

**THE EFFECT OF PLAYFUL EXPERIENCES ON THE PLASTICITY AND  
METAPLASTICITY OF THE BRAIN**

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## ABSTRACT

The influence of play behavior on the brain was investigated through plasticity and metaplasticity methodology. Regions in both cortical and sub-cortical areas were investigated. Animals in both studies either experienced play with juvenile partners or did not experience play by being paired with an adult. Play experience alone was shown to affect the plasticity in the prefrontal cortex, although it did not show structural changes to sub-cortical regions. If animals were given nicotine after play experiences, the affects of play in the prefrontal cortex were abolished. In addition, playful behaviors appear to prime some sub-cortical regions of the brain for expression of later plasticity. Thus, play appears to alter the structure of multiple brain areas, but do so in different ways.

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## LIST OF ABBREVIATIONS

AID	dorsal agranular insular cortex
BDNF	brain-derived neurotrophic factor
BLA	basolateral nuclei
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CeA	central nucleus
Cg3	cingulate area 3
DRN	dorsal raphe nucleus
IC	intercalated nucleus
IL	infralimbic cortex
mPFC	medial prefrontal cortex
NAcc	nucleus accumbens
OFC	orbital frontal cortex
PAG	periaqueductal gray
PFC	prefrontal cortex
PL	prelimbic cortex
Pnd	postnatal day
VTA	ventral tegmental area

## Chapter 1

### 1.1. General Introduction

For most animals, social interaction is a necessary tool for proper development, with the role of play being the most prominent during the juvenile period. While studies on human play exist, the majority of our understanding of play is derived from animal studies. A plethora of animals have been studied with regard to social play (horses, bears, birds, monkeys, mongooses, etc), but rats have long served as the best model in understanding behavior and its relationship to the brain (Whishaw & Kolb, 2005). Rough and tumble play is the most common type of play in rats (Bolles & Woods, 1964; Poole & Fish, 1975), constituting 4% of the daily time budget. In fact, rats spend approximately 2-3% of their daily energy playing and also eat 7% more than animals that do not play regularly (Siviy & Atrens, 1992). Given the costs incurred by play, it seems logical that there must be compensatory benefits that offset those costs.

Play has not only been shown to be a pleasurable experience, with rats emitting 'happy' vocalizations in anticipation of (Knutson, Burgdorf, & Panksepp, 1998) and during (Burgdorf, Panksepp, Beinfeld, Kroes, & Moskal, 2006) playful bouts, but also serves as a rewarding behavior (Calcagnetti & Schechter, 1992; Humphreys & Einon, 1981; Normansell & Panksepp, 1990). In addition to being a rewarding behavior, play also has a direct effect on the brain. Playful experiences have been shown to release growth factors (e.g., BDNF) (Gordon, Burke, Akil, Watson, & Panksepp, 2003) and affect endogenous opioids (Panksepp, Jalowiec, DeEskinazi, & Bishop, 1985; Vanderschuren, Stein, Wiegant, & Van Ree, 1995a, 1995b) in the brain, as well as affect

*neural plasticity* in specific prefrontal cortex regions (Bell, Pellis, & Kolb, 2010; Markham, Morris, & Juraska, 2007).

The term neural plasticity is described as the ability of neurons to alter both shape and function based upon experiences. This idea of experience-based changes to brain structures was first brought about in the 1890's (James, 1890; Tanzi, 1893) and was reintroduced years later (Hebb, 1947; Konorski, 1948; Ramon y Cajal, 1928). Neural plasticity takes place at the synapse, which is the point of communication between different cells. Plasticity can be studied at the anatomical level via changes in length or amount of branching of dendrites as well as the number of spines on the dendrites. Possibly the best-known study exemplifying plasticity was performed by Hubel and Weisel (1970), in which the cortical mapping of sight was investigated. One eye of kittens was sown shut and it was hypothesized that the hemisphere which controlled that eye would show no activity, but surprisingly, it was shown that the hemisphere was actually processing information from the open eye. The brain had reorganized itself to change the function of the tissue. As this example exemplifies, the brain has the ability to not only change aspects of cellular anatomy, but also the function of brain tissue. It is now known that neural plasticity can be affected by a variety of environmental experiences (Kolb, Forgie, Gibb, Gorny, & Rowntree, 1998) including exposure to psychoactive drugs (Robinson & Kolb, 2004).

Recent work has investigated how a specific environmental experience, play behavior, may also affect plasticity. As previously mentioned, the experience of play has a role in shaping the prefrontal cortex, specifically in the orbital frontal cortex (OFC) and the medial prefrontal cortex (mPFC), although these areas are affected by different

aspects of the experiences arising from play. While the OFC is sensitive to the amount of partners, the mPFC is affected by the actual experience of play (Bell, et al., 2010). What remains unknown is whether these play-induced changes extend beyond the prefrontal cortex (PFC) and how these play-induced changes interact with other plasticity-inducing experiences.

(1) Since the prefrontal cortex has shown altered neuronal morphology via playful experiences, we are interested in what happens to sub-cortical areas that are strongly connected to the prefrontal cortex. For this study, sub-cortical regions of the fear system will be investigated. If rats are socially isolated, and therefore not able to play during their juvenile period, they show an inability to regulate their emotional responses to fearful or stressful situations (Arakawa, 2003; Da Silva, Ferreira, Carobrez Ade, & Morato, 1996; Lukkes, Mokin, Scholl, & Forster, 2009; Von Frijtag, Schot, van den Bos, & Spruijt, 2002). Anatomically, there are strong connections between the PFC and primary brain regions in the fear system; the amygdala and hippocampus. Therefore, the first study will focus on determining if the plastic changes seen in the PFC are associated with plastic changes in these sub-cortical areas.

(2) There are multiple experiences that induce plasticity on neural systems during a lifetime and these experiences may occur simultaneously or in sequence. Thus, changes induced by one set of experiences can modulate or attenuate those of other experience – these interactions among plasticity inducing experiences is known as metaplasticity. A PFC modified by play experience in the juvenile period is likely to encounter plasticity inducing experienced later in life, so the question is how to these plastic changes interact. The term metaplasticity was first coined by Abraham and Bear (1996), and is described

as the “plasticity of synaptic plasticity”. Although first described at the synaptic level, such plasticity can also involve larger anatomical units, such as the length and branching of the dendrites (Kolb, Gibb, & Gorny, 2003; Muhammad & Kolb, 2011). A general model of metaplasticity consists of combining the effects of exposure to one experience at one stage of life with exposure to another experience later in life, so as to study the effect of the early experience on later brain plasticity. Therefore, the second study will examine play-induced plasticity influences the plasticity in response to another experience known to affect neural plasticity (exposure to nicotine) later in life.

### **1.1.1 The Anatomy of brain areas studied in this thesis**

#### **1.1.1.1 The Fear System**

Along with the prefrontal cortex, the hippocampus and amygdala are major structures within the neural circuitry that regulates fear. These structures compose the “higher” functioning component of the fear system, with the “lower” areas, including the hypothalamus and periaqueductal gray (PAG). The fear system is organized hierarchically, with the cortex not necessarily needed for a general expression of fear (Panksepp, Normansell, Cox, & Siviy, 1994; Shewmon, Holmes, & Byrne, 1999), but, rather, serving a regulatory function (Ochsner & Gross, 2005; Sullivan & Gratton, 2002).

#### *1.1.1.2. Prefrontal cortex*

The prefrontal cortex (PFC) is the most anterior part of the frontal lobe and, in rats, is divided into two separate parts; the orbital frontal cortex and the medial prefrontal cortex (Leonard, 1972). These two areas are not only connected to each other, but also to other areas in the “lower” functioning areas of the fear system. Both the OFC and mPFC have bidirectional connections with the amygdala (for a review, Cardinal, Parkinson,

Hall, & Everitt, 2002) and indirect connections to the hippocampus (e.g., Vertes, Hoover, Szigeti-Buck, & Leranth, 2007). The PFC also has connections with brainstem projections, for example, there are strong connections to the PAG (Holstege, Bandler, & Saper, 1996).

#### *1.1.1.3. Hippocampus*

The PFC has connections with the hippocampus, with the majority of these involving either the subiculum or *Cornu Ammonis 1* (CA1). For the purpose of this thesis, I will focus on the CA1 region. Area CA1 is the most dorsal part of the hippocampus and has strong, excitatory connections to both the mPFC and the OFC (Jay & Witter, 1991), although no direct connections exist from the PFC (Sesack, Deutch, Roth, & Bunney, 1989). In addition, CA1 also projects to the amygdala, specifically the BLA region (Canteras & Swanson, 1992). The hippocampus also has neurons which simultaneously project to both the PFC and the amygdala (Ishikawa & Nakamura, 2006).

#### *1.1.1.4. Amygdala*

The amygdala is known to be the epicenter of emotion and is comprised of 13 nuclei in the rat brain. The specific nuclei of focus, is the basolateral nuclei (BLA). The basolateral nuclei are comprised of the lateral, basal and accessory basal nuclei, and together they house roughly 137,000 cells (Tuunanen & Pitkanen, 2000). Of these cells, there are two types found in the BLA; pyramidal-like cells (Class I) and nonspiny stellate-like cells (Class II) (reviewed in Sah, Faber, Lopez De Armentia, & Power, 2003), with our focus being on Class II cells. The BLA has many efferent and afferent connections to other brain regions. Inputs to the BLA nuclei include, sensory cortices and prefrontal cortex, and outputs include the medial temporal lobe, nucleus accumbens,

prefrontal cortex and hippocampus (reviewed in Sah, et al., 2003). Within the amygdala, a majority of the BLA connections go to the excitatory central nuclei (CeA), although there are also connections to the inhibitory intercalated nuclei (IC). Inputs received from the PFC are excitatory (glutamatergic) (Brinley-Reed, Mascagni, & McDonald, 1995) and have connections to two nuclei in the amygdala; the BLA and the IC. Information from the BLA is sent to the CeA (excitatory), which is then relayed to corresponding areas. In contrast, information sent directly to the IC is relayed to the CeA, but since these projections travel through the IC, the projection to the CeA is inhibitory.

