.

In summary, we report that a transient decrease in NCoR expression within the developing amygdala leads to an increase in juvenile social anxiety-like behavior in males, an increase in anxiety-like behavior in adult males and a disruption in social discrimination behavior in adult males. These data indicate that NCoR plays a critical role in the developing amygdala in organizing both social and anxity-like behavior.

Figure Legends

Figure 1

A transient decrease in NCoR expression within the developing amygdala was found to significantly increase social anxiety-like behavior in juvenile males. Animals infused with NCoR siRNA during early development showed an increased latency to enter the dark side of the light/dark chamber as compared to control animals when adult male shaving were present in the dark side. * = $p<0.05$ (control siRNA n=8, NCoR siRNA n=13). Error bars represent SEM.

Figure 2

A transient decrease in NCoR expression within the developing amygdala was found to significantly increase anxiety-like behavior in adult males. A. Animals infused with NCoR siRNA showed an increase in anxiety-like behavior as compared to controls in both the elevated plus maze and B. the light/dark box. * = $p<0.05$ (control siRNA n=8, NCoR siRNA n=13). Error bars represent SEM.

Figure 3

A. A typical pattern of social discrimination was observed with the control males, specifically, control males spent significantly more time investigation the anogenital region of the novel juvenile rat than the familiar juvenile rat. B. Interestingly, males with a decrease in NCoR mRNA expression within the developing amygdala failed to discriminate between the novel and familiar juvenile rat. * = $p<0.05$ (control siRNA n=8, NCoR siRNA n=13). Error bars represent SEM.

Figures

Figure 1

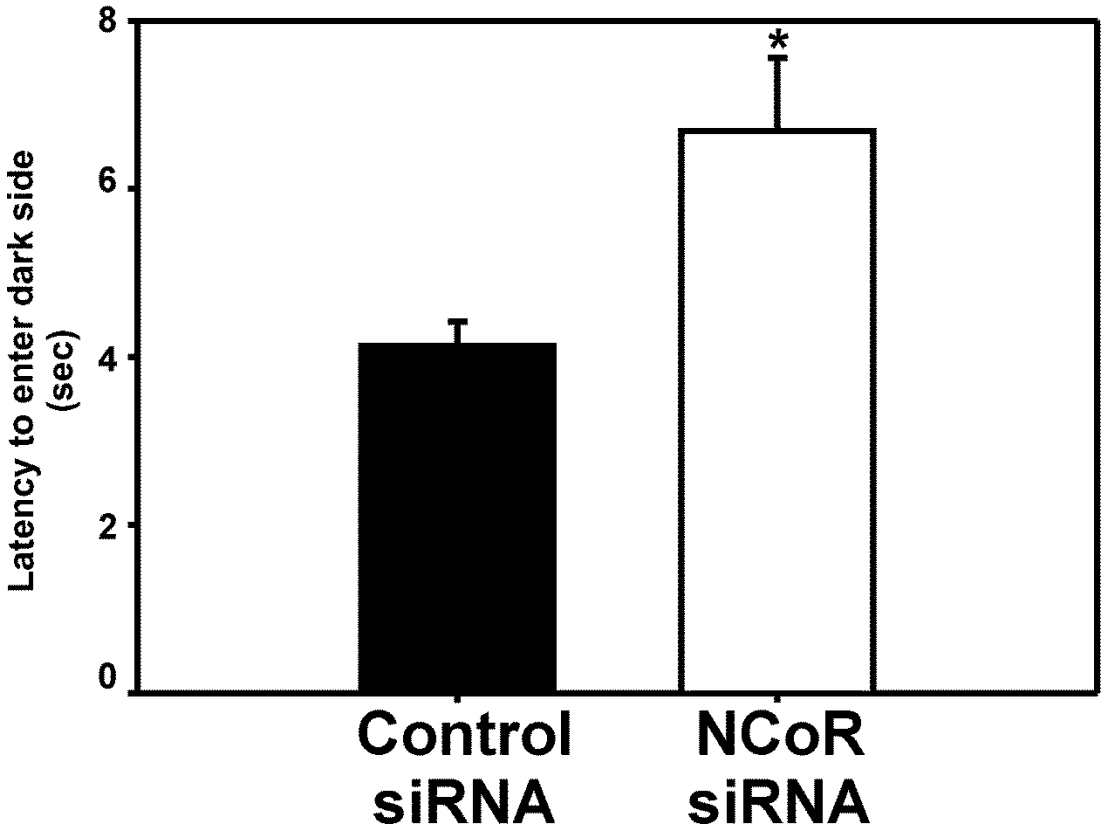
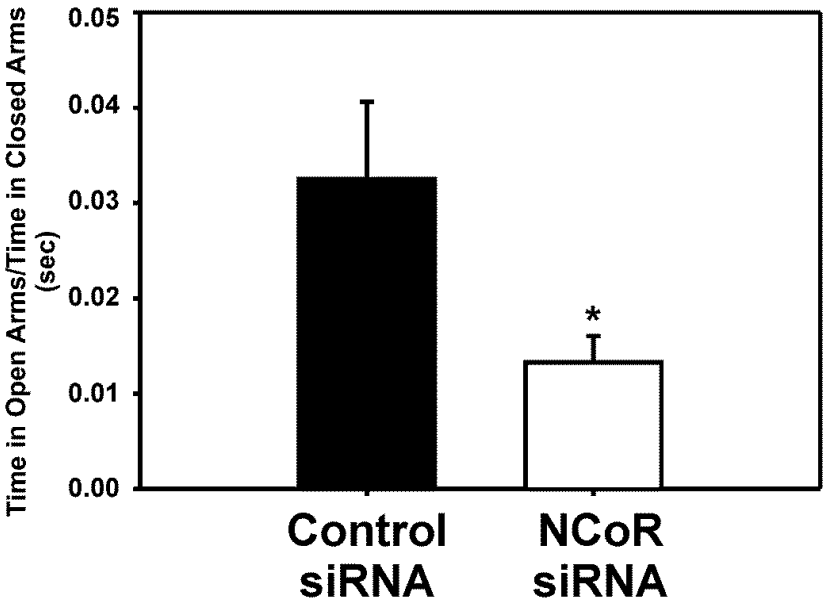


