

**THE ANATOMICAL AND BEHAVIOURAL CORRELATES OF EXPERIENCE**

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## **DEDICATION**

I would like to dedicate this work to my husband Claude, my son Travis, and my grandson T.J (Travis Junior). I feel very blessed to have all of you in my life, standing behind me and supporting me through the worst of times. Claude and Travis, I thank for your unquestionable belief in me and your support when I decided to go on after my Masters. You both showed me so much patient and love throughout this process, and I am sure I tried the limits of both many times over the last four years. You have been my biggest fan club. T.J. you were a bright spot, coming into this world and reminding me just how important and precious life is, and I know that I am truly blessed.

## **ABSTRACT**

### **The Anatomical and Behavioural Correlates of Experience**

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The effects of experience on developing and mature prefrontal brain circuitry and behaviour were investigated in rats using Ritalin, complex housing, and learning as tools. The results showed that Ritalin altered prefrontal cortical (PFC) circuitry and produced abnormal play behaviour and cognitive deficits into adulthood. Moreover, early stimulant exposure inhibited the robust anatomical changes typical after complex housing, demonstrating that early drug experience compromised cortical plasticity. Ritalin also altered adult PFC circuitry, but without enduring behavioural effects. Thus, it appeared that the mature brain was better able to compensate under adverse conditions. Nonetheless, novel experiences altered adult PFC circuitry with individual tasks producing unique patterns of change. Therefore, similar to other cortical regions the PFC was modified by experience. What was unique, however, was that experience-induced plasticity in the PFC appeared to be transient in adult animals, suggesting that once the task was learned the PFC was no longer required.

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## **CHAPTER 1**

### **Literature Review**

## **I. An Overview of Brain Development**

The outcome of years of research has brought about the awareness that both ‘nature’ and ‘nurture’ are equally important in shaping who we are, albeit varying in influence at different times during development. On the ‘nature’ side, brain development and maturation occurs in a predetermined manner following a sequence of events with seemingly little impact from the external environment. For example, synaptogenesis occurs during a particular time line of development regardless of seemingly major disruption such as injury, pharmacological interventions or deprivation. On the nurture side, developmental processes such as synaptic connectivity depend heavily on experiential input, with lack of appropriate input having devastating and perhaps permanent effects on the mature brain. The influence of experience and genetics are not distributed equally in all regions, however. For example, a twin study of elderly men found that the size of the hippocampus , a region of the brain involved in learning and memory processes, was determined less by genetics (40%) than experiential factors or the combination of genetics and experience (60%) [103]. In contrast, 80% of the variance in corpus collosum volume could be attributed to genetic influences. Although these differences are not equal among the various regions, the relative input of the environment on hippocampus size illustrates the notion that although nature supplies all of the components necessary for brain development and maturation to occur, experiential factors influence and in essence mold the ‘final’ product.

### ***The influence of Experience***

The term experience, as used in the literature relates to both internal and external environmental factors. Examples of environmental factors would include things like

hormones, the prenatal environment, and maternal/paternal behaviour, rearing conditions, learning, social interactions, and exposure to drugs and other chemicals, all of which have been shown to influence brain development and structure either prenatally, postnatally, or both [66]. Thus, these experiential factors have the capacity to modify and alter developing, as well as existing brain circuitry. Early experience as well as the postnatal environment through the lifespan, and the organisms' interaction with it, will shape the final structure of the brain and in essence create the individual. To understand how experiential factors may impact brain structure and connectivity in and beyond the womb requires first a review of the gross organization and structure of the organ in question.