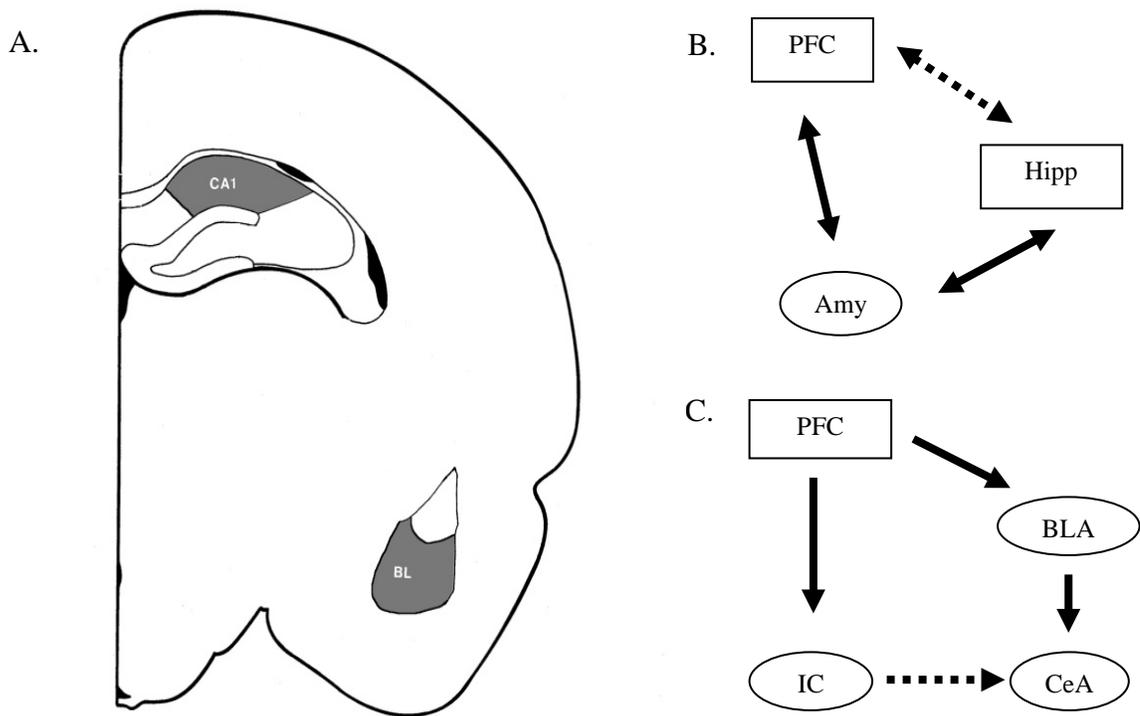


Figure 1.1. *Brain regions and circuits involved in the processing of fear. Figure A: A coronal section highlighting the location of area CA1 of the hippocampus and the BLA nucleus of the amygdala. Figure B: A simplified diagram of the connectivity of the fear system between the prefrontal cortex, amygdala and hippocampus (dotted line signifies an indirect connection). Figure C: A simplified diagram of the connectivity within the amygdala (dotted line signifies inhibitory influence).*

### **1.1.2. The Reward System**

The mesolimbic reward pathway is involved with the rewarding aspects of drugs. This pathway begins in the ventral tegmental area (VTA) and has dopaminergic projections to the striatum, amygdala, hippocampus, nucleus accumbens (NAcc) and the PFC. For the purpose of this thesis, I will focus on the PFC and NAcc as regions of interest within the reward system. In addition to receiving dopaminergic inputs from the VTA, the PFC also sends projections back to the VTA (Kalsbeek, De Bruin, Feenstra, & Uylings, 1990) and to the NAcc. Both the PFC and NAcc have been associated with regulating addiction and have been suggested to be involved in play behavior.

#### *1.1.2.1. Orbital Frontal Cortex*

The orbital frontal cortex is located along the rhinal fissure and forms the anterior insular area in the rat. Although there are subdivisions of the OFC, for the purpose of this thesis, I will focus only on area AID (Zilles, 1985). The OFC has connections to the mPFC and is highly connected with other areas outside of the PFC. These include connections with a plethora of sensory areas (olfaction, taste, vision, somatic sensory), limbic areas (amygdala, hippocampus), and midbrain structures (for a review, Price, 2006).

#### *1.1.2.2. Medial Prefrontal Cortex*

The medial prefrontal cortex is located medially in the PFC and surrounds the most anterior part of the anterior dorsal cingulate cortex. There are 5 main subdivisions of the mPFC, which include; anterior cingulate cortex, the shoulder cortex, lateral orbital areas, prelimbic cortex (PL) and the infralimbic cortex (IL) (Zilles, 1985). For this thesis however, I will refer to mPFC as a whole. The mPFC has projections to most sub-cortical

regions of the brain, including the amygdala, PAG, VTA, and, NAcc and receives projections from many areas including hippocampus, amygdala, VTA, and NAcc (for a review, Heidbreder & Groenewegen, 2003).

### 1.1.2.3. Nucleus Accumbens

The nucleus accumbens (NAcc) is part of the ventral striatum and is divided into two functional areas; the core and the shell (Voorn, Gerfen, & Groenewegen, 1989; Záborszky et al., 1985). Each area has its own afferent and efferent connections, but for the purpose of this study, we will refer to the nucleus accumbens as a whole. NAcc receives inputs from limbic system structures (i.e., hippocampus, amygdala, PFC) and projects to the motor system. This region serves as an intricate part of the reward system and lesions of this area abolish drug-seeking behavior (Ikemoto & Panksepp, 1999).

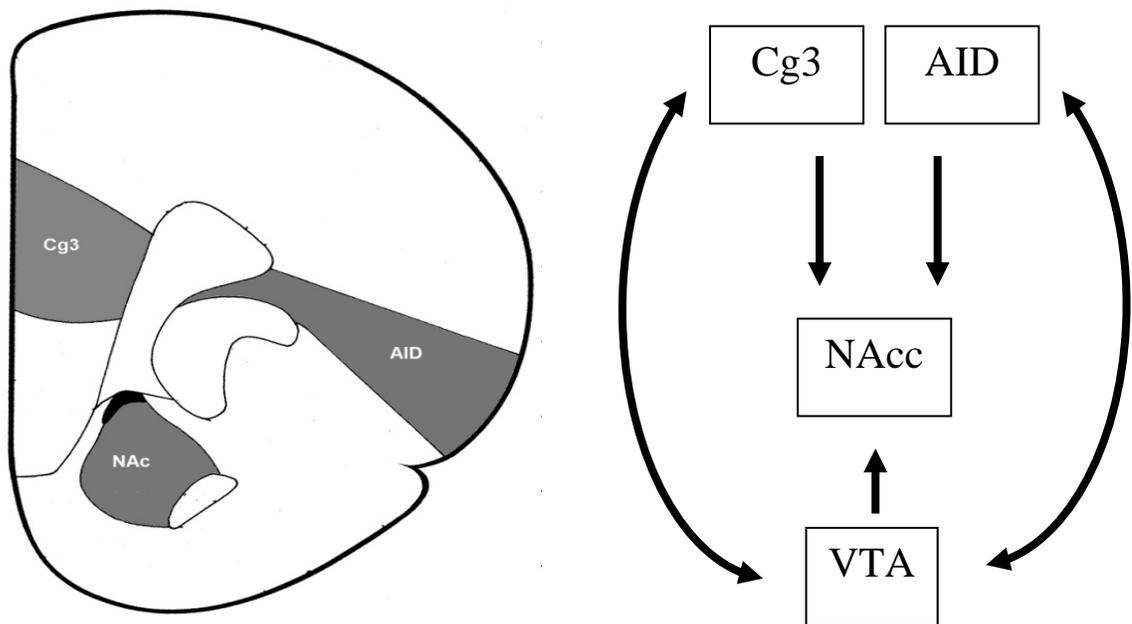


Figure 1.2. *Brain regions involved in the reward system. To the left: A coronal section showing the location of Cg3, AID and NAcc. To the right: A simplified version of the reward system.*

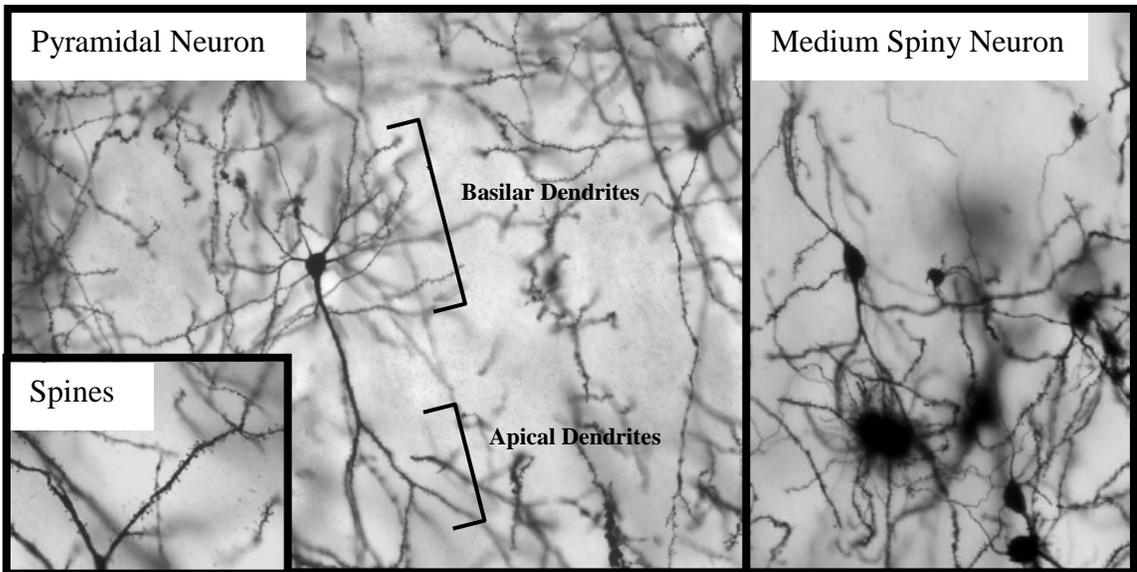


Figure 1.3. *Photomicrographic examples of the cells investigated (pyramidal, medium spiny) and also the spines emanating off of each dendrite. Included are the two separate grouping of dendrites (apical and basilar).*

## 1.2. Summary: The Objectives for Thesis

The present studies were designed to further enhance the understanding of the effects playful experiences on the brain. The following objectives were accomplished in this study:

- To investigate whether there is play-induced plasticity in sub-cortical brain regions within the fear system (amygdala and hippocampus), which have strong, reciprocal connections with the PFC, an area known to exhibit plastic changes following playful experiences.
- To investigate whether play-induced plasticity produced in the juvenile period affects drug-induced plasticity in cortical and sub-cortical regions in adulthood.

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## **Chapter 2**

### **Play's Anatomical Influence on Fear Circuitry**

## **Abstract**

The experience of play has been shown to affect plasticity in cortical regions of rats, but it is unknown if these play-induced changes also occur in sub-cortical regions. Given the connections between the cortical areas and certain sub-cortical regions, it is possible that those sub-cortical regions could also be induced to change arising from playful experiences. The fear system is a likely candidate due to strong connections between the PFC and the main regions in the fear circuit, which are the amygdala and hippocampus. Four groups were tested for plastic changes: two playful groups which were either paired with 3 juveniles or 1 juvenile, and two non-playful groups paired with either 3 adults or 1 adult. Neurons were measured for total length and branching of the dendrites. There were no significant differences between any of the groups in either the hippocampus or amygdala, suggesting that play behavior was not directly affecting the plasticity in these sub-cortical regions.

## 2.2. Introduction

Play in rats consists of attack, defense and counter attack. A playful attack is aimed at nuzzling the nape of the play partner with the snout, while aggressive fighting is aimed at biting and attacking the lower flanks of the animal (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). There are three main defensive tactics used in playful interactions; evasion, partial-rotation and full-rotation to supine. Partial rotation consists of the animal staying on all four paws or hind legs, while rotating to face the attacker. The full-rotation usually requires that the animal rotate fully onto its back (supine), therefore being able to block the attacker with all four paws. These defensive behaviors also follow a unique age-specific timeline in their frequency of use, involving a shift at weaning from mostly using the partial-rotation tactic to mostly using the full-rotation to supine tactic during the juvenile period. In males, a shift back to mostly using the partial rotation tactic occurs at puberty and on into adulthood (Pellis, 2002; Pellis & Pellis, 1990, 1997). Normal playful behaviors are modulated by the PFC, through the orbital frontal cortex and medial prefrontal cortex. Animals displaying OFC lesions are unable to modify their behavior based upon the identity of their social partner (Pellis et al., 2006), while animals with mPFC lesions are unable to initiate movements (Hauber, Bubser, & Schmidt, 1994), and this is reflected in the reduced use of the most complex play tactics in rats with mPFC lesions (Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009).