Figure 2

A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0.03
0.02
0.01
0.00
*
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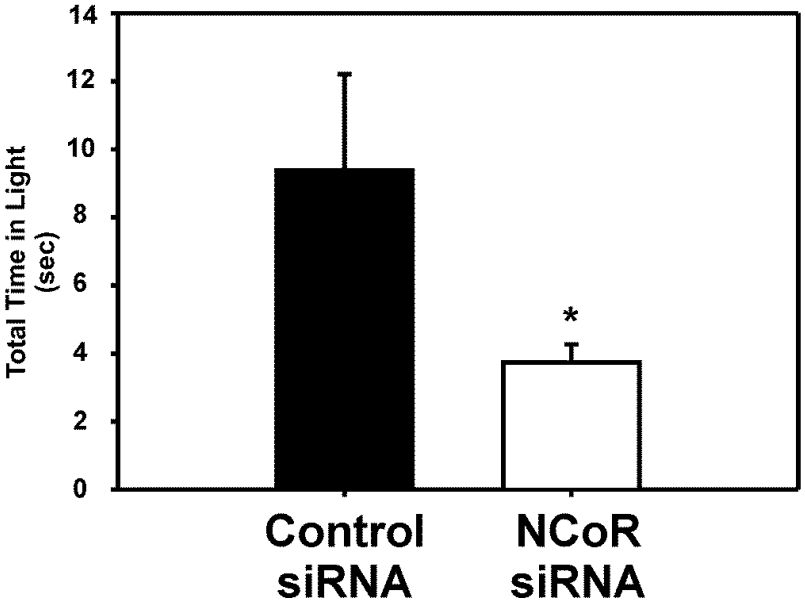
NCoR siRNA

B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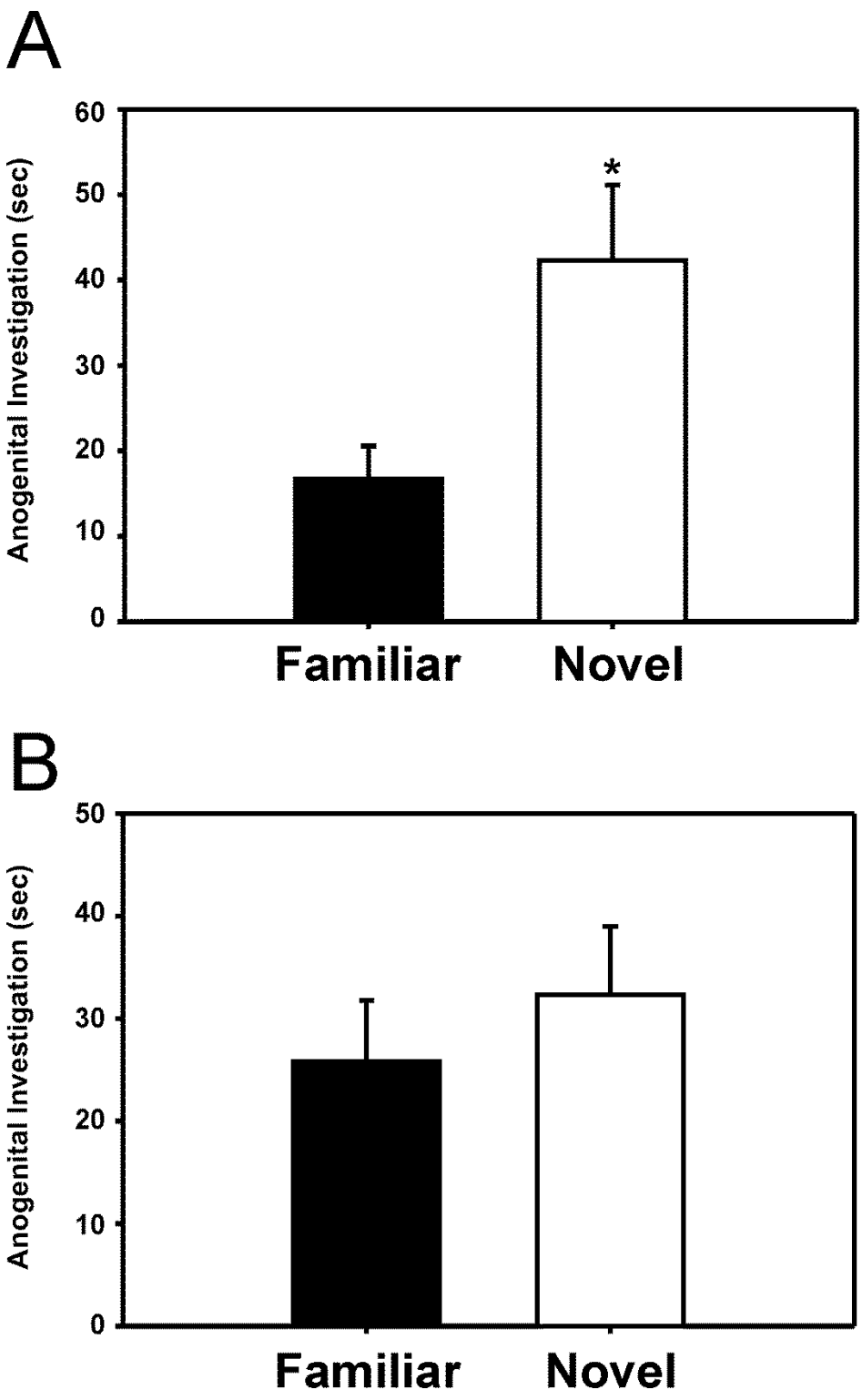
12
10
8
6
4
2
0
*
Control siRNA
NCoR siRNA

Figure 3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Anogenital Investigation (sec)
60
50
40
30
20
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0
*
Familiar
Novel
B
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50
40
30
20
10
0
Familiar
Novel

Chapter 5: Simulated maternal grooming disrupts sexually dimorphic NCoR and Kaiso gene expression within the developing amygdala

Abstract

Coregulatory proteins are known to be important for steroid receptor and non-steroid receptor control of gene expression. We previously found that the expression levels of the corepressor, NCoR, are differentially expressed between males and females during early brain development, and that NCoR is critical in the typical organization of juvenile social play behavior in males, anxiety-like behavior in both sexes and social recognition in adult males. As coregulatory protein levels are important in a variety of amygdala dependent behaviors, we wanted to assess the potential impact of the early environment on conregulatory proetin expression, such as NCoR and proteins that interact with NCoR. To test this, we examined the possibility that subtle variations in maternal grooming could alter expression levels of coregulatory associated proteins within the developing amygdala. Interestingly, the sex difference in NCoR expression was eliminated by simulated maternal grooming. In females, an increase in the somatosensory stimuli associated with maternal grooming during development reduced the levels of NCoR mRNA within the amygdala to male-like levels. Increased somatosensory stimuli did not alter NCoR mRNA expression levels in males. NCoR is known to interact with the methyl-binding protein, Kaiso and has recently been shown to interact with Disrupted in Schizophrenia 1 (DISC1) to regulate gene transcription.. We now report females express higher levels of Kaiso and DISC1 mRNA as compared to males in the amygdala on PN10. We also report that increased somatosensory stimuli associated with maternal grooming during development reduced the levels of Kaiso mRNA within the amygdala to male-like levels, similar to effects on NCoR. In contrast to NCoR and Kaiso, DISC1 expression levels were not altered by increases in the somatosensory stimuli associated with maternal grooming in either sex. Taken together, these data indicate that while NCoR, Kaiso and DISC1 mRNA levels are

sexually dimorphic in the developing brain, only NCoR and Kaiso levels are responsive to increases in the somatosensory stimuli associated with maternal grooming. Therefore, numerous factors such as sex and maternal care may interact to create a diverse epigenetic environment within the developing brain that can have lasting consequences on brain and behavior.