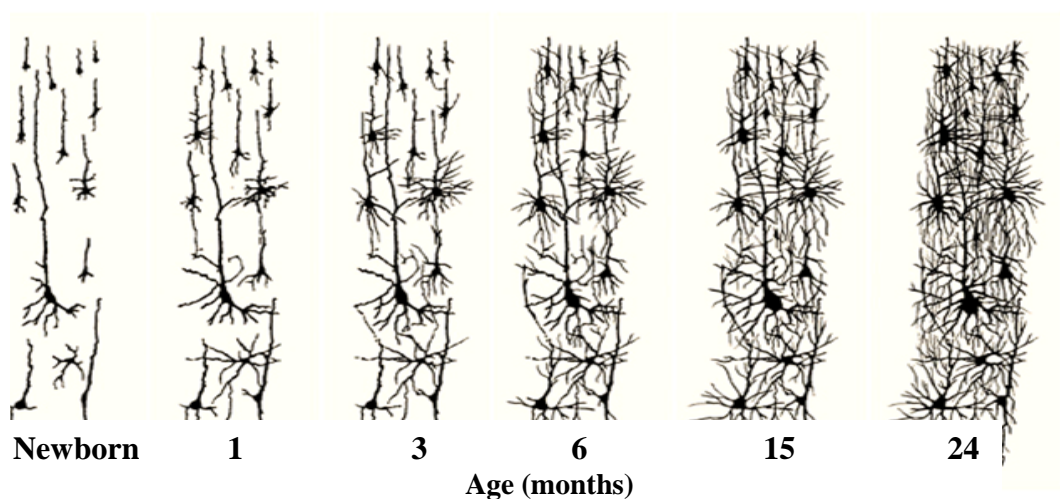
### ***Brain Development***

The mammalian brain is composed of billions of neurons. Although the estimates range greatly, a widely reported estimate is about 80 billion neurons in the human brain. In one of the recent estimates the total number of neurons was on the order of 21.5 billion on average in the neocortex alone [91]. Each neuron may make thousands of connections with other neurons (estimates of about 10,000 per neuron), making the total number of connections to be about  $10^{14}$ .

### **Cortical development**

Once born (neurogenesis), these neurons must make their way to their final destination where they will differentiate into their perspective phenotypes. In the case of cortical neurons, the final destination will be in one of a number of layers (laminae) that make up the cortex. The laminar organization of the mammalian cortex is well-conserved across species (e.g., [61]). The cerebral cortex typically consists of six layers of varying

volume and depth depending on the cortical region. Layer I is the outermost layer of the cortex and contains no cell bodies, layer II, contains small granule neurons, layer III houses a number of cell types including pyramidal neurons, layer IV, also known as the internal granular layer, contains granule cells that receive sensory information from the thalamus, layer V consists predominately of large pyramidal neurons and is the major output layer of the cortex and, finally layer VI, that forms the innermost border between the cortex and the underlying white matter, is a non-descript layer in terms of cell type. Although the cell body (soma) of specific neuron populations may arise in a given layer their processes extend into other layers. Through these interlayer connections neurons within each layer make up the cortical columns of the cortex that subserve similar functions. Figure 1 illustrates how neurons begin as rather ‘simple’ cells within their perspective layers with little overlap, but as development continues neuronal processes become more complex, extending across and through cortical layers. Once differentiated



**Figure 1.1.** An illustration of the maturation of processes of cortical neurons (in this case from near Broca’s area of the cortex). Neurons begin as simple cells with little overlap between layers. During maturation cells become increasingly complex and processes overlap into other layers. Adapted from *Biological Foundations of Language* (pp. 160 - 161), by E. Lenneberg, 1967, New York: Wiley, From Kolb, B. & Whishaw, I.Q *Fundamentals of Human Neuropsychology* (5th ed), Worth, 2003.

the cells begin to develop processes (axons and dendrites) that extend out from the soma. And in the case of excitatory neurons (pyramidal and stellate neurons), protrusions (spines) that will eventually facilitate communication between cells begin to develop out from these processes. Although a neuron will have only one axon, most neurons have an abundance of dendritic ‘trees’/branches from which they interact with other cells.

During early prenatal development brain cells are developing at a rate of about 250,000 per minute (Cowan (1979), cited in [79]), reaching well over the estimated 80 billion by the 20th gestational week in humans [91]. After the peak period of neurogenesis, neuronal numbers begin to decrease through a process of neuronal pruning, and by the end of the third trimester only about 50% of the original neurons are left viable. In humans, synaptogenesis begins prenatally (occurs postnatally in rodents), creating the beginnings of what will be the adult pattern of organization. Synaptogenesis continues postnatally, with new connections now being shaped by the external, as well as internal environment. In fact, most of the connections made postnatally will be, to a large degree, a product of the environment and the organisms’ interactions within it. Thus, the mammalian brain continues to develop long after birth and, in the case of the prefrontal cortex, continues to do so into early adulthood [27]. Brain development then is an active process that, once set in motion by genetic factors, becomes increasingly individualized by the influence of an organisms’ unique experience. For a more in depth description and review of the human cortex see [60] and, for the rat brain see [78].