At the cellular level, these same regions of the PFC are affected by different aspects of play behavior accrued during the juvenile period. Specifically, the development of dendritic branching is affected in both basilar and apical fields depending

on the specific brain region. The arborization of the basilar fields increases in the OFC (dependent on the number of social partners experienced) while the apical dendrites in the mPFC decreases (dependent on the experience of play) (Bell, et al., 2010).

### **2.2.1. Play Trains Emotional Control**

It has been hypothesized that animals who play are better able to handle unexpected events compared to those who do not (Spinka, Newberry, & Bekoff, 2001). Evidence suggests that some of the peculiar behaviors engaged in during play are specifically evolved for such training. For example, juvenile rats perform a puzzling behavior during play fighting called anchoring. During a full rotation defensive maneuver, the attacking rat will usually hold the defending rat down with the front paws, while they steady themselves with their back paws. Although, ~ 20% of the time, the attacking animal will place all four paws on top of the bottom animal, leaving it vulnerable to an unexpected outcome of being pushed off (Foroud & Pellis, 2003). This unusual behavior suggests that rats are preparing themselves to be able to handle unexpected events.

### **2.2.2. The Effects of Social Isolation**

If deprived of playful experiences at an early age, an animal will display a variety of behavioral abnormalities, with the most prominent being an inability to properly regulate emotions. One method widely used to understand these behavioral changes is to socially isolate an animal and compare their behavior to animals which have not been isolated. Such isolation has profound effects not only on behavior, but also on the development of various brain systems. Interestingly, most studies have identified the most sensitive period for these effects to be between 21 and 40 days following birth,

which falls within the peak period of play. An important issue that arises is whether the play performed in this sensitive period is the critical experience affecting the behavioral and neural changes, after all when socially isolated, the rats are deprived of many experiences.

In a beautiful set of experiments, Eison and others have demonstrated that play is the critical experience missing when isolated. Rats were housed in isolation during the juvenile period (25-40) but were allowed to engage in play for 1 hour a day and this was sufficient to eliminate the cognitive deficits usually associated with the effects of social isolation (Eison, Morgan, & Kibbler, 1978). In addition, if rats were paired with either a non-playful, drugged juvenile (Eison, et al., 1978), a non-playful adult (Eison, et al., 1978), or another juvenile separated by mesh (Holloway & Suter, 2004; Pellis, Field, Smith, & Pellis, 1997; van den Berg et al., 1999), as a partner during the juvenile period, the deficits typically seen with complete social isolation are still present. These experiments clearly indicate that while other social experiences may still be important, those arising from play are critical to the effects of isolation.

One of the major deficits seen in rats reared without the opportunity to engage in social play as juveniles is their inability to regulate their responses to stressful or fearful stimuli. This deficit appears in both social and nonsocial situations.

If socially isolated rats, when adults, are placed in a colony with a dominant male, they will fail to show submissive postures as a reaction to repeated aggressive attacks (bites to the rump), fail to change behavior based upon individual identity, as well as fail to engage in stress-relieving behavior after such an encounter (Von Frijtag, et al., 2002). It also appears as if isolated animals are frightened by social interactions. If

rhesus monkeys are socially isolated and presented with a wooden model of a female in a coitus position, the male monkeys were able to properly execute the normal mounting and foot clasp behaviors. However, if presented with a live female, such males fail to mount correctly, often attempting to mount from the front of side and failing to apply a foot clasp on the females ankle even if they do mount from the rear (Deutsch & Larsson, 1974). This suggests that social isolation does not affect the ability to perform behaviors, rather, it disables proper functioning of behaviors when paired with live partners.

If placed on an elevated plus-maze (a test for anxiety) a socially isolated rat will show an increase in anxiety, staying in the closed arm longer compared to socially housed animals (Da Silva, et al., 1996), therefore showing an over-reaction to a stressful non-social context. In addition, if rats are deprived of juvenile play, they will exhibit a higher fear response to a novel open field (Arakawa, 2003) and also express increased anxiety-like behaviors in adulthood to a brightly-lit open field (Lukkes, et al., 2009).

The effects of play experiences on the regulation of fear and its impact on later social and non-social behavior has been shown not only in rats and monkeys, but also in cats. For example, in one study, kittens were reared in one of four conditions: (1) mother-only, (2) mother and juvenile, (3) surrogate mother only, or (4) surrogate mother and juvenile. They were housed in these conditions from 2 days - 6 weeks, while testing occurred from 8 weeks – 28 weeks. Kittens paired with surrogate-only were hyperactive and did not venture to higher levels in the play arena (compared to surrogate-juvenile group). Also, mother-only and surrogate-only groups engaged in more object play as well as more slapping, licking and biting than juvenile-paired groups (Guyot, Bennett, & Cross, 1980).

While the behavioral data strongly suggest a change in the ability to regulate fear, it remains to be determined how these overt effects are mediated by changes at the level of the neural mechanisms that mediate fear.

The hippocampus and amygdala are major structures involved in the neural circuitry that regulates fear and both are highly connected to the PFC. Ablation of the hippocampus in rats been shown to result in less freezing behavior, a fear-induced defensive behavior, when confronted with a cat (Blanchard & Blanchard, 1972), while ablation to the amygdala abolishes both innate fears (Klüver & Bucy, 1937; Prather et al., 2001) and loss of conditioned fears (Weiskrantz, 1956) in monkeys. Both of these brain regions are believed to be involved in different aspects of the fear system. The hippocampus is thought to be important for contextual factors related to the expression of fear and its extinction (Sotres-Bayon, Bush, LeDoux, 2004), while the amygdala serves as a relay station for processing relevant fear-inducing stimuli.

It also appears as if both the OFC and mPFC (for a review, Ongur and Price, 2000) have a regulatory role in the expression of fear/stress based on the extensive connections with the amygdala. In fact, depending on the specific endpoint of the connection in the amygdala, the mPFC connections (for a review, Quirk & Beer, 2006) can be translated as either excitatory or inhibitory, therefore either reinforcing or inhibiting the fear response, as needed. In addition to these connections, the mPFC seems to be critical for the expression of learned fears (Corcoran & Quirk, 2007).

Based on the behavioral and neural evidence, I predict that the changes in the prefrontal cortex that arise due to the experience of play during the juvenile period, should also produce correlated changes in the amygdala and hippocampus.

### **2.2.3. Amygdala**

The areas under investigation include the basolateral nuclei (BLA) of the amygdala and area CA1 of the hippocampus. As previously mentioned, the BLA receives inputs directly from the PFC (as well from as other areas) and is also reciprocally connected to the hippocampus (area CA1). Chemical inactivation of the BLA has been shown to impair learned fear responses (Ribeiro et al., 2011). Given that the BLA is the main locus of communication between the PFC and the amygdala, the BLA is also likely to be affected by playful experiences and so may also exhibit changes in neuronal anatomy.

### **2.2.4. Hippocampus**

Within the hippocampus, CA1 has direct connections to the PFC (Jay & Witter, 1991) and has direct connections with the amygdala (Canteras & Swanson, 1992) . Interestingly enough, it has been shown that electrophysiological synchronization exists between the lateral nucleus (included in the BLA) and CA1 after fear conditioning (Seidenbecher, Laxmi, Stork, & Pape, 2003). Area CA1 has also been shown to undergo plastic changes via fear conditioning ( Li et al., 2005). Therefore, as for the BLA, it is possible that play-induced plasticity seen in the PFC may also be reflected in by neural changes in CA1.

## **2.3. Methods**

\*The brains tested were originally derived from another study completed in our laboratory. Therefore, the *subjects*, *experimental groups* and *histology* sections are a rendering of that project (Bell, et al., 2010).

### **2.3.1. Subjects\***

A total of 96 female Long-Evans hooded rats were used in this study and born at the University of Lethbridge, Canadian Centre for Behavioural Neuroscience. On postnatal day 21 (pnd), 30 of those subjects were then randomly selected into one of four groups (see *experimental groups* below) and were then housed in those groups. Rats were kept in 46cm×25cm×20cm polyethylene tubs with processed corncob as bedding, and maintained at a constant 21–23 °C on a 12:12 light–dark cycle. Food and water were provided ad libitum. All animals were handled and cared for in accordance with the Canadian Council for Animal Care (CCAC) regulations.

### **2.3.2. Experimental Groups\***

Subjects were separated into four separate conditions; no peer play (housed with adult), single peer play (housed with another juvenile), multiple peer play (housed with three other juveniles), or the adult condition (housed with three adults). Subjects were weaned at 21 days old and were then separated into their respective conditions, where they remained until postnatal day 60. Animals reared with adults have little opportunity to engage in play behaviors since adults usually avoid engaging in play with juveniles (Einon, et al., 1978), while those paired with a single juvenile would experience normal play. Pairing with three other juveniles was believed to result in a greater play experience from the contagion affect (Hole & Einon, 1984; Pellis & McKenna, 1992) . Subjects were handled for 10 minutes every three days for 10 minutes to obtain familiarity.

### **2.3.3. Histology\***

On postnatal day 60, subjects were deeply anesthetized using 0.6 ml of 3.4% sodium pentobarbital and then were perfused with 9% saline and their brains were collected. All brains were prepared using the modified Golgi-Cox procedure (Gibb & Kolb, 1998). Following collection, brains were then placed in Golgi-Cox solution and after 14 days, were placed in 30% sucrose solution for seven days. The brains were then cut into 200 micron ( $\mu\text{m}$ ) sections using a vibrating microtome and placed on 2% gelatin-dipped glass slides. Slides were placed in an airtight and darken container for three days, stained, cover slipped and left to dry for approximately 2 weeks.

### **2.3.4. Anatomy**

In order to quantify neuronal morphology, cells were traced onto a paper using a *camera lucida*. A total of five cells (three minimum) were selected from each hemisphere in each area, with the mean score of each measure being used for analysis. In order for cells to be selected, they needed to meet certain criterion: (1) Cell was fully impregnated (2) Cell was not overlapping other cells.

### **2.3.5. Areas measured and their quantification**

For the analysis of the hippocampus, Layer III pyramidal neurons from area CA1 were traced. The basilar portion of the cell was drawn only, owing to the lack of apical branches. Two methods of analysis were used, Sholl analysis and branch order analysis. Sholl analysis (Sholl, 1956) was used to determine the total dendritic length by overlaying a transparency of concentric circles onto the drawing of the neuron and counting the number of dendrites crossing at each circle. Branch order analysis (Coleman

& Riesen, 1968) an estimate of dendritic complexity, was calculated by counting the number of bifurcations on each specific dendrite.

For analysis of the amygdala, spines of nonspiny stellate-like (Class II) cells in BLA were traced. Owing to the enlarged size of the cells in amygdala cells (compared to pyramidal cells), we were unable to fully capture the entire cell for analysis. Third-order branches were selected for spine density, due to the fact that these branches are usually the last one and for need of consistency of measurement across cells. Each section was between ~750-1250  $\mu\text{m}$  and was traced in high magnification (1600x). Spine density was then calculated by the total number of spines, divided by the total length of traced branch.

## **2.4. Results**

### **2.4.1. Hippocampus**

A univariate ANOVA revealed no difference in length [ $F(1, 47) = .051, p = .823$ ] or branching [ $F(1, 47) = .073, p = .788$ ] between hemispheres. Therefore, hemisphere, as a factor, is not included in subsequent analyses.