Introduction

An exciting line of research has indicated that variations in the early social environment cause changes in epigenetic factors within the developing brain. For example, it appears that differences in maternal care influences glucocorticoid receptor (GR) and ERα expression by altering corresponding promoter methylation (Weaver et al., 2004;Champagne et al., 2006). As rat mothers typically groom the anogenital region of males more than females (Moore and Morelli, 1979;Moore, 1984), it is possible that variations in maternal care may be contributing to sex differences in gene expression.

Simulated maternal grooming (SMG) has been used to model a component of maternal care, the somatosensory stimulation of the anogenital region. This paradigm is unique in that it can be used to control for other factors of maternal care and to focus on the tactile stimulation of licking and grooming. Recently, ERα expression and DNA promoter methylation patterns were found to be sexually dimorphic (Kurian et al., 2010;Edelmann and Auger, 2011) and interestingly, SMG was found to increase ERα promoter methylation and expression in females

to male-like levels within the developing preoptic area and amygdala (Kurian et al., 2010;Edelmann and Auger, 2011).

Nuclear receptor function within the brain is regulated at the level of DNA through the balanced recruitment of coregulatory complexes. Coactivators are known to cause increases in gene expression; whereas, corepressors, such as Nuclear Receptor Corepressor (NCoR), cause decreases in gene expression. While NCoR has been characterized as a corepressor of nuclear receptors, it has also been shown to participate in DNA methylation-induced gene repression via its direct or indirect interactions with methyl-binding proteins. Understanding the function and potential differential expression of these coregulatory proteins may be critical in understanding how relatively simple environmental stimuli, such as the early social environment, can have complex effects within the developing brain.

We now report that the sex difference in NCoR expression (Chapter 2 data) was eliminated by simulated maternal grooming. In females, an increase in the somatosensory stimuli associated with maternal grooming during development reduced the levels of NCoR mRNA within the amygdala to male-like levels, but did not alter NCoR mRNA expression levels in males. As NCoR is known to interact with the methyl-binding protein, Kaiso (Yoon et al., 2003) and has recently been shown to interact with Disrupted in Schizophrenia 1 (DISC1) to regulate gene transcription (Sawamura et al., 2008), we also investigated the effect of simulated maternal grooming on Kaiso and DISC1 mRNA expression levels. We now report that there is a sex difference in Kaiso and DISC1 mRNA expression on postnatal day 10 (PN10) within the amygdala. In addition, we report that SMG reduced female levels of Kaiso mRNA within the amygdala to male-like levels. In contrast to NCoR and Kaiso, DISC1 expression levels were not

altered by increases in the somatosensory stimuli associated with maternal grooming in either sex. Taken together, these data indicate that while NCoR, Kaiso and DISC1 mRNA levels are sexually dimorphic in the developing brain, only NCoR and Kaiso levels are responsive to increases in the somatosensory stimuli associated with maternal grooming.

Methods

Animals

Adult female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were mated in our animal facility and allowed to deliver normally. Cages were checked regularly to determine the day of birth (postnatal day 0, PN0). Animals were maintained on a 12 hour light/12 dark light cycle and food and water were available ad lib. This research was approved by the University of Wisconsin Animal Care and Use Committee. Animals in this experiment were previously used in the following publication (Edelmann and Auger, 2011).

Simulated Maternal Grooming (SMG) Paradigm

The SMG paradigm was performed as previously described (Edelmann and Auger, 2011). Briefly, pups received simulated maternal grooming (SMG) or control handling three times per day, one in the light cycle and two in the dark cycle, until the day of sacrifice. During treatment, pups were removed from the dam immediately prior to treatment, kept under a warming lamp and on a warm heating pad while away from the dam, and immediately returned to the dam following treatment. SMG consisted of 3 rounds of 10 strokes to the anogenital region with a soft nylon-bristled brush. Control pups were handled in the same manner but did not

receive stimulation from the paintbrush. Each experimental group included animals from at least four litters and animals from each litter were mixed and then distributed equally among groups. Litters were culled to no more than 12 pups. On PN0, one foot from each pup was tattooed with a small amount of India ink for future identification.

SMG Tissue Collection

Animals were sacrificed via rapid decapitation 2 hours after the second round of SMG/control handling on postnatal day 10 (PN10). Group sizes consisted of 8 control females, 9 SMG females, 12 control males, and 11 SMG males. The amygdala was microdissected with razor blades and immediately frozen in isopentane on dry ice. To dissect the amygdala, the whole brain was placed ventral side up on a cold surface. Using a razor blade, two coronal cuts, one caudal to the optic chiasm and one caudal to the hypothalamus, were made. This section of tissue was placed rostral side up and a cut was made along the optic tract followed by another cut at approximately 60 deg to form an approximate triangle. Both sides of the amygdala were collected, pooled, and frozen. The tissue was stored at −80 °C until homogenization.

Quantification of mRNA

Total RNA was isolated from snap-frozen tissue using the AllPrep DNA/RNA Mini Kit (Cat. #80004, Qiagen, Valencia, CA). RNA concentrations were determined using the Qubit Quantification Platform (Cat. #Q32857, Invitrogen, Carlsbad, CA) and cDNA was generated with ImProm-II Reverse Transcription System (Cat. #A3800, Promega) according to manufacturer recommendations in an Eppendorf MasterCycler Personal PCR machine. Samples were stored at −80 °C. Real-time RT-PCR was conducted with a Stratagene Mx3000P™ real-

time PCR system. cDNA was amplified using Sybr® Green I (S7563, Invitrogen), GoTaq Colorless Master Mix (Cat. #M7132, Promega), and ROX (Cat. #12223012, Invitrogen) was used as a passive reference dye to control for baseline fluorescence in each sample. All primers were found to have efficiencies between 90% and 110%. The amplification protocol is as follows: an initial melting step at 95 °C for 2 min followed by 40 cycles of a 95 °C melting step for 30 s, a 60 °C annealing step for 30 s, and a 72 °C elongation step for 30 s. Following amplification, a dissociation curve analysis was used to ensure purity of the products, a single peak was observed for all products. Relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). HPRT was used as a housekeeping gene for all comparisons. The following are the primer sequences: NCoR: Forward AGGTGAGCTGGCAGGACTTA, Reverse AGATAAAGGGCCTCCTCCAA, Kaiso: Forward GGAGCAGGCATGGAGAGTAG, Reverse GGAATTTTCGGTCTTCCACA, DISC1: Forward TCATCGGAGCGGGAAGGGCT, Reverse GCGTGTTTTGTGGGCGGCTTC, HPRT: Forward CCGCTGTCTTTTAGGCTTTG, Reverse CCGCTGTCTTTTAGGCTTTG.