Although the brain may be considered ‘mature’ sometime in early adulthood the brain, and especially the neocortex, continues to be malleable to some extent throughout

life, allowing for a degree of change that will facilitate learning and memory (see [66,68]).

### ***Brain Plasticity***

The brain has an exceptional capacity for change. It is this ability to change in response to experiential factors, an essential characteristic of the brain that is commonly referred to as brain ‘plasticity,’ that allows the individual to adapt and learn from experience. To do this the brain must have an ‘imprint’ of sorts, of past experiences that can be used to update responses for use in similar, future circumstances. Thus the brain carries traces of past experiences that will influence future behaviour.

Although the brain retains a certain level of plasticity throughout the lifespan, there are periods during development when the capacity for change is much higher than in the mature brain. A prime example is during late prenatal and early postnatal cortical development in primates (for review [42]), as well as non-primate species, such as rats (for review [66]). During this period there is an exceptionally high level of plasticity that is thought to be related to an overabundance of synapses that are undergoing pruning and reorganization (see [58]). To be malleable however, means being vulnerable to negative influences as well.

### ***‘Critical’ vs ‘sensitive’ developmental periods***

During the developmental process there are both critical and sensitive periods [8]. The term ‘critical period’ refers to a developmental period when exposure to particular stimuli is required for systems or circuits to develop appropriately. Some of the earliest

and most influential evidence for the existence of ‘critical periods’ came from the Nobel prize winning studies of Hubel and Wiesel [54]. The researchers identified a critical period in visual development, showing that normal development of ocular dominance columns required visual stimulation at a specific period in development. The absence of such stimuli resulted in irreparable visual deficits when the visual deprivation occurred during a particular window of development, with little affect either earlier or later in development.

In contrast, a ‘sensitive period’ refers to a period of increased sensitivity to experiential factors. So, although there are periods when the brain is either more or less sensitive to a particular stimulus, the greatest impact of such an experience occurs during a certain time frame or window [8]. For example, in the rat there are periods in early postnatal when the brain is more (first week of life) or less (second week of life) vulnerable to insult as shown in a series of studies by Kolb and colleagues (see [67,70,71]). The proposed mechanisms attributed to the age-related plasticity include variations in the availability/expression of various growth factors, the developmental processes occurring at the time of injury (e.g., synaptogenesis, gliogenesis), as well as the pre-injury environment (see [68]). The effects of early stressors (maternal separation) on brain structure also vary along developmental time lines that correlate with the development/maturation of the endocrine stress system [18]. For example, Bock and colleagues found opposing changes in neuronal response of the prefrontal cortex to stress in young rats exposed to maternal separation prior (reduced spine density) compared to after (increases spine density) the stress hyporesponsive period of the developing adrenal system. So, although ‘critical’ inputs may not be necessary for normal development of

the prefrontal cortex during these stages, ongoing developmental processes create an environment of increased sensitivity to external factors. Further, the location and influence of these experiences in the brain will be determined for the most part by the systems that they activate [6].

Adolescence appears to be another ‘sensitive’ period of development. A large body of research suggests that the brain may be particularly sensitive to environmental influences during this maturation period (for review [2,4,6]). Interestingly, adolescence coincides with ongoing proliferation and maturation of neuronal processes in regions believed to be involved in higher cognitive function (e.g.,[105]) in the rat, as well as in humans [29]. Therefore, it would be predicted that the PFC, a region that subserves higher cognitive function would be at greatest risk for negative environmental influences, such as drug exposure, and indeed this appears to be the case (see [5]).

These examples do not suggest that experience cannot also leave a lasting impression on the mature brain as well. In fact there are times when the adult brain may be more susceptible to environmental factors. For example, Meaney and colleagues [19], found that prolonged periods of elevated corticosterone (a model that mimics chronic stress) in young and adult rats resulted in cognitive deficits in adulthood following adult but not early exposure.