#### **2.4.1.1. Sholl analysis**

A two-way ANOVA (Play, Partners) revealed no main effect of play [ $F(1, 45) = .669, p = .418$ ] nor partners [ $F(1, 45) = .000, p = .992$ ]. There was also no interaction between the two [ $F(1, 45) = .885, p = .352$ ].

A repeated measures test was conducted to compare length of dendrites. Mauchley's  $W$  indicated a violation of sphericity, therefore  $df$ 's were corrected with Greenhouse-Geisser. This test revealed no significant effect of play [ $F(3.269, 147.090) = .744, p = .538$ ], partners [ $F(3.269, 147.090) = .756, p = .531$ ] nor their interaction [ $F(3.269, 147.090) = .144, p = .944$ ].

#### 2.4.1.2. Branch Order analysis

A two-way ANOVA (Play, Partners) revealed no main effect of play [ $F(1, 45) = 2.143, p = .150$ ] or partners [ $F(1, 45) = .087, p = .770$ ]. There was also no interaction between the two [ $F(1, 45) = .812, p = .372$ ].

A repeated measures test was conducted to compare the amount of branching of dendrites. Mauchley's  $W$  indicated a violation of sphericity, therefore  $df$ 's were corrected with Greenhouse-Geisser. This test revealed no significant difference in play [ $F(3.009, 135.418) = 1.280, p = .284$ ], partners [ $F(3.009, 135.418) = .894, p = .446$ ] nor an interaction of the two [ $F(3.009, 135.418) = .690, p = .560$ ] on branching distance from soma.

#### 2.4.2. Amygdala

A univariate ANOVA revealed no significant difference between hemispheres in spine density [ $F(1, 45) = .030, p = .862$ ]. Therefore, hemisphere will not be included in the analysis.

Table 2.1. Mean ( $\pm$ SEM) alterations to the dendritic morphology of the hippocampus (CA1) and the amygdala (BLA).

	Play		No Play	
	Three Partners	One Partner	Three Partners	One Partner
<i>A. CA1</i>				
Sholl	90.454 $\pm$ 1.954	87.506 $\pm$ 3.123	85.000 $\pm$ 4.190	87.886 $\pm$ 2.705
Branch	36.381 $\pm$ 0.873	35.576 $\pm$ 1.338	33.240 $\pm$ 1.795	34.828 $\pm$ 1.159
<i>B. BLA</i>				
Spines	6.908 $\pm$ 0.229	6.530 $\pm$ 0.302	7.433 $\pm$ 0.428	6.602 $\pm$ 0.370

The effects of playful interactions or amount of partners revealed no significant differences to the length or branching of dendrites in either area CA1 or the amount of spines on dendrites in the BLA nucleus.

#### 2.4.2.1. Spine Density

A two-way ANOVA (Play, Partners) revealed no significant differences between play [ $F(1, 43) = .771, p = .385$ ] nor partners [ $F(1, 43) = 3.152, p = .083$ ]. There was also no interaction between the two [ $F(1, 43) = .442, p = .510$ ].

Table 2.2. *Total sum of brains per group*

<b>Group</b>	<b>Hemispheres</b>	<b>Full Brains</b>
Enriched	23	9
Limited	9	4
Little	12	6
Very Little	5	2

Total number of hemispheres and entire brains which met criterion to be included in analysis.

#### 2.5. Discussion

The proposed hypothesis posited changes to the morphology of neurons in the amygdala and hippocampus due to the experience of play behavior. This hypothesis was derived from behavioral data which suggests irregularity in the fear system due to lack of play, and anatomical data which suggests that the amygdala and hippocampus are connected to the PFC and therefore, likely influenced by changes in the PFC.

The current data do not appear to support this hypothesis (table 2.1). Although strong connections do exist between PFC regions, amygdala and hippocampus, several reasons exist that may explain the lack of plasticity due to play in the sub-cortical regions: (1) some of the groups compared only small sample sizes, (2) the subcortical areas focused on and/or what was measured may have been incorrect, (3) play-induced

plasticity may not be transmitted to sub-cortical regions, rather remain a unique property of cortical areas.

The brains used for this study had been stained nearly 5 years ago and some slides were extremely faded. Some to the extent that for several hemispheres and brains, insufficient cells with the quality needed for analysis could be obtained (table 2.2). For inclusion in the analysis, each hemisphere had to yield a minimum of three cells, so a number of the brains were not included in the statistical analysis. This being the case, it is possible that with a more adequate sample, significant effects of play on these sub-cortical brain regions may have been identified. Mitigating this possibility is that there were no trends in the data suggesting this may be the case. If the data are thus true reflection of the situation, that play does not have an impact on anatomical properties of the neurons from sub-cortical brain regions associated with the regulation of fear, then, given the evidence that play experience affects the development of the ability to regulate fear, other explanations are needed to account for the negative results.

One possible explanation is that the areas investigated are not the areas that are changed by play experience. Our focus on CA1 of the hippocampus is primarily based on the strong anatomical connections with the OFC, mPFC and amygdala. Although CA1 is strongly connected to these areas and is involved in the fear circuitry, its involvement is primarily on the context in which the fear-inducing stimuli appear. There may have been no structural changes in this area for a multitude of reasons. First, in accordance with changes to the dendritic arborization in the PFC, I neglected to investigate changes to the amount of dendritic spines. In fact, we know that CA1 dendritic spines do change in regard to particular experiences, such as running (Stranahan, Khalil, & Gould, 2007).

Furthermore, other areas in the hippocampus may be more sensitive to play-induced plasticity. Area CA3 may be a good candidate to look for change due to plastic changes being seen as a result of stress (McLaughlin, Gomez, Baran, & Conrad, 2007; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Watanabe, Gould, & McEwen, 1992), although this area does lack connections to the amygdala and PFC.

Data do support the idea that the amygdala is sensitive to play, for instance, the size of the amygdala predicts the amount of play in primates (Lewis & Barton, 2006) and, in rats, if lesioned, it has been shown that play is reduced (Daenen, Wolterink, Gerrits, & Van Ree, 2002). The investigation of the BLA nucleus is also based upon strong, reciprocal connections from the PFC regions and also the hippocampus. A difficulty in the study of neuronal morphology in the amygdala is the large size of the cells. In order to counteract this problem, the focus was placed on the spine density of the dendrites. Different experiences and stimuli have been shown to affect spine density of amygdala cells, so making spine density a suitable anatomical feature to measure. Some studies have attempted to draw the cell body, but from my experience, there are two major limitations in trying to do so. First, the cell body is so large that you cannot fully capture the cell using standard light microscopy. Second, if trying to digitize the cell and use computer software for analysis, the ability to judge dendritic connectivity to the cell being drawn is compromised. Therefore, even though limited, spine density is the best method used to derive cell structural changes, and again the negative evidence found here may reflect that the BLA is not changed by play experience.

Another nucleus of the amygdala in which changes could be induced by play, is the central nucleus (CeA). Within the amygdala, the CeA serves as a relay station to other

more “primitive” areas of the fear system (e.g., PAG). The CeA also receives information from the IC and BLA. As mentioned before, the prefrontal cortex has connections to both the BLA and CeA, which then influence messages to the CeA. Generally, connections from the PFC to the BLA are excitatory but messages to the IC excite inhibitory neurons, which in turn, inhibit the signal for fear in the CeA. Therefore, if there would be an irregularity in how the CeA responds to these messages, a down-flow effect in the expression of the fear system would arise.

The final possibility is that the data reveal the real situation, whereby neural plasticity induced by play not does extend to sub-cortical areas. It is possible that the play-induced changes exerted on the PFC are sufficient to regulate the function of sub-cortical components of the fear system without requiring any further anatomical changes in those brain areas. Since the PFC has excitatory and inhibitory control over the amygdala, it is logical to hypothesize that the higher system could potentially exert complete regulatory control over the fear circuit.

This theory is further strengthened by studies investigating the regulation of controllable and uncontrollable stressors. The theory placed forth centers around the dorsal raphe nucleus (DRN) which provides 5-HT innervations to limbic and forebrain structures involved in the emotional aspect of behavior (Jacobs & Azmitia, 1992). If animals are placed in a situation where they encounter uncontrollable stress, deficits ensue, such as heightened fear/anxiety. Activation of the DRN has been shown to promote these deficits caused by uncontrollable stress (Grahn et al., 1999; Maswood, Barter, Watkins, & Maier, 1998) . If presented with mild controllable stressors early in life, rats will not show deficits to uncontrollable stress, as rats without that experience

would. Changes in the function of the DRN do not appear to explain this increased ability to exert this increased controllability, but rather, it appears to be the mPFC (Amat et al., 2005; Amat, Paul, Zarza, Watkins, & Maier, 2006), and most specifically the PL region of the mPFC (Baratta et al., 2009), that does so. The DRN receives virtually all its inputs from the mPFC and these connections result in the inhibition of the DRN cells. These experiments suggest that the PFC controls the DRN, which in turn affects the response to uncontrollable stressors.

While the above possibilities attempt to explain the presence or absence of morphological changes in neurons, an alternative is that experience-induced changes at the neuronal level occur at the functional rather than anatomical level. That is, the experience-induced changes may occur at the neurochemical level. An example is illustrated by oxytocin, a peptide, best known for its involvement in breast-feeding and mother-child bonding, but is now known to be involved more generally in social relationships and doing so by mediating emotional responses. For example, if pups are not licked as often by their mother (social interaction), they show weakened oxytocin receptors in the fear system, while if full nursing behavior and licking is given, rats are better able to handle fearful situations (Caldji et al., 1998). Oxytocin effects are also seen in humans. For example, men who sniffed oxytocin were better at non-verbal emotional verification (Domes, Heinrichs, Michel, Berger, & Herpertz, 2007). Oxytocin also has a role in social recognition (Popik, Vetulani, & van Ree, 1992). Social recognition is influenced when small doses of oxytocin is administered to the olfactory bulb, hippocampus or the amygdala. In fact, if oxytocin-free mutant rats are injected with

oxytocin into the amygdala, their social recognition deficits are reversed (Ferguson, Aldag, Insel, & Young, 2001).

Oxytocin is known to lower blood pressure (Petersson, Alster, Lundeberg, & Uvnäs-Moberg, 1997), reduce cortisone release and subsequent anxiety-like behaviors (Windle, Shanks, Lightman, & Ingram, 1997), and act upon the HPA axis and also affect the amygdala (Huber, Veinante, & Stoop, 2005; Viviani & Stoop, 2008). In fact, there are dense oxytocin receptors in the amygdala as well as other limbic areas (for a review, Gimpl & Fahrenholz, 2001) and this may be an avenue which regulates the fear system. Thus, play-induced changes in the functioning of neurochemicals such as oxytocin, may provide an avenue by which plasticity occurs in sub-cortical systems, but would go undetected at the anatomical level studied in this thesis.

In conclusion, behavioral evidence shows a clear discrimination in emotional regulations between animals who experience playful situations and those who do not. Our findings suggest that play does not affect the plasticity of the amygdala (BLA) or hippocampus (CA1), although there are other possibilities which could explain the behavioral deficits in emotional regulation. It may be that the areas of focus were the incorrect areas of change, plasticity is only seen in the PFC and is strong enough to regulate sub-cortical regions, or that plasticity does not occur but the variations are neurochemical and not neural morphological. Future research needs to investigate each of these possibilities due to the theoretical implications of them.