Statistical Analysis

Statistical comparisons were made between the four treatment groups using a two-way analysis of variance. All post hoc tests were conducted using Tukey's *post hoc* comparison tests.

Results

NCoR gene expression

To examine if maternal stimuli alters NCoR mRNA within the developing amygdala, we examined the impact of SMG in both males and females at PN10. We found a significant interaction of sex by treatment ($F(1,33) = 5.28$, $p<0.05$, Figure 1). Post hoc analysis using Tukey's post hoc comparison tests reveled that control females had significantly higher levels of NCoR mRNA than control males ($p<0.05$, Figure 1). Post hoc analysis also showed that SMG females have significantly lower levels of NCoR mRNA than control females ($p<0.05$, Figure 1). SMG did not appear to alter NCoR mRNA in males.

Kaiso gene expression

To examine if maternal stimuli alters Kaiso mRNA within the developing amygdala, we examined the impact of SMG in both males and females at PN10. We found a significant effect of treatment ($F(1,33) = 13.72$, $p<0.001$, Figure 2) and interaction of sex by treatment ($F(1,33) = 4.97$, $p<0.05$, Figure 2). Post hoc analysis using Tukey's post hoc comparison tests reveled that control females had significantly higher levels of Kaiso mRNA than control males ($p<0.05$, Figure 1). Post hoc analysis also showed that SMG females have significantly lower levels of Kaiso mRNA than control females ($p<0.001$, Figure 2). SMG did not appear to alter Kaiso mRNA in males.

DISC1 gene expression

To examine if maternal stimuli alters DISC1 mRNA within the developing amygdala, we examined the impact of SMG in both males and females at PN10. We found a significant effect of sex ($F(1,33) = 10.57$, $p<0.01$, Figure 3), but SMG did not appear to have an effect on DISC1 mRNA levels. Post hoc analysis using Tukey's post hoc comparison tests reveled that control

females had significantly higher levels of DISC1 mRNA than control males ($p<0.05$, Figure 3). Post hoc analysis also showed that SMG females have significantly higher levels of DISC1 mRNA than SMG males ($p<0.05$, Figure 3).

Discussion

As rat mothers typically groom the anogenital region of males more than females (Moore and Morelli, 1979;Moore, 1984), we investigated the effects of increased maternal stimuli on NCoR and various factors with which it is known to associate. We report that variations in the somatosensory stimuli associated with maternal grooming during the neonatal period alter expression levels of NCoR and Kaiso, but do not appear to affect expression levels of DISC1. Specifically, we confirmed our previous findings that females express higher levels of NCoR mRNA as compared to males in the developing amygdala. We also find that increased maternal stimuli decreases NCoR mRNA expression levels in females, but does not appear to affect males. Similarly, we show that females express higher levels of Kaiso mRNA as compared to males in the developing amygdala, and that increased maternal stimuli decreases Kaiso mRNA expression levels in females, but does not appear to affect males. Therefore, it appears that the early maternal environment can modulate the expression levels of important epigenetic factors, effectively eliminating sex differences in these factors. That is, providing females with more typical male-like levels of maternal stimuli resulted in male-like expression of these factors. As the amygdala has been found to be critical in the formation of many behaviors, it is possible that the early maternal environment shapes these behaviors by altering factors that may play a role in their organization. As males receive more maternal grooming compared to females, and providing females with additional simulated maternal grooming produces male typical levels of

coregulatory protein expression, these data suggest that differences in maternal care directed at offspring produce sex differences in coregulatory expression and therefore brain development.

As coregulatory protein expression is critical for steroid receptor action, the differential expression of these coregulatory proteins is likely to alter neuronal responses to circulating steroid hormones. The differential exposure to circulating steroid hormones and the action of these hormones on nuclear receptors have been shown to result in many of the lasting structural and behavioral differences between males and females (Blaustein and Olster, 1989;Cooke et al., 1998). During development, male rodents are exposed to higher levels of testosterone (Weisz and Ward, 1980) as compared to females, this variation in exposure leads to differences in neurogenesis, cell death, and cell migration within the brain (Cooke et al., 1998), and ultimately lasting sex differences in behavior. Nuclear receptor function within the brain is regulated at the level of DNA through the balanced recruitment of coregulatory complexes. Coactivators are known to cause increases in gene expression; whereas, corepressors, such as Nuclear Receptor Corepressor (NCoR), cause decreases in gene expression. While NCoR has been characterized as a corepressor of nuclear receptors (Horlein et al., 1995), it has also been shown to participate in DNA methylation-induced gene repression via its direct or indirect interactions with methyl-binding proteins, such as Kaiso (Yoon et al., 2003;Bird and Wolffe, 1999;Klose and Bird, 2006). Understanding the function and potential differential expression of these coregulatory proteins may be critical in understanding how relatively simple stimuli can have complex effects within the developing brain. NCoR has recently been shown to be sexually dimorphic, that is females have been shown to express higher levels of NCoR mRNA as compared to males, and critical in the organization of typical juvenile social play behavior, anxiety-like behavior and to impair

social recognition in adult males (Chapter 2, Jessen et al., 2010, Chapter 4). We now confirm that NCoR mRNA expression levels are higher in females than in males and show that females also express significantly higher levels of Kaiso mRNA. We also report that the somatosensory stimuli associated with maternal grooming decreases NCoR and Kaiso mRNA expression levels to male-like levels. It is possible the typical maternal grooming levels influence levels of these epigenetic factors and consequently influence the behaviors that NCoR has been found to be critical in organizing, such as juvenile social play behavior, anxiety-like behavior and social recognition. Specifically, we suggest that the mothers' differential behavior directed at male versus female offspring changes the levels of coregulatory proteins and thereby likely shapes the way neurons respond to fluctuations in circulating steroid hormones.