The distinction then is that a ‘critical’ period has a definitive beginning and end, whereas a ‘sensitive’ period is the point of greatest experiential impact although not necessarily without impact at other time frames as well [8]. Determining ‘critical’ and ‘sensitive’ periods is important to the investigation of environmental influences on brain structure and function, as they exemplify that the developmental process itself is a

determining factor in the brain's response to any given experience. It also implies that with late developing cortical regions such as the PFC, there is a prolonged period of susceptibility to the influences of experiential factors.

## **II. Prefrontal Cortex**

### ***Executive function***

The prefrontal cortex (PFC) is the control center for cognitive function, engaged during tasks that require 'higher' information processing. As Miller and Cohen [86] point out, the PFC is not required for simple behaviours or those behaviours defined as inflexible, such as behaviours that are innate or automated responses to a particular stimulus. Indeed, decorticate rats are able to perform the rudimentary components of most behaviours, but appear to lose the capacity to extend and build upon these as would be required for more complex activities [114]. Rather, the PFC with its unique and extensive interconnections has the challenging task of choosing appropriate actions to achieve a given goal against a backdrop of competing, incoming stimulus information. A unique characteristic of the PFC is that it sends and receives information from all other sensory and motor cortical regions, and many subcortical areas as well (see[86]). These connections allow the PFC to carry out a variety of cognitive tasks which fall under the umbrella phrase 'executive function'.

The term 'executive function' is used to cover an array of cognitive functions that include planning, sustained attention, 'rule' learning, goal-directed behavior, behavioural inhibition, behavioral sequencing, and decision making [34]. A defining characteristic of 'executive function' is the ability to make comparisons or contrasts, deciding to choose

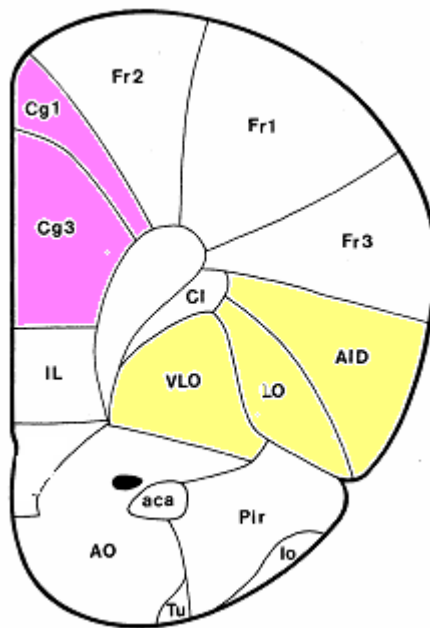
one behaviour/goal over another. That each of these functions can vary within and across situations is what provides humans with an almost infinite repertoire of behaviors and characterizes individual differences. Further, the ability to select appropriate responses to stimuli/events and put it in the correct social context is fundamental in complex societies, and indeed damage to the PFC produces obvious anomalies in social behaviour [7] .

### ***Defining the Prefrontal in the rat***

Whether or not the rat PFC is comparable to that of primates is debatable. Nonetheless, the rat PFC is comprised of heterogeneous regions of cortex that to some extent can be delineated by anatomy, connectivity, as well as function. Dalley and colleagues [34] have defined the prefrontal cortex (PFC) in the rat based on Rose and Wooley's (1948, cited in [34]) definition of the prefrontal region as areas of the cortex with reciprocal connections with the medial dorsal nuclei (MD) of the thalamus and Uylings and van Eden [108] description. The authors propose that the rat PFC can be divided into three general regions that include the medial, lateral, and ventral prefrontal cortex. Proposed sub-regions of the medial prefrontal cortex (mPFC) include the precentral, anterior cingulate, prelimbic, and infralimbic cortical regions. The lateral PFC includes the lateral orbital cortex (LO) as well as the dorsal and ventral agranular cortex (AID & AIV, respectively). The third region consists of the remaining ventral cortical area above the olfactory bulb and is subdivided into the ventral (VO) and ventral lateral (vLO) orbital prefrontal cortex.