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**Chapter 3**  
**The Metaplasticity of Play**

## **Abstract**

The prefrontal cortex is known to undergo plastic changes in response to a variety of experiences, including the experience of play and to drugs, such as nicotine. The objective of this study was to determine whether experience with play in the juvenile period influenced neural plasticity induced by nicotine in adulthood. Animals were either paired with three other juveniles (play condition) or one adult (no play condition) and reared in these conditions over the juvenile period. As young adults, half of each group was given nicotine. Neural plasticity in the prefrontal cortex and nucleus accumbens was measured by length and branching of dendrites. Plasticity of the prefrontal cortex due to play was consistent with previous experiments in regards to changes in Cg3, but exposure to nicotine produced the same changes whether the animals had played or not. In contrast, there was no independent effect of play or nicotine on the nucleus accumbens, but for rats that had played, there was a large increase in dendritic branching when exposed to nicotine. These findings suggest that drug exposure later in life can diminish the effects of play in some brain regions, while in others, play primes the tissue for an exaggerated plastic response.

### **3.2. Introduction**

Early experiences are known to largely affect not only the behavior of rats, but also their neural morphology. A wide variety of environmental experiences, such as prenatal stress (Muhammad & Kolb, 2011a), tactile stimulation (Kolb & Gibb, 2010; Muhammad & Kolb, 2011b), enriched environment (Kolb, Gorny, Söderpalm, & Robinson, 2003), play (Bell, et al., 2010), and exposure to psychoactive drugs (for a review, Robinson & Kolb, 2004) have shown to promote reorganization of the brain via neural plasticity. While each of these experiences has been shown to be capable of inducing neural plasticity, how different experiences combine their effects on such neural reorganization is less understood.

The idea that experience-based plasticity at one age influences the plasticity that can be induced by other experiences at another age, is known as metaplasticity. The majority of work on metaplasticity involves an early exposure to a drug, followed by an enhanced experience later in life. For example, when rats were administered stimulants and then placed in an enriched environment two weeks later, the neural morphology resembled the effects of the drug compared to the enriched environment (Hamilton & Kolb, 2005; Kolb, Gorny, Li, Samaha, & Robinson, 2003). This follows the definition that earlier experiences may have lasting effects on plasticity. However, less is understood of how such enriched experiences affect the plasticity of later drug exposure.

Studies by Muhammed et. al., (2011) have begun to investigate how experiences early in development influences the response to subsequent experiences. In these studies, rats were exposed to either prenatal stress or tactile stimulation during infancy (first three weeks post birth), followed by exposure to amphetamine, a psychoactive

stimulant, as adolescents or adults. Neuroanatomical findings from this study suggest that prior experience blocked the changes in both cortical (OFC and mPFC) and sub-cortical (NAcc) regions associated with amphetamine exposure (Muhammad, Carroll, & Kolb, In Submission; Muhammad, Hossain, Pellis, & Kolb, 2011; Muhammad & Kolb, 2011; Muhammad & Kolb, 2011a, 2011b). These studies further suggest that metaplastic changes are sensitive to initial experiences in development, although it does not mention if environmental enrichment produces the same effects if given after infancy.

Juvenile rats will readily engage in playful behaviors when given the opportunity to do so with the largest amount of play occurring during the prime of their juvenile phase (30-40 days (Panksepp, 1981; Pellis & Pellis, 1987, 1990, 1997). As previously mentioned, play appears to affect the neural plasticity in both regions of the prefrontal cortex (OFC & mPFC). Playful experiences alter the arborization of these areas with the basilar dendrites of the OFC increasing due to experiencing social interactions with multiple partners and the apical dendrites of the mPFC decreasing due to the actual experience of engaging in play irrespective of the amount of partners (Bell, et al., 2010). Because this behavior occurs predominantly during the juvenile period and the presence of play-induced plasticity in cortical areas, play behavior may be useful for investigating metaplasticity when initial experiences occur during the juvenile stage. Therefore, this chapter will investigate metaplasticity by examining the effects of play-induced plasticity on later drug exposure.

Drug induced plasticity is normally seen when the brain is exposed to psychoactive stimulants (for a review, Robinson & Kolb, 2004), such as amphetamine and nicotine, and in the current study, nicotine was used. It is well known that nicotine is

the addicting component of tobacco and is the addictive agent in cigarettes. In accordance with other addictive drugs, nicotine activates the mesolimbic dopamine system by releasing dopamine from the ventral tegmental area (VTA), which in turn, affects the nucleus accumbens and PFC. In fact, a single dose of nicotine is enough to increase dopamine in the nucleus accumbens stemming from the VTA (Di Chiara & Imperato, 1988; Imperato, Mulas, & Di Chiara, 1986; Schilstrom, Nomikos, Nisell, Hertel, & Svensson, 1998). Nicotine has also been shown to evoke neural plasticity in both cortical and sub-cortical regions of the brain. Of interest, nicotine induces plasticity in the prefrontal cortex, in the same regions play-induced plasticity has been reported. While plasticity related to play involves a decrease in length of the apical dendrites in the mPFC and an increase of basilar dendrites in the OFC (Bell, et al., 2010), nicotine-induced plasticity shows an increase of length on apical and basilar dendrites in the mPFC, whereas changes are unknown in the OFC (Brown & Kolb, 2001).

In addition to PFC plasticity, the nucleus accumbens is also an area of interest for drug-induced plasticity. It has been shown that nicotine neuro-chemically affects the nucleus accumbens (Pontieri, Tanda, Orzi, & Chiara, 1996) and produces alterations to plasticity via an increases in dendritic length, branching and also spines (Brown & Kolb, 2001; McDonald et al., 2007). While the effect of nicotine on the nucleus accumbens is well understood, it has yet to be determined whether changes to this area due to play behavior occur. This is of interest due to the fact that playful experiences are widely regarded as reinforcing and rewarding behaviors. Therefore, we hypothesize that because of the rewarding nature of playful behaviors, these behaviors alone will induce plasticity in the nucleus accumbens.

The goal of this experiment is two-fold, First, to replicate the findings of play induced plasticity in the PFC by Bell et. al., (2010) and extend the analysis to determine whether play experience alters the morphology of the nucleus accumbens. Second, to investigate metaplasticity by examining whether play-induced plasticity arising in the juvenile period influences the plasticity induced by subsequent drug exposure in cortical and sub-cortical regions. If the model of change represented by Muhammad et al., (2011) is valid, then our results should show that our primary experience (play behavior) block the effects of later drug exposure (nicotine) in cortical areas. In addition, we suspect that play behavior alone will influence plasticity in the nucleus accumbens, and this experience-based plasticity will then influence later plasticity known to occur via nicotine exposure.

### **3.3. Methods**

#### **3.3.1. Subjects**

A total of 72 female Long-Evans hooded rats were used in this study. Out of these animals, 60 were born at the University of Lethbridge, Canadian Centre for Behavioural Neuroscience, while their mothers (12) were originally from Charles River. On postnatal day (pnd) 22, all animals were randomly selected into one of two groups and one of two conditions (see *experimental groups* below) and were then housed in those groups for the remainder of the experiment. Rats were kept in 46cm×25cm×20cm polyethylene tubs with processed corncob as bedding, and maintained at a constant 21–23 °C on a 12:12 light–dark cycle. Food and water were provided ad libitum. All animals were handled and cared for in accordance with the Canadian Council for Animal Care (CCAC) regulations.

### **3.3.2. Experimental Groups**

Subjects were randomly separated into two groups; one group had no opportunity for peer play (housed with an adult and no peers) and the other had lots of opportunity for peer play (housed with three other juveniles and no adults). Animals reared with adults have little opportunity to engage in play behaviors since adults usually avoid engaging in play with juveniles (Einon, et al., 1978), while those paired with three other juveniles would experience both playful experiences and multiple partners. Subjects were weaned at 22 days old and were then separated into their respective play conditions until pnd 70. After this age the animals were randomly subdivided into two additional groups: a control condition (no nicotine) or an experimental condition (nicotine).

### **3.3.3. Nicotine Administration**

Animals were either injected with nicotine (0.3 mg/kg) or saline subcutaneously into the nape of the neck, for 10 consecutive days. All injections took place in a small room near the colony room and occurred between an approximate 3 hour time span in the afternoon.

### **3.3.4. Histology**

On postnatal day 95, subjects were deeply anesthetized using 0.6 ml of 3.4% sodium pentobarbital and then were perfused with 9% saline and their brains were collected. All brains were prepared using the modified Golgi-Cox procedure (Gibb & Kolb, 1998). Following collection, brains were then placed in Golgi-Cox solution and after 14 days, were placed in 30% sucrose solution for seven days. The brains were then cut into 200 micron ( $\mu\text{m}$ ) sections using a vibrating microtome and placed on 2% gelatin-

dipped glass slides. Slides were placed in an airtight and darkened container for three days, stained, cover slipped and left to dry for approximately 2 weeks.

### **3.3.5. Anatomy**

In order to quantify neuronal morphology, cells were traced onto paper using a *camera lucida*. A total of five cells (three minimum) were selected from each hemisphere in each area, with the mean score of each measure being used for analysis. In order for cells to be selected, they needed to meet two criteria: (1) the cell was fully impregnated with stain, and (2) the cell was not overlapping other cells.

### **3.3.6. Areas and Quantification**

Layer III pyramidal neurons were traced from Zilles (Zilles, 1985) area Cg3 (mPFC) and AID (OFC). For area Cg3, both apical and basilar dendrites were drawn, while only basilar were drawn from AID due to lack of intact apical fields. In addition, medium spiny neurons were traced from the nucleus accumbens. Two separate methods of analysis were used to obtain information of dendritic morphology; Sholl analysis and branch order analysis. Sholl analysis (Sholl, 1956) was used to determine the total dendritic length by overlaying a transparency of concentric circles onto the drawing of the neuron and counting the number of dendrites which crossed each circle (16 circles). In order to estimate the complexity of branching of each dendrite, branch order analysis (Coleman & Riesen, 1968) was used, where complexity is calculated by counting the number of bifurcations on each specific dendrite.

### 3.4. Results

#### 3.4.1 AID

A univariate ANOVA revealed no differences of hemisphere on length [ $F(1, 40) = .129, p = .721$ ] or branching [ $F(1, 40) = .150, p = .701$ ], therefore hemisphere was not included as a factor in subsequent analyses.

##### 3.4.1.1. Sholl analysis

A two-way ANOVA (Drug, Play) revealed no significant main effect of drug [ $F(1, 38) = .387, p = .537$ ], play [ $F(1, 38) = 2.612, p = .114$ ] nor drug by play interaction [ $F(1, 38) = .154, p = .697$ ].

A repeated measures test was conducted to compare length of dendrites. Mauchley's  $W$  indicated a violation of sphericity, therefore  $df$ 's were corrected with Greenhouse-Geisser. There were no significant differences of specific points for drug [ $F(2.892, 109.915) = .228, p = .870$ ], play [ $F(2.892, 109.915) = .856, p = .463$ ] nor drug by play interaction [ $F(2.892, 109.915) = .327, p = .798$ ].

##### 3.4.1.2. Branch order analysis

A two-way ANOVA (Drug X Play) revealed no significant main effect of drug [ $F(1, 38) = .289, p = .594$ ], play [ $F(1, 38) = .773, p = .385$ ] nor an interaction of the two [ $F(1, 38) = .633, p = .431$ ].

A repeated measures test was conducted to compare the amount of branching of dendrites. Mauchley's  $W$  indicated a violation of sphericity, therefore  $df$ 's were corrected with Greenhouse-Geisser. No significant differences were seen between drug [ $F(3.275, 124.433) = .228, p = .892$ ], play [ $F(3.275, 124.433) = 1.281, p = .283$ ] nor an interaction of the two [ $F(3.275, 124.433) = .530, p = .678$ ].