Recently, NCoR has been shown to interact with Disrupted in Schizophrenia 1 (DISC1) to modulate CRE-mediated gene transcription (Sawamura et al., 2008). DISC1, which was originally discovered in a Scottish pedigree with increased prevalence of schizophrenia and other mental illnesses, is thought to confer risk for many mental illnesses including schizophrenia-spectrum disorders, bipolar disorder, major depressive disorder, and autism-spectrum disorders (St et al., 1990;Millar et al., 2000). DISC1 is widely expressed throughout human, primate, and rodent brains, but is primarily seen in the hippocampus, amygdala, hypothalamus, cortex, and cerebellum (Austin et al., 2003). DISC1 appears to modulate the different stages of neurodevelopment and ensures proper progenitor cell growth, differentiation, and migration to the developing brain (Sawamura et al., 2008;Perkins et al., 2005). As DISC1 was reported to be associated with NCoR, we decided to examine if maternal stimuli also alters the expression of this important molecule. Interestingly, we show that female rats express higher levels of DISC1

mRNA during development. This sex difference in DISC1 mRNA expression during early development may partially explain the sex differences in the onset and severity of schizophrenia in humans, as males both have an earlier onset and show an increase in severity in the disorder (Hafner et al., 1998). In addition, we show that DISC1 mRNA expression levels do not appear to be altered by differences in the early maternal environment. While we have shown that variations in the early social environment may impact sex differences in the developing brain and thereby may influence the development of some disorders, DISC1 expression levels were not disrupted by changes in the somatosensory stimuli associated with maternal grooming. However, considering that the disorders associated with disrupted DISC1 function are extremely severe, it is not unexpected that subtle variations in maternal care do not alter expression levels.

In summary, these data indicate that variations in the early maternal environment may be an additional mechanism by which sexual differentiation of the amygdala is regulated. Specifically, we have shown that increases in the somatosensory stimuli associated with maternal grooming alters expression levels of the epigenetic factors NCoR and Kaiso, effectively eliminating the sex differences in these factors. Therefore, it is possible that the early maternal environment may influence the development of both typical and atypical behavior by altering factors that may play a role in the organization of these behaviors. These data also support the idea that differences in early social interactions contribute to sex differences in the brain.

Figure Legends

Figure 1

Sex differences and SMG regulation of NCoR expression within PN10 neonatal brain. Females express significantly higher levels of NCoR mRNA within the developing amygdala. SMG significantly decreased NCoR mRNA expression levels in females, but not males. * = $p<0.05$. Error bars represent SEM.

Figure 2

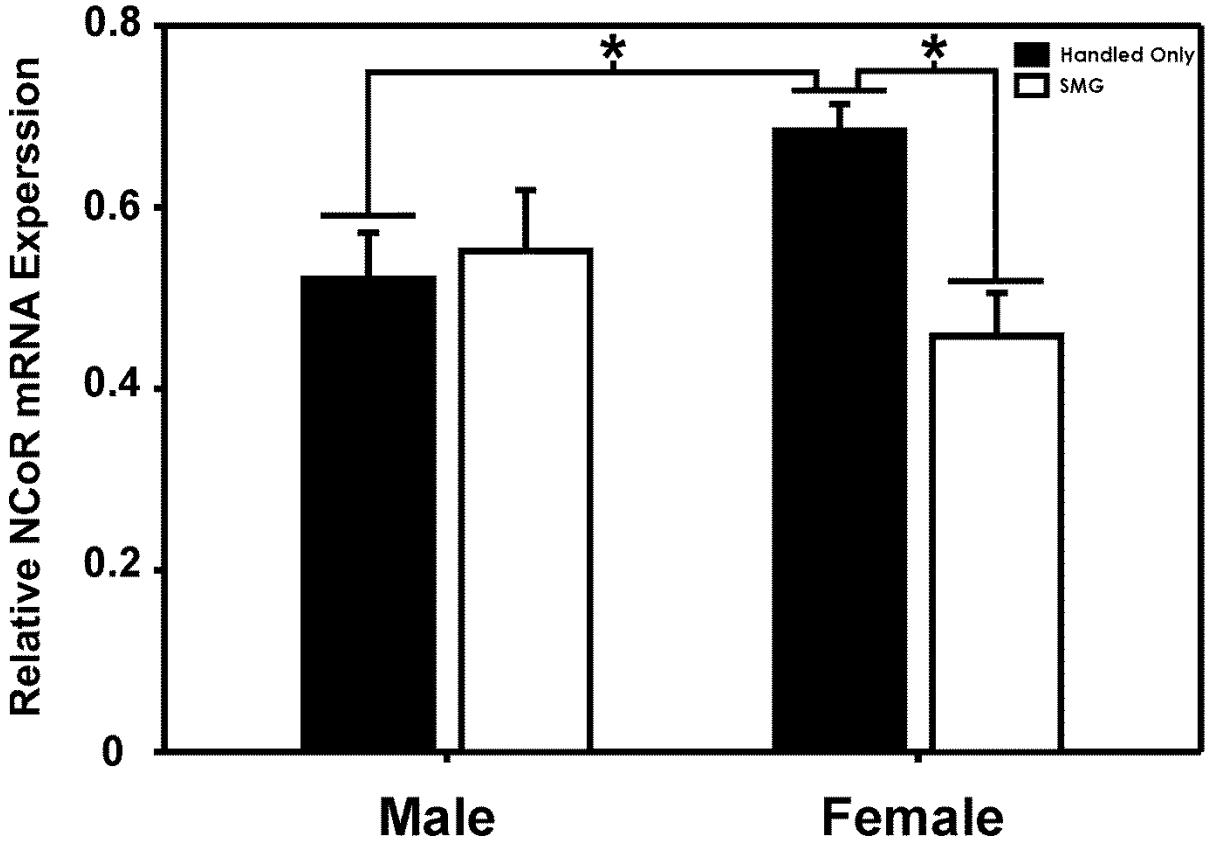
Sex differences and SMG regulation of Kaiso expression within PN10 neonatal brain. Females express significantly higher levels of Kaiso mRNA within the developing amygdala. SMG significantly decreased Kaiso mRNA expression levels in females, but not males. * = $p<0.05$. Error bars represent SEM.

Figure 3

Sex differences and SMG regulation of DISC1 expression within PN10 neonatal brain. Females express significantly higher levels of DISC1 mRNA within the developing amygdala. SMG did not significantly affect DISC1 mRNA expression levels in males or females. * = $p<0.05$. Error bars represent SEM.