Zilles [121] however, argues against the inclusion of the infralimbic cortex within the mPFC, pointing to differences in cortical depth, the number of cortical layers, and

connectivity to midline nuclei of the thalamus that set the infralimbic apart from the medial regions above. Rather, Zilles [120,121] assigns the midline cortex above the infralimbic cortex as the cingulate cortex with subregions I–III representing the mPFC. In this schema region III of the cingulate cortex (Cg3) replaces the prelimbic (PL) cortex illustrated in the rat atlas of Paxinos & Watson [94]. For the purpose of this dissertation the nomenclature of Zilles [120] will be adopted when referring to regions of the PFC (Figure 2).



**Figure 1.2.** An illustration of a coronal section modified from Zilles [120] and Schoenbaum, Roesch, & Stalnaker [100]. The yellow area depicts the OFC (with the inclusion of the AI) and the pink area shows the regions that are included in the mPFC.

It should be noted here however, that although the orbital prefrontal cortex (lateral, ventral and medial) in the rat is defined as the ventral cortex located immediately dorsal to the olfactory bulb, owing to the reciprocal connections to the MD, the AID can be considered as part of the homologue of the OFC in the primate. Therefore, the term OFC would also include the AID, referring to the region of the prefrontal cortex that

share similar connections and functions in the primate and rat as illustrated by Schoenbaum and colleagues [100] and Kolb and colleagues [77].

The intention of this review is not to debate the viewpoints put out above relating to the division of the prefrontal cortex, but rather to inform the reader as to some of the issues that remain to be resolved through further investigation of the functions that these regions subserve. The reader should also be cognizant of the fact that although there are substantial anatomical variations in the PFC across species, similarities including connectivity and function do exist that allow for generalizations as well [34]. So, although the rat prefrontal cortex may be less anatomically specialized compared to the primate PFC, it does subserve animal-like cognitive executive functions and as such offers a ‘simpler’ model from which to investigate cognitive processing and mechanisms that can then be extrapolated to the more complex functions of the primate PFC [24].

### ***Function***

Determining specific functions within subregions of the PFC is made difficult owing to the extensive interconnections across subregions of the PFC, as well as the overlap of functions that subserve cognitive processing. For example, working memory, long associated with PFC function and executive function, requires various components of cognitive processing that are likely carried out by different subregions [34]. So, holding information ‘on line’, a necessary function of working memory that requires that relevant ‘rules’ and cues related to the immediate task remain available for use as demonstrated in delay tasks, also requires cognitive functions including switching, attention, and inhibition, processes that are subserved by the Cg3, IL and OFC [34].

Nonetheless, specific behaviours can be attributed to regions of the mPFC and OFC that can provide insight into the cognitive processing affiliated with these regions, and in this regard lesion studies have been invaluable in assessing the regional-specific contributions of the prefrontal cortex.

### ***Medial prefrontal cortical lesions***

The medial prefrontal cortex has been associated with a wide range of behaviours. For example, although emotion is typically associated with the amygdala the expression of learned fear requires the Cg3, as shown by focal excitotoxic lesions to this region [33]. Behavioural flexibility or ‘switching’ from one characteristic to another also requires the involvement of the Cg3 and IL as shown in lesion studies using non-match and match to place paradigms of the T-maze and the five choice serial reaction time task (5-CSRTT) ([34,37], respectively). Deficits in temporal memory or temporal ordering, which requires the memory of not only what has been seen or done in the past but the order that they occurred as well, are also shown in rats using the eight-arm radial arm maze [63] and a modified object recognition task [87] following lesions of the mPFC. Further, Walton and colleagues [113] have shown a role for the mPFC in effort-based decision making as well. In this task, normal rats will switch to a lesser reward when the effort to receive a larger reward becomes too demanding. Rats with lesions of the Cg3 and IL, however, are unable to switch and continue to expend a great a deal of effort to receive the larger reward. Finally, the mPFC also appears to be important in developing appropriate strategies for solving maze problems involving spatial navigation [69]. This list is not all

inclusive but rather a selection to demonstrate the variations of tasks that require the involvement of the mPFC.