### 3.4.2. Cg3 Apical

A univariate ANOVA failed to reveal significance between hemispheres in either length [ $F(1, 43) = 3.761, p = .059$ ] and branching [ $F(1, 43) = 3.171, p = .082$ ].

Consequently, hemisphere was not considered a factor in subsequent analyses.

#### 3.4.2.1. Sholl analysis

A two-way ANOVA (Drug, Play) revealed a main effect of drug [ $F(1, 41) = 6.290, p = .016$ ], but not of play [ $F(1, 41) = 3.334, p = .075$ ] nor an interaction between the two [ $F(1, 41) = 1.209, p = .278$ ]. Pair wise comparison revealed that the main effect of drug increased length in the Nicotine-Play group ( $p = .013$ ). Furthermore, pair wise comparisons also revealed that in the control group, play alone decreased length ( $p = .052$ ), and also revealed a significant difference between the control-play group and nicotine-no play group ( $p = .003$ ) (Figure 3.1).

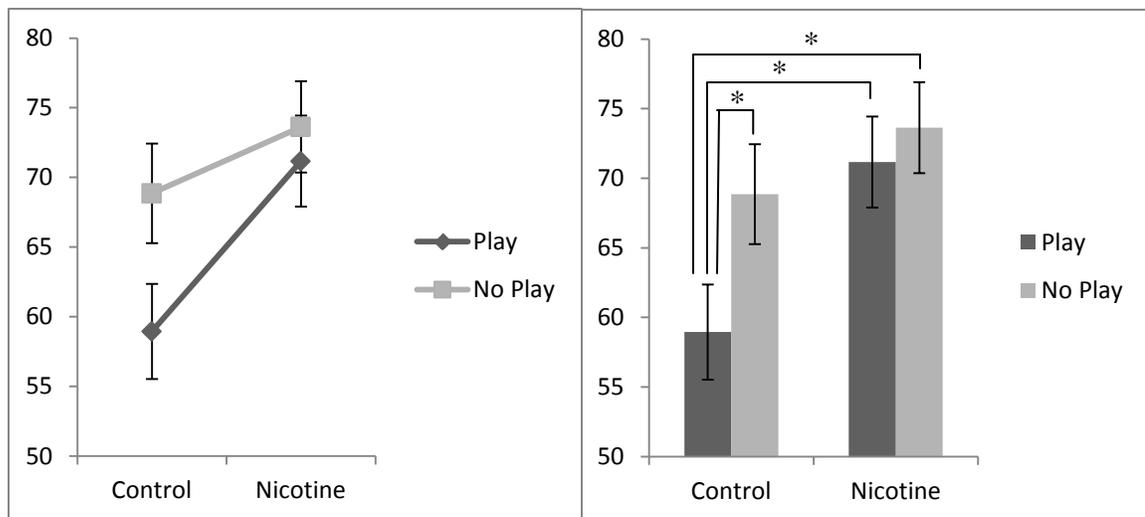


Figure 3.1. Mean ( $\pm$  SEM) Dendritic length per condition (Drug, Play) of the Cg3 apical region of the medial prefrontal cortex. Control-Play compared to Control-No Play ( $p = .052$ ), Nicotine-Play ( $p = .013$ ), and Nicotine- No Play ( $p = .003$ ) showing a decrease in branching. There was no difference between Control-No Play and nicotine groups.

A repeated measures test (Drug X Play X Length) was conducted to compare length of dendrites. Mauchley's W indicated a violation of sphericity, therefore df's were corrected with Greenhouse-Geisser. Repeated measures failed to reveal significant differences for drug [ $F(1.795, 73.610) = 2.469, p = .097$ ], but did reveal a significant difference for play [ $F(1.795, 73.610) = 3.175, p = .053$ ], and no significant interaction [ $F(1.795, 73.610) = .531, p = .571$ ]. The significance for play is marked by a large difference in the final Sholl ring (Figure 5.2)

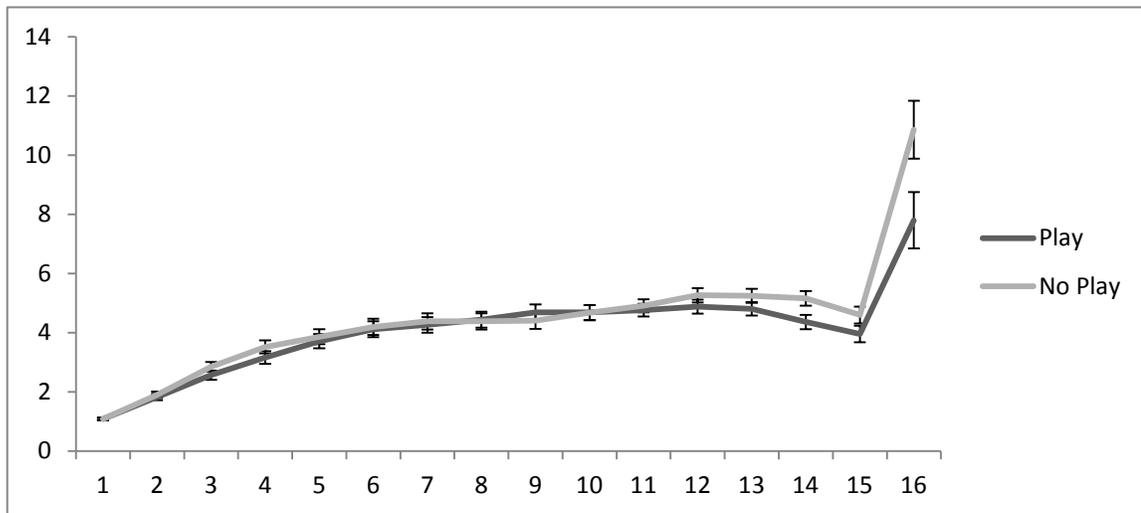


Figure 3.2. Mean ( $\pm$  SEM) on changes in dendritic length from soma. Sholl rings are represented with 16 representing the final Sholl ring 16 and any extending beyond that ring.

#### 3.4.2.2. Branch Order analysis

A two-way ANOVA (Drug, Play) failed to reveal a significant main effect of drug [ $F(1, 41) = 3.248, p = .079$ ], play [ $F(1, 41) = 3.285, p = .077$ ] nor interaction between the two [ $F(1, 41) = .006, p = .938$ ] on amount of branching from soma.

A repeated measures test (Drug X Play X Branching) was conducted to compare length of dendrites. Mauchley's W indicated a violation of sphericity, therefore df's were

corrected with Greenhouse-Geisser. It revealed no significant differences in drug [ $F(3.347, 137.236) = .381, p = .788$ ], play [ $F(3.347, 137.236) = .928, p = .437$ ] or an interaction between the two [ $F(3.347, 137.236) = .594, p = .638$ ].

### **3.4.3. Cg3 Basilar**

A univariate ANOVA revealed no difference between hemispheres in either length [ $F(1, 43) = .206, p = .652$ ] or branch order [ $F(1, 43) = .127, p = .724$ ]. Therefore, hemisphere was not included in further analysis.

#### *3.4.3.1. Sholl analysis*

A two-way ANOVA (Drug, Play) revealed no significant difference in play [ $F(1, 41) = 2.970, p = .092$ ], but did reveal a significant increase in length in the drug group compared to the control group [ $F(1, 41) = 4.370, p = .043$ ]. Pair wise comparison revealed that this increase was seen in the nicotine group which had experienced play behavior ( $p = .029$ ) and also for the group which had not experienced previous play behavior ( $p = .009$ ) (Figure 3.3). There was no significant interaction between the drug and play [ $F(1, 41) = 1.126, p = .295$ ].

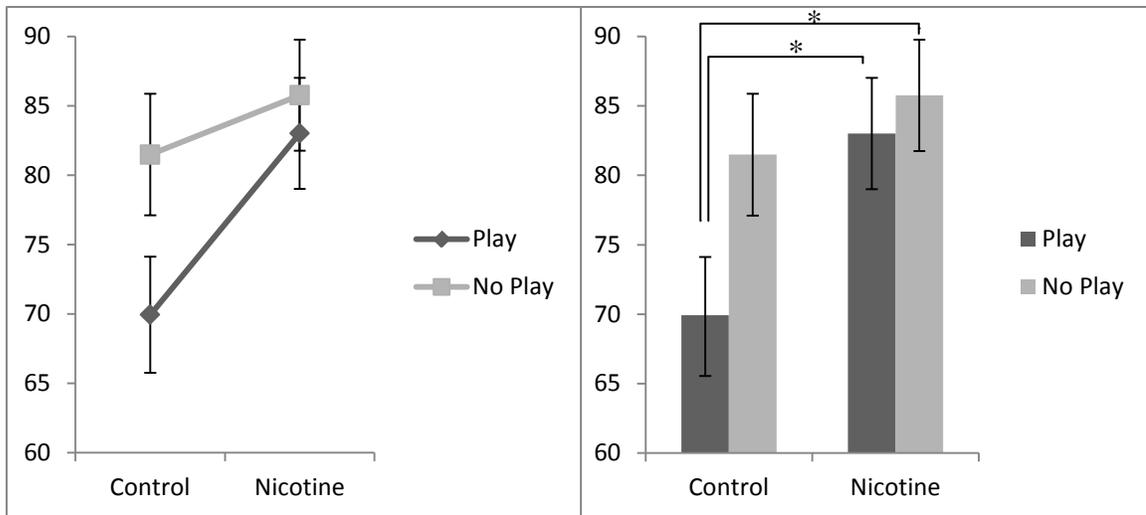


Figure 3.3. Mean ( $\pm$  SEM) length per condition (Drug, Play) of the Cg3 basilar region of the medial prefrontal cortex. Control-Play compared to Nicotine-Play ( $p=.029$ ), and Nicotine-No Play ( $p=.009$ ) showing a decrease in branching. In addition, Control-Play compared to Control-No Play did not reach significance, although revealed a strong trend towards significance ( $p=.064$ ).

A repeated measures test (Drug X Play X Length) was conducted to compare length of dendrites. Mauchley's W indicated a violation of sphericity, therefore  $df$ 's were corrected with Greenhouse-Geisser. This repeated measures test indicated no significant changes between drug [ $F(15, 105.565) = 2.366, p = .084$ ], play [ $F(15, 105.565) = 2.656, p = .061$ ] nor an interaction between the two [ $F(15, 105.565) = 10.185, p = .185$ ].

#### 3.4.3.2. Branch Order analysis

A two-way ANOVA (Drug, Play) revealed no significant main effect of play [ $F(1, 41) = 3.100, p = .086$ ], although it did reveal a significant main effect of drug [ $F(1, 41) = 4.510, p = .040$ ] with nicotine increasing branching. Pair wise comparison revealed that this difference was seen in both the group that received nicotine and playful experiences ( $p = .011$ ) and also the group which did not receive playful experience ( $p = .008$ ). Pair wise comparison also revealed an increase in branching in control animals who had not

experienced play compared to animals who had experienced play ( $p=.026$ ) (Figure 3.4). There was no significant interaction between drug and play [ $F(1, 41) = 2.604, p = .114$ ].