Figures

Figure 1



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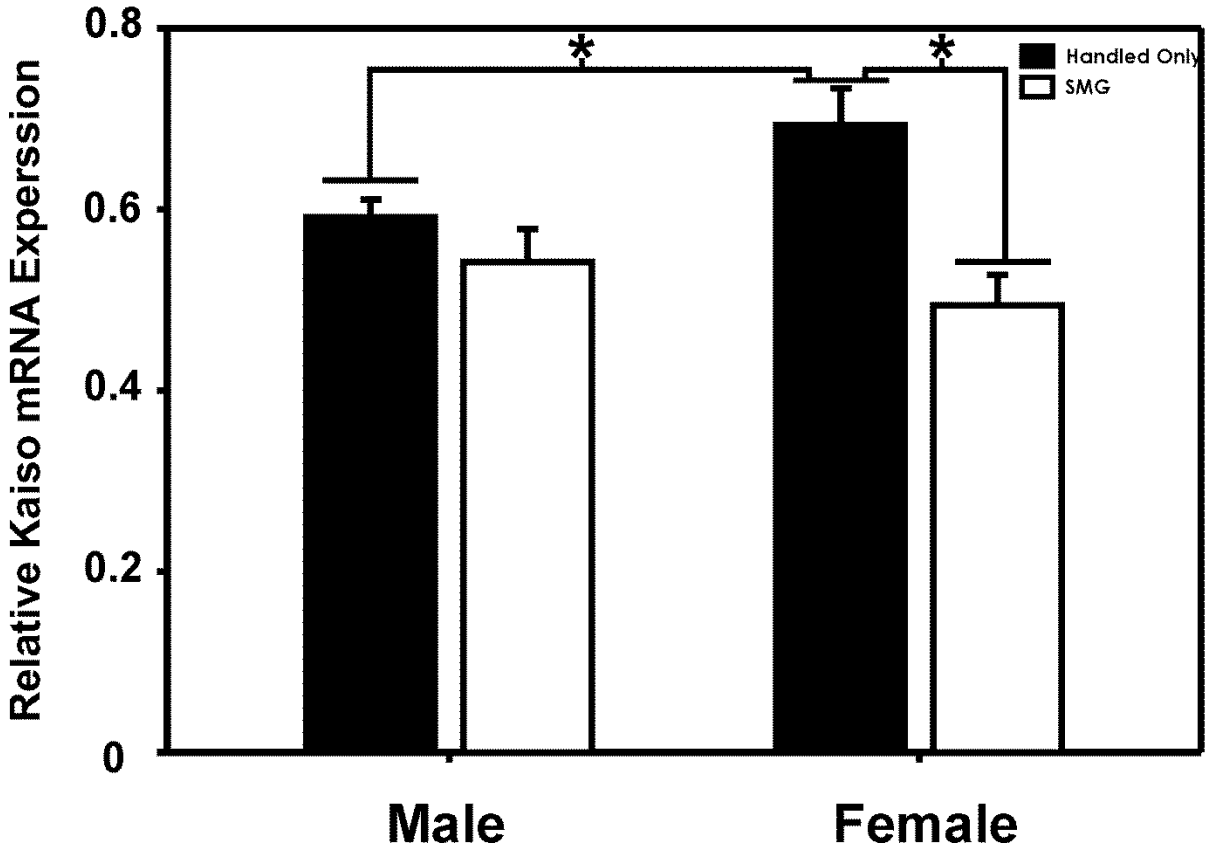
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0.4
0.2
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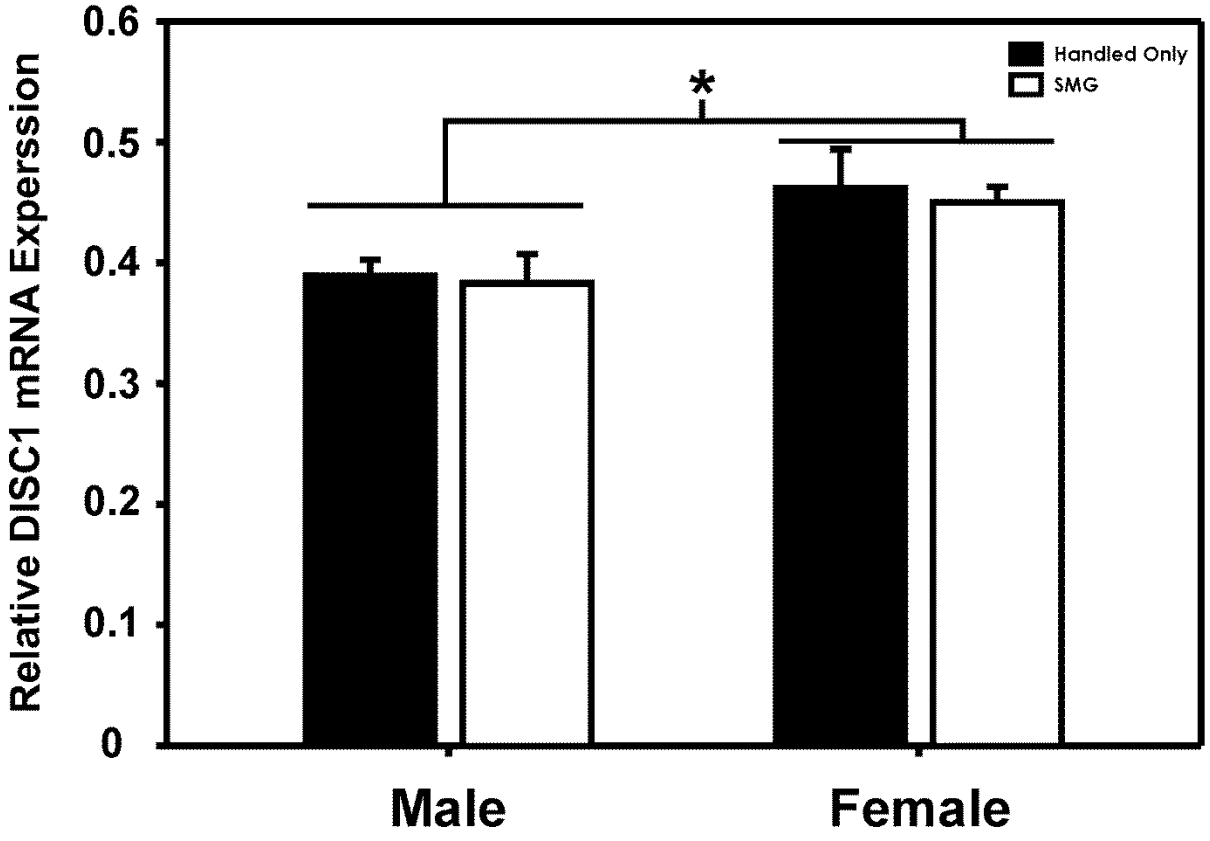
Relative Kaiso mRNA Experssion
*
*
Handled Only
SMG
Male
Female

Figure 3


Relative DISC1 mRNA Expression
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0.5
0.4
0.3
0.2
0.1
0
*
Handled Only
SMG
Male
Female

Chapter 6: Discussion

Discussion

Findings

The data presented in the preceding chapters examined the role of particular epigenetic factors during early postnatal brain development and their lasting impact on programming juvenile and adult behavior. Specifically, we examined sex differences in coactivators and corepressors in the developing amygdala, the organizational role of NCoR within the developing brain, and environmental influences on expression levels of NCoR and epigenetic factors with which it has been found to be associated. NCoR has been implicated in various neurological disorders and this research was conducted with the hope that it might contribute to a better understanding of both typical and atypical development. Importantly, we provide evidence that a subtle, brief, disruption in the expression of NCoR within the developing amygdala can lead to long term increase in anxiety-like behavior throughout an individual lifespan even after NCoR levels are return to normal levels.