### *Lesions of the Orbital prefrontal cortex*

As with the mPFC, lesions to the OFC produce a variety of behavioural deficits although the range may not be as extensive. For example, lesions of the OFC produce many behavioural anomalies that can be associated with deficits in inhibitory control/behaviour. Rats with OFC lesions have difficulty in reversal tasks, showing perseverate behaviour [30]. Unlike the mPFC however, the deficit in reversal following OFC lesions does not appear to be related to changes in perceptual learning, but rather an inability to switch behaviour when the value of expected outcome changes [102]. Thus, Schoenbaum and colleagues [101] point out that the OFC may play a more important role in executive function than previously thought. The researchers argue that behavioural inhibition is a necessary component of executive function that requires inhibiting ‘natural’ or automated responses in favour of a response more appropriate based on additional information and its relevancy to the intended goal.

Aside from reversal learning and inhibitory control, the OFC is probably most often associated with social behaviour, and plays a similar role across species (e.g. [65]). The underlying deficit may be one of perception and production of social signals [95]. The accurate perception of social signals and the need to follow ‘rules’, are essential components of normal play behaviour. The OFC plays a role not necessarily in the production of play, but rather the ability to follow species-typical play behaviour patterns[95]. So, not only do young rats with OFC lesions fail to show appropriate

partner-related changes in play behaviour, but their ‘ignorance’ of socially acceptable behaviour may also be inferred from the tendency of non-lesion rats to reject them as perspective play mates. Again, this review is not exhaustive but highlights some of behaviours that can be attributed to the OFC and related circuitry.

### ***Connectivity***

Aside from the connectivity to the thalamus already described, the PFC has extensive cortico-cortical connections with the posterior parietal cortex and sensory cortices. Subcortical structures including the nucleus accumbens and striatum also have reciprocal connections, direct and indirect with the PFC. Further, there are extensive connections with the amygdala, hippocampus (especially the Cg3 and CA1 subfield), and hypothalamus, all of which contribute to the extensive range and variability of behaviour exhibited by the PFC. Moreover, the PFC sends and receives projections from the major nuclei of the neuromodulatory systems (including the cholinergic and monoamines).

Neuromodulators play a significant role in PFC functioning. For example, when attention processes are activated by PFC-related tasks there is an increase of ACh into the PFC [92], and not surprisingly then the depletion of acetylcholine (ACh) in the PFC produces deficits in attention processing [106]. Serotonin (5-HT) has also been implicated in information processing of the PFC, with 5-HT depletion in the PFC producing deficits in reversal learning in the monkey, a task related to OFC functioning in both rat and monkey studies[32]. Finally, both dopamine (DA) and norepinephrine (NE) are essential to cognitive processes in the PFC. Moreover, optimal functioning of the PFC requires that specific levels of DA and NE are available during cognitive

functions. An over abundance or depletion will produce cognitive deficits [9,11]. So, for example over stimulation of the D1 receptor in the prefrontal cortex has been associated with cognitive deficits in both rats and monkeys (see [88]).

### **III. Experience**

It would be impossible to discuss the influence of experience on brain structure without at least a brief mention of some of the most influential scientists that have driven this line of inquiry. Some of the early contributions date back to the historical work of Ramon Y Cajal and his foresight in proposing that the neuron was the functional unit of the brain. The work of Hebb extended this proposal by adding that early experience that could influence performance learning would occur through the strengthening (efficacy) of existing synapses [51]. Just over a decade later Bennett and colleagues (e.g.,[14]) were the first to actually demonstrate that the brain could be structurally altered by experience, citing changes in gross morphology such as brain weight and cortical thickness as well as changes in acetylcholine levels of animals housed in enriched environments. At about this time, Hubel and Wiesel [53,116] had also released the first evidence of activity-dependent structural plasticity. In these studies Hubel and Wiesel discovered that denying sensory input in one eye of the cat during specific periods of development resulted in a significant reduction in the number of neurons in the visual cortex activated by later visual input to the once occluded eye. It was not until the early 1970's, however that Greenough and colleagues (e.g.[48]) were able to demonstrate the first evidence of morphological changes at the cellular level. The researchers found that rats exposed to complex environments or training showed alterations in dendritic morphology and spine

density using Golgi staining techniques. In the 1980's Kolb and colleagues applied the principles of brain plasticity discovered through lesion studies, namely that the brain has the ability to reorganize to compensate for injury, to investigate the effects of enrichment on the injured brain. Kolb proposed that as similar mechanisms of plasticity were initiated by learning and injury, enriched housing may prompt these mechanisms during developmental times when the brain was less able to compensate for injury (see [71-73]).