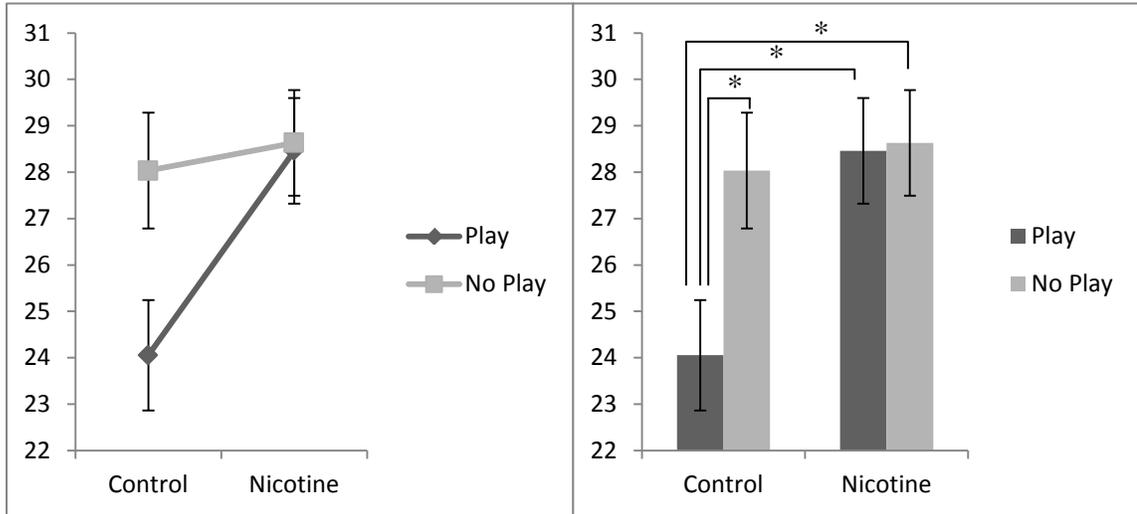


Figure 3.4. Mean ( $\pm$  SEM) Branch order per condition (Drug, Play) of the Cg3 basilar region of the medial prefrontal cortex. Play-no nicotine compared to No-play-no nicotine ( $p=.026$ ), Play-nicotine ( $p=.011$ ), and No-play-nicotine ( $p=.008$ ) showing a decrease in branching. There was no difference between No-play control and nicotine groups.

A repeated measures test (Drug X Play X Branching) was conducted to compare amount of branching from soma. Mauchley's W indicated a violation of sphericity, therefore df's were corrected with Greenhouse-Geisser. The repeated measures analysis revealed no significant difference in specific branches with a main effect of drug [ $F(5, 142.945) = 2.080, p = .096$ ], play [ $F(5, 142.945) = 1.732, p = .155$ ], nor an interaction of drug and play [ $F(5, 142.945) = .287, p = .862$ ].

#### 3.4.4. Nucleus accumbens

A univariate ANOVA revealed no difference between hemispheres in length [ $F(1, 40) = .010, p = .919$ ] or branching [ $F(1, 40) = .245, p = .623$ ]. For this reason, hemisphere was not included as a factor in subsequent analyses.

##### 3.4.4.1. Sholl analysis

A two-way ANOVA (Drug, Play) revealed no main effect of drug [ $F(1, 38) = .094, p = .761$ ], play [ $F(1, 38) = 1.922, p = .174$ ] nor an interaction between the two [ $F(1, 38) = 3.491, p = .069$ ].

A repeated measures test (Drug X Play X Length) was run to compare length of dendrites. Mauchley's  $W$  indicated a violation of sphericity, therefore  $df$ 's were corrected with Greenhouse-Geisser. The repeated measures test revealed no significant difference between drug [ $F(3.069, 116.616) = .563, p = .644$ ], play [ $F(3.069, 116.616) = .363, p = .784$ ] but did show an interaction of the two [ $F(3.069, 116.616) = 2.606, p = .054$ ].

##### 3.4.4.2. Branch Order analysis

A two-way ANOVA (Drug, Play) failed to reveal a significant main effect of drug [ $F(1, 38) = 2.129, p = .153$ ], but did reveal a significant effect of play [ $F(1, 38) = 7.203, p = .011$ ], with branch order increasing in the playful group. Pair wise comparison of play revealed that this difference is present in the play group exposed to nicotine, for which there is a significant increase in branching ( $p = .025$ ). Furthermore, pair wise comparison also revealed that there was a decrease in branching in both the Control-No Play (.005) and Nicotine-No Play (.003) group compared to Nicotine-Play group (Figure 3.5). There was no significant interaction seen between play and drug [ $F(1, 38) = 3.105, p = .086$ ].

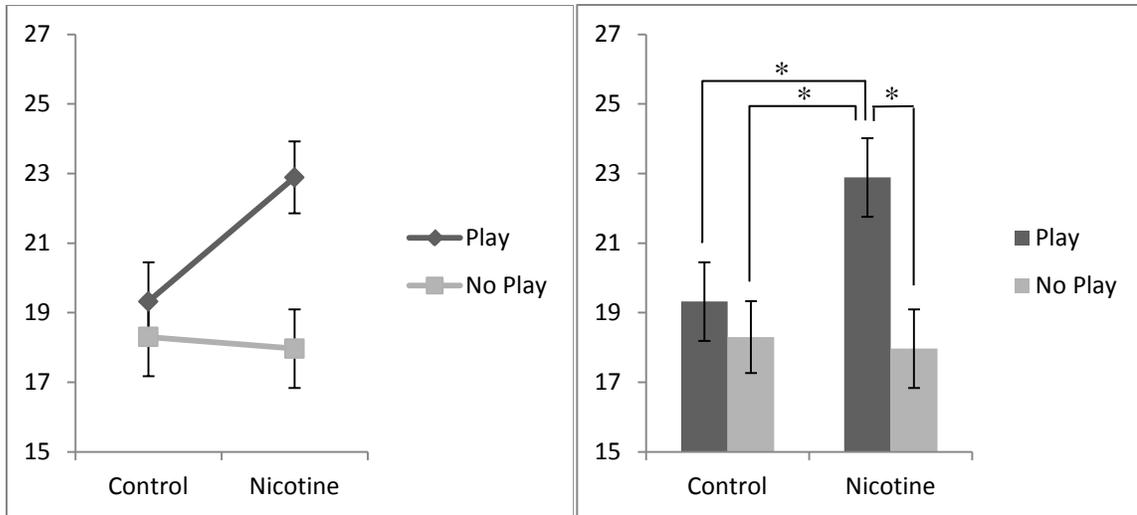


Figure 3.5. Mean ( $\pm$  SEM) Branch order per condition (Drug, Play) in the nucleus accumbens. Nicotine-Play compared to Control-No Play ( $p=.005$ ), Control- Play ( $p=.025$ ), and Nicotine-No Play ( $p=.003$ ) showing a decrease in branching. There was no difference between No-play control and nicotine groups.

A repeated measures test (Drug X Play X Branching) was run to compare branching order. Mauchly's W indicated a violation of sphericity, therefore df's were corrected with Greenhouse-Geisser. Repeated measures failed to reveal a significant difference in play [ $F(3.551, 134.953) = .536, p = .792$ ], although it did reveal significance in the drug group [ $F(3.551, 134.953) = 4.000, p = .029$ ], showing an increase on length due to nicotine in the first three-fourths of distance points from soma via Sholl analysis (Figure 3.6).

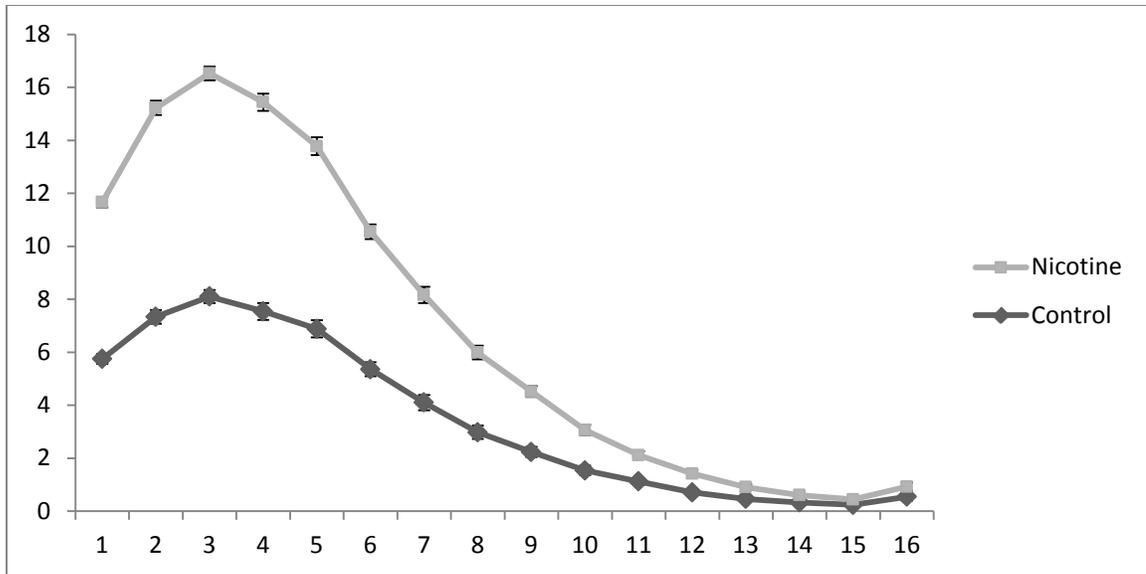


Figure 3.6. *Mean ( $\pm$  SEM) on changes in dendritic length from soma in the nucleus accumbens. Sholl rings are represented with 16 representing the final sholl ring 16 and any extending beyond that ring.*

### 3.5. Discussion

The effect of playful experiences followed by later drug exposure (nicotine) on the dendritic morphology (length and branching) of cortical (OFC & mPFC) and sub-cortical (NAcc) brain regions were investigated. Previous reports have generalized a model of metaplasticity that suggests that if two consecutive enrichment experiences (environmental or drug exposure) are administered, the results on the metaplasticity of the two will mirror those of the first experience. For example, experiences occurring during development (prenatal stress or tactile stimulation) have been shown to mask the plasticity induced by drugs later in life (Muhammad, et al., In Submission; Muhammad, et al., 2011; Muhammad & Kolb, 2011; Muhammad & Kolb, 2011a, 2011b). The present study was based on this model of metaplasticity and so it was predicted that the plasticity

present in subjects exposed to play and then to nicotine, should resemble that present in subjects exposed to play alone.

It was reported that the experience of play has plastic changes on the OFC and mPFC in rats, specifically that the OFC is sensitive to the number of partners experienced and the mPFC is sensitive to the experience of play itself (Bell, et al., 2010). Our results for the mPFC are consistent with the data reported in Bell et. al., (2010), with a decrease seen in the dendritic length in apical field (figure 3.1) for the rats reared with playful peers. Different to that report however, the current study found a decrease in the dendritic length (figure 3.3) and also in branching (figure 3.4) in the basilar field. Also, unlike the previous study, the current study revealed no significant differences in dendritic morphology in the OFC arising from exposure to multiple partners. These findings suggest that the social influences on the OFC may be more subtle than those on the mPFC. For example, while the mPFC may be highly sensitive to the experiences derived from play, the OFC may require not only multiple partners, but also, some additional social experiences with those partners. These additional components of the experiences needed to affect neural anatomy of the OFC remain to be determined.

Given the robust effects of play on the mPFC in the present study, it is reasonable to evaluate the added effects of later exposure to nicotine. If the proposed theory for metaplasticity is correct, then the morphology of the mPFC neurons from animals exposed to nicotine following play, should resemble the above changes arising from playful experiences. This study does not support this prediction.

If subjects received play experiences and later drug exposure to nicotine, the morphological changes in mPFC neurons resembled those changes derived from nicotine.