In chapter 2, we report that in the amygdala on postnatal day 1 (PN1) males express higher levels of SRC-1 and CBP mRNA, but that this differences is no longer present by postnatal day 10 (PN10). In contrast, females express a greater relative amount of NCoR mRNA as compared to males on PN1, interestingly this difference is still present on PN10. The specific function of the differential expression of these coregulatory proteins within the developing amygdala is yet to be elucidated; however, our data indicates that the differential expression might serve to modify steroid-mediated sex differences in the brain.

In chapter 3, we used real-time PCR to show that NCoR mRNA levels are sexually dimorphic, females express higher levels of NCoR mRNA within the developing amygdala and hypothalamus, and that NCoR mRNA levels are reduced by estradiol treatment. To investigate

the functional role of NCoR on juvenile social behavior, we infused siRNA targeted against NCoR within the developing rat amygdala and assessed the enduring impact on juvenile social play behavior, sociability, and anxiety-like behavior. As expected, control males exhibited higher levels of juvenile social play than control females. Reducing NCoR expression during development further increased juvenile play in males only. Interestingly, decreased NCoR expression within the developing amygdala had lasting effects on increasing juvenile anxiety-like behavior in males and females. These data suggest that the corepressor, NCoR, functions to blunt sex differences in juvenile play behavior, a sexually dimorphic and hormone-dependent behavior, and appears critical for appropriate anxiety-like behavior in juvenile males and females.

In chapter 4, we show that the effects of decreased NCoR expression within the developing amygdala on anxiety-like behavior persist until adulthood in males. To further examine the functional role of NCoR on social behavior, we examined the effects of decreased NCoR expression within the developing amygdala on juvenile social anxiety-like behavior and adult social discrimination behavior. We now show that decreased NCoR expression during amygdala development results in increased juvenile social anxiety-like behavior and disrupts social discrimination behavior in adults. These data suggest that NCoR is critical for the development of appropriate anxiety-like and social behavior that is controlled by the amygdala. As NCoR also participates in DNA methylation-induced gene repression via its direct or indirect interactions with methyl-binding proteins, it is possible that these effects were due to an abnormal interaction of NCoR with nuclear receptors or with methyl-binding proteins.

In chapter 5, we examined the potential impact of the early environment on NCoR expression using a simulated maternal grooming paradigm. Interestingly, the sex difference in NCoR expression was eliminated by simulated maternal grooming. In females, an increase in the somatosensory stimuli associated with maternal grooming during development reduced the levels of NCoR mRNA within the amygdala to male-like levels. Increased somatosensory stimuli did not alter NCoR mRNA expression levels in males. NCoR is known to interact with the methyl-binding protein, Kaiso and has recently been shown to interact with Disrupted in Schizophrenia 1 (DISC1) to regulate gene transcription. We now report that there is a sex difference in Kaiso and DISC1 mRNA expression on postnatal day 10 within the amygdala. Similar to the sex difference in NCoR mRNA expression levels, females express higher levels of Kaiso and DISC1 mRNA as compared to males. We also report that increased somatosensory stimuli associated with maternal grooming during development reduced the levels of Kaiso mRNA within the amygdala to male-like levels, similar to effects on NCoR. In contrast to NCoR and Kaiso, DISC1 expression levels were not altered by increases in the somatosensory stimuli associated with maternal grooming in either sex. Taken together, these data indicate that while NCoR, Kaiso and DISC1 mRNA levels are sexually dimorphic in the developing brain, only NCoR and Kaiso levels are responsive to increases in the somatosensory stimuli associated with maternal grooming. Therefore, numerous factors such as sex and maternal care may interact to create a diverse epigenetic environment within the developing brain that can have lasting consequences on brain and behavior.

Taken together, these data suggest a dynamic model of chromatin remodeling which may drive the organization of the developing rat brain.

Summary

Sexual differentiation of the neonatal rat brain is regulated by dynamic processes occurring at the level of DNA, resulting in sexually dimorphic gene expression. Steroid hormone receptors act partly in the developing brain by recruiting coregulatory proteins and other transcription factors that lead to altered gene expression. Recent data indicate that epigenetic processes play an important role in sexual differentiation of the brain. The dynamic interaction between epigenetic factors and chromatin leads to functional plasticity within the brain that may explain the diversity of responses to endogenous and exogenous signals. Subtle variations in these epigenetic factors may also partially explain individual differences in the development of neurological and mental health disorders.

The amygdala is known to be important for modulating juvenile social play (Meaney et al., 1981b), aggression (Pinel et al., 1977) and anxiety (Hitchcock and Davis, 1986;Shibata et al., 1986). Therefore, programming or possibly reprogramming of gene expression within the amygdala is likely to impact numerous socio-emotional functions. We now show that both coactivators and corepressors are sexually dimorphic in the developing amygdala. Specifically, males express higher levels of SRC-1 and CBP than do males and females express higher levels of NCoR mRNA than do males. These differences may be partly due to differences in hormone exposure as treatment with estradiol benzoate decreased the expression of NCoR in females to male like levels (Jessen et al., 2010b). This is interesting as differences in hormones levels due to endogenous or exogenous sources, such as early-life adversity, may alter NCoR expression levels within the developing amygdala and thereby change the cell or tissue's response to further cues.

NCoR was one of the first corepressors to be identified through its interaction with thyroid hormone receptors (Horlein et al., 1995), however, it was also reported to interact with androgen receptors (Cheng et al., 2002;Yoon and Wong, 2006), estrogen receptors (Lavinsky et al., 1998), and progestin receptors (Liu et al., 2002). NCoR has also been shown recently to interact directly, as well as indirectly, with methyl-binding proteins (Yoon et al., 2003;Cukier et al., 2008;Kokura et al., 2001;Alland et al., 1997). In particular, NCoR is reported to interact directly with Kaiso and MeCP2 (Kokura et al., 2001;Yoon et al., 2003;Cukier et al., 2008) and may interact indirectly with methyl-binding proteins through its association with the Sin3 corepressor complex (Alland et al., 1997). Methyl-CpG binding proteins bind to methylated DNA and can initiate the recruitment of nuclear corepressors and histone deacetylase to form repressor complexes. These repressor complexes can inhibit gene expression by removing acetyl groups from histones leading to condensation of the chromatin and gene repression (Klose and Bird, 2006). Corepressor complexes recruited by methyl-binding proteins include multi-protein Sin3, NuRD, CoREST, and the NCoR/SMRT repressor complexes (Cunliffe, 2008). It is likely that different combinations of these corepressor complexes allows for increased diversification of function and thereby phenotypical variations.

While the functional role of corepressors in the developing brain is still being elucidated, we now show that a transient, targeted disruption of NCoR within the developing amygdala has lasting consequences on juvenile social play, anxiety-like behaviors and social recognition. Interestingly, our lab has also found that transiently decreasing the expression MeCP2 within the amygdala during the neonatal period results in altered social behavior later in life (Kurian et al., 2008). However, transient reductions in MeCP2 within the developing amygdala decreased juvenile social play in males to female levels, while transient reductions in NCoR hyper-

masculinized juvenile social play in males. These data suggest that different epigenetic factors or their combinations modify divergent phenotypes. While the alterations in juvenile social play occurred only in males, the transient reduction of NCoR had lasting consequences on increasing anxiety-like behavior in both males and females. These data indicate that transient disruptions in corepressors complexes within the developing brain can have sexually dimorphic consequences on juvenile social behavior and have alterations in anxiety-like behavior in both sexes. It is important to consider that sex differences in epigenetic processes may not always induce sex difference, but may be there to reduce sex differences. This is best illustrated with the NCoR data in which lowering NCoR expression levels within the developing amygdala further enhanced male-typical juvenile play behavior in males, but to atypical levels (Jessen et al., 2010b). Therefore, NCoR may play a role in reducing sex differences in areas of the brain were sex differences could be detrimental.

An exciting line of research has shown that variations in the early social environment cause changes in epigenetic factors within the developing brain. For example, it appears that differences in maternal care influences glucocorticoid receptor (GR) and ERα expression by altering corresponding promoter methylation (Weaver et al., 2004;Champagne et al., 2006). As rat mothers typically groom the anogenital region of males more than females (Moore and Morelli, 1979;Moore, 1984), it is possible that variations in maternal care may be contributing to sex differences in gene expression. Our lab has recently reported that differences in maternal grooming directed at males versus females produces sex differences in DNA promoter patterns. While differences in DNA methylation interfere with transcription factor binding to the genome, DNA methylation serves more efficiently to enhance binding of proteins that decrease gene expression. Therefore, we investigated if maternal stimuli also altered the expression of proteins

that are recruited to methylated DNA. We report that variations in the somatosensory stimuli associated with maternal grooming during the neonatal period alter expression levels of NCoR and Kaiso, but do not appear to affect expression levels of DISC1. It appears that the early maternal environment can modulate expression levels of important epigenetic factors, effectively eliminating sex differences in these factors. That is, providing females with more typical male-like levels of maternal stimuli resulted in male-like expression of these factors. As the amygdala is a critical site for socioemotional behavior, these data suggest that differences in mother-infant interactions may modify social or emotional processes by altering epigenetic factors.

Directions for Future Research

These data begin to address many questions and at the same time suggest an abundance of new questions.

What genes does NCoR disruption affect?

The findings in chapters 3 and 4 indicate that a targeted and transient disruption in NCoR in the developing amygdala results in a variety of behavioral outcomes, such as disruptions in juvenile social play behavior, anxiety-like behavior and social discrimination. It would be interesting to examine the genes that this disruption affects both immediately after the infusion and later during the juvenile and adult periods. It would be important to look at the methylation status of these genes, mRNA expression as well as protein expression to truly understand the effects. This would allow us to better understand the mechanisms by which NCoR disruption leads to these differences in behaviors.

What are the consequences of over-exposure to steroid hormones during early development?

Most studies investigating the impact of steroid hormones during early development focus on castration and increased hormones in females. In chapter 3, we show that disruptions in NCoR during early development leads to many different behavioral outcomes. As NCoR is thought to function as a corepressor of nuclear receptors, this disruption would possibly lead to an increase in nuclear receptor action. It would be interesting to examine the effects of increases in steroid hormones (above typical male levels), such as testosterone and estradiol during this period on some of these same behaviors.

What is the function of SMRT in the developing amygdala?

The findings in chapter 2 indicate that SMRT mRNA expression levels are not sexually dimorphic, however, it would still be interesting to investigate the function of SMRT in the developing amygdala. SMRT may be involved in the organization of some of the same behaviors with which NCoR appears to be involved or if SMRT has a completely different function.

Are NCoR expression levels disrupted by other environmental stimuli?

The findings in chapter 5 indicate that NCoR and Kaiso levels are disrupted by changes in the early maternal environment. It would be very interesting to investigate if other external stimuli affect the expression levels of NCoR and other epigenetic factors. The early neurodevelopment period has been implicated as a critical period for the development of many neurodevelopmental disorders (e.g. schizophrenia). It would be important to investigate if

different maladapative stimuli would result in differences in these epigenetic factors during this period.

Conclusions

Understanding epigenetic mechanisms, which underlie sexually dimorphic gene expression and function, is critical for further understanding both typical and atypical neurodevelopment. Furthermore, sexual differentiation of the brain is a powerful model to study epigenetic mechanisms, as brief exposure to steroid hormones can reorganize the brain and have lasting consequences on behavior. Perhaps more important, studying the male versus female brain provides a natural model of risk or resilience for some mental health disorders. For example, autism spectrum disorders, Rett syndrome, attention deficit hyperactivity disorder and schizophrenia, have sex differences in prevalence, time of onset and/or severity. As these disorders are believed to have an epigenetic component, sex differences in epigenetic processes may alter ones risk or resilience to develop a particular disorder. Therefore, it will be very interesting to further examine sex differences in epigenetic mechanisms and how these differences contribute to typical differentiation of the brain and social behavior, but also how they may confer sexually dimorphic risk or resilience for developing neurological and mental health disorders. That is, these differences may not produce a disorder, but may alter the way neurons respond to later insults that are associated with behavioral disorders. It will also be important to further determine how exogenous or endogenous cues shape sex differences during development, as well as typical and atypical brain function and behavior.

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