Thus, it appears as if the beneficial neural changes induced by play in the mPFC were abolished by later nicotine exposure. This finding is extremely surprising because previous research investigating metaplasticity suggest that the plasticity of secondary experiences are overshadowed by the primary experience, whether that experience is environmental or exposure to a drug. Our data suggest that this model does not apply to play experiences followed by exposure to drugs.

In addition to investigating the plasticity of prefrontal regions, the nucleus accumbens was also investigated. It was hypothesized that the nucleus accumbens would be sensitive to the experience of play because of rewarding nature of play and the role of the accumbens in reward. This prediction was not supported by the present study; with the results being that there were no structural changes (length or branching) in the nucleus accumbens related to play experience.

Although no structural changes were seen in the NAcc related to only the experience of play, the influence of playful experiences interacted with the plasticity to later nicotine exposure in the NAcc. Past research on the plasticity of the nucleus accumbens has shown that the dendritic morphology of cells is sensitive to drug exposure (for a review, Robinson & Kolb, 2004). The present study did not find an independent effect of nicotine exposure on the nucleus accumbens. The unexpected finding was that those rats experiencing play as juveniles had a significant increase in dendritic arbor when exposed to nicotine (figure 3.5), suggesting that playful experiences are a precursor to later plasticity in NAcc due to nicotine .

The present findings thus suggest that play is priming the nucleus accumbens to enhance later plasticity, therefore reinforcing the idea of metaplasticity. The functional consequences of these changes in the nucleus accumbens remain to be determined.

The priming by the play experience that results in an enhanced morphological change in the nucleus accumbens following nicotine exposure may represent a protective response, where the changes reduce the risk of addiction. Or, possibly, the opposite may be true. Additional studies are needed to determine if play experienced rats are more or less susceptible to becoming addicted to drugs like nicotine or amphetamine.

This study has indicated the different roles of metaplasticity if play experiences are followed by later exposure to nicotine. In stark contrast of the generalized model of metaplasticity, later nicotine exposure appears to block the pattern of neural change imposed by play earlier in development. Also in contrast to the generalized model, with regard to the nucleus accumbens, the two consecutive plasticity-inducing experiences have an additive/multiplicative interaction, changing the neural morphology beyond that of either experience alone. Therefore, the present study suggests that metaplasticity is revealed in different aspects, with different brain areas and different experiences interacting and changing in different ways.

### 3.6. Results

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**Chapter 4**  
**General Discussion**

This thesis investigated the effect of play on the plasticity of specific cortical and sub-cortical brain regions, as well as the effect play has on the metaplasticity, where play and drug exposure was administered consecutively. Taken together, playful experience appears to have a direct effect on some cortical systems and a priming effect on some sub-cortical systems.

Bell et. al., (2010) showed that social experiences in the juvenile period influence the plasticity of the prefrontal cortex, affecting both the mPFC (Cg3) and the OFC (AID). The forms of the neural changes and the specific experiences responsible for them differed in these two areas of the PFC. First, in the mPFC there was a decrease in the length of the basilar dendrites, whereas in the OFC, there was an increase in apical dendrites. Second, the critical social experience important for the changes in the mPFC was engaging in play with a peer, whereas the critical social experience for the OFC was engaging socially with multiple partners. The data from the present study are consistent with these morphological changes in mPFC, although it is not as clear for changes seen in OFC. The OFC is an extremely sensitive area in the cortex and is highly connected to other brain regions and receives inputs from multiple areas (Price, 2006). Our results indicate that the plasticity of the OFC may not be as sensitive to a specific factor (partners) as its prefrontal counterpart, the mPFC, to the experience of play.

As the Bell et al. (2010) reports, the increase in OFC branching did not discriminate between 0-1 partners but experienced morphological alterations to 3 partners. It may be that the OFC is sensitive to interactions with multiple partners, with some threshold level of particular kinds of social interactions with those many partners being necessary to induce such changes. The present study did not analyze the amount or

type of social interactions (including play behavior) experienced. If a threshold to induce plasticity in the OFC exists, it is possible that excessive interaction with one partner from a triad may have diminished the impact of experiencing interactions with multiple partners. Further studies are needed to investigate what the crucial experiences may be for inducing plasticity of the OFC to fully understand how it is affected by social interactions.

With the verification that play behavior has an influence on the development of the PFC, the effect of play on the plasticity of sub-cortical regions was investigated. Animals that do not possess a prefrontal cortex, or indeed, have the whole cortex removed, still engage in playful behavior (Pellis, Pellis, & Whishaw, 1992). This suggests that sub-cortical regions are involved, and rather imperative, in the production of play and, therefore, could be subjected to feedback from the play experience and be modified by that experience. That is, play-induced plasticity may extend to sub-cortical areas.

The experiment focused on investigating the main sub-cortical components of the fear system for alterations in the neural morphology arising from play experience. The hippocampus (CA1) and amygdala (BLA) were chosen as sites of investigation due to their role in expression of fear and also for their strong anatomical connections between each other and the prefrontal cortex. It is well-known that both the amygdala (Clugnet & LeDoux, 1990; Racine, Milgram, & Hafner, 1983) and hippocampus (Bliss, 1979) are very susceptible to plasticity, although our results suggest that these areas are not directly sensitive to play experiences.

The experience of play has shown to directly affect the plasticity of cortical areas, while appearing to not affect the plasticity of sub-cortical areas. In order to investigate the extent of “playful plasticity”, the metaplasticity of play on later experiences was studied. That is, does experience with play in the juvenile period affect the way cortical and sub-cortical brain areas respond to plasticity-induced experiences in adulthood? The experience of choice for the second experience was exposure to a drug (nicotine). Drug exposure has been shown to induce strong plasticity on the brain and specifically, nicotine has been shown to induce changes to PFC. Therefore, in the second experiment, animals were first exposed to playful interactions, and then later exposed to nicotine.

Since our results of play-induced plasticity failed to reveal differences in the OFC, it was unlikely that changes would be seen in this area due to drug exposure, and this is indeed the case. It does not appear as if either play experience or later drug exposure significantly affected the morphology of OFC neurons. In contrast, the mPFC responded to both play and nicotine with morphological changes. Moreover, the plasticity induced in the mPFC by playful experiences was masked by later exposure to nicotine. This suggests one of two things: (1) the benefits derived by play in shaping the mPFC are lost by subsequent exposure to drugs like nicotine, or (2) the large changes in neural morphology in the mPFC of a well-played brain exposed to nicotine are somehow compensatory, diminishing the negative impact of nicotine exposure.

In the metaplasticity experiment, in addition to the PFC, the nucleus accumbens was investigated for possible changes in the neural morphology. Play behavior is well known to be a pleasurable experience and rewarding to rats. In fact, rats will learn to navigate a maze when rewarded with play (Humphreys & Einon, 1981; Normansell &

Panksepp, 1990) and will readily return to an arena previously used as a playful environment compared to one which has not (Calcagnetti & Schechter, 1992). Since the nucleus accumbens is known to be the “reward center”, play-induced plasticity of this area could be suspected to occur. Our findings did not support the prediction of play-induced plasticity of the nucleus accumbens.

The general consensus within the play literature is that this behavior is rewarding. For example, rats will readily produce “happy” vocalizations (50-kHz) in anticipation of playful interactions (Knutson, et al., 1998), during a playful interaction (Burgdorf et al., 2008), or in an arena frequently used by other rats, suggesting the “seeking” of social contact (Brudzynski & Pniak, 2002). In addition, these vocalizations were positively correlated to the rewarding effects of play behavior (Burgdorf, et al., 2008). The production of 50-kHz ultrasonic vocalizations also works via the mesolimbic dopamine system (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007). This evidence strongly supports the idea that play is seen as a rewarding behavior for animals.

Clearly, the reward mechanisms that include the nucleus accumbens are involved in promoting playfulness. The lack of change in the morphology of the neurons in the nucleus accumbens following play experience suggests that if play does have a feedback influence on the function of this nucleus (or some other associated system) the changes are not expressed at this gross cellular level. Even so, the evidence from the present study suggests that the experience of play can prime the cells of the nucleus accumbens for response to later experiences.

Play experience alone did not affect the plasticity of the nucleus accumbens, although later drug exposure (nicotine) did produce changes in plasticity, but only if first

experiencing play. Our data reinforces previous work describing drug-induced plasticity in the nucleus accumbens, but suggests that these alterations in plasticity will only occur if the animal had experienced play behaviors during their juvenile period.

Together, these studies strongly suggest that the experience of play affects the neuronal structure of both the cortical and sub-cortical areas of the brain, although they appear to work in different ways. Play alone directly affects the cortex, but it does not appear to directly affect sub-cortical regions (hippocampus, amygdala, nucleus accumbens). Although, play does appear to prime some sub-cortical regions for later metaplastic change. This ability raises multiple questions, which will need to be addressed in future studies.

While the results are intriguing, it still remains to be understood which particular experiences derived from play are inducing the plasticity and metaplasticity shown by the rats which played. In the present experiments, the animals in the play groups experienced two distinct factors: the experience of play, and pairing with age-matched conspecifics. This fact leaves the possibility of not only that play may promote these effects, but also the possibility that just the pairing with a same-aged partner may be sufficient to induce these changes. Moreover, if it is play that is important, what aspect of the play is essential, the amount experienced or particular experiences occurring in some forms of play and not other forms? Therefore, this issue needs to be studied further in order to tease out which variable is crucial for inducing the dendritic alterations seen.

The idea that play behavior has shown beneficial changes to the cortex (mPFC) and negative affects to the nucleus accumbens may lead to the theory that while playful experiences have positive effects, they may also have negative effects (drug addiction),

although that assumption may be premature. The current thesis did not investigate sensitivity to nicotine exposure, which is necessary to determine if the animals would be more readily addicted to the drug.

In order to fully understand if play may influence the addictive nature of the nucleus accumbens, other drugs must be tested. The ideal choices of possible drugs are other psychostimulants. Psychostimulants (amphetamine, cocaine, etc.) are known to permanently alter brain structure (Y. Li, Kolb, & Robinson, 2003; Robinson & Kolb, 1999, 2004) and also work upon the nucleus accumbens. In fact, cocaine has been shown to alter neuronal morphology in both the nucleus accumbens and neocortex (Robinson, Gorny, Mitton, & Kolb, 2001). By understanding the effect of other psychostimulants when animals are first exposed to play, it will be able to distinguish if play followed by drug exposure will lead to the results mentioned in this thesis, or if the plasticity is sensitive to only nicotine.

The ability of play to impact the metaplasticity of the nucleus accumbens can be not only investigated via later drug exposure, but also via later environmental experiences of different kinds. It has been shown that certain experiences have the ability to alter the morphology of the nucleus accumbens (Kolb, Gorny, Söderpalm, et al., 2003). Exposure to enriched experiences, as well as exposure to a variety of drugs, needs to be investigated following the same methodology used here to fully understand the effect that play has on the metaplasticity of the nucleus accumbens.

The findings from this thesis suggest that the experience of play alone only affects cortical regions. In addition to directly influencing brain regions, playful experiences also appear to prime some sub-cortical regions of the brain. This would explain our lack of

significance of change in the sub-cortical regions of the fear system. It is possible that play primes these systems in a manner that permits them to respond to unpredicted and stressful situation more effectively, which could involve plasticity in the morphology of their composing neurons as part of an improved emotional regulation. These results thus reinforce our understanding of the role of play in being able to alter the structure, and perhaps function, of multiple regions in the brain, both cortical and sub-cortical.

## 4.1 References

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