These studies that began with Cajal and continue today in the labs of Kolb, as well as Greenough and many others, provide invaluable evidence in support of the contention that all experiences, including injury, learning, drug use, and even development itself, shape and modify brain connectivity/structure. How and where these changes occur is not clear.

When searching for the mechanisms that might support experience-induced plasticity it makes sense to look for changes that occur concurrently with the experience. Indeed, molecular changes have been identified that occur during learning. For example, it has been found that both levels of CREB (cAMP response element-binding) and  $\Delta$ FosB (delta Fos B), transcription factors that alter gene expression, are increased during learning, including addiction (see for review [62,89,90]). There is also accumulating evidence of experience-induced changes in neurotrophic factors, such as BDNF (brain derived neurotrophic factor) and FGF-2 (basic fibroblast growth factor) that may mediate learning [43,83]. Although the above is only a small sample of the changes in proteins and gene expression that might occur during learning, there may be a common theme in all molecular changes, which is that they are relatively transient. Yet, many of the behavioural effects associated with learning are enduring and it would be expected that

they would need to be supported by changes in the brain that are also relatively permanent [90]. This argument would also pertain to changes in neuromodulators, such as altered catecholamine levels, discussed earlier. So, once again although NE and DA may facilitate learning and memory processes and perhaps even initiate events that would produce more permanent changes, their influence is also transient.

It is argued that structural changes that include altered neuronal morphology are excellent candidates when looking for a mechanism that would support the permanency of learning and alterations in brain circuitry [90]. Indeed, models of learning, such as enriched/complex environments, formal training, and psychostimulant use all show alterations in structural morphology that would meet the requirements of relative permanency needed to support long term adaptations in behaviour.

### ***Enriched/complex environments***

The terms ‘enriched’ or ‘complex’ as used in relation to housing are interchangeable and require further definition. It is recognized that enrichment can refer to any environment that in some form or the other enhances or increases the amount of stimulation offered by the experience. In the studies cited in this review the terms ‘complex’ and ‘enriched’ housing refers to housing conditions that provide the potential for increased stimulation through exploration and interaction with objects placed within the enclosure. Further, the enclosure itself is considered ‘enriched’ in that it is much larger relative to the standard housing typical of the perspective laboratory, increasing exploratory behaviour. It is also typical for animals in complex environments to be group

housed, and depending on the standard housing practices of the perspective lab, control groups may or may not be group-housed (see Diamond for review [36]).

Complex housing is undoubtedly the most studied model in the investigation of experience-induced plasticity in the laboratory. The robust effects of differential housing on structural morphology in a number of cortical areas, as well as the ease of its application have likely added to its popularity. Another benefit is that it can be applied in diverse study subjects, adding to the testability of the task in diverse populations.

### Early Enrichment

The belief that in general the young brain is highly malleable and influenced by environmental factors is exemplified by the effects of differential housing on neuronal morphology. Beginning with the early experiments by Greenough and colleagues it was recognized that complex housing produced increases in the dendritic fields of the occipital, parietal and temporal cortices of post-weaning rats (e.g., [48,74,110,112]). Housing-induced alterations in dendritic morphology in rats are not restricted to the post-weaning period, however. Venable and colleagues [109] found significant increases in the number and length of dendrites in the occipital cortex of rats that spent time in complex environments beginning 10 days after birth until weaning (postnatal day 24). Differential housing also influences dendritic morphology in the visual, sensory, and auditory cortices in young pigs [56]. In a study investigating the effects of ‘indoor’ versus ‘outdoor’ rearing on dendritic morphology researchers found that outdoor rearing conditions produced increases in branching of neurons in the auditory cortex, whereas the indoor rearing condition produced increases in branching in all three cortical regions.

### Adult Enrichment

In a number of studies by Greenough and colleagues [44,45] they established that both the middle aged and aged laboratory rat displayed robust changes in neuronal morphology in the visual and sensory cortex. The location of dendritic changes that occur in the visual cortex of the adult also appear to be comparable to those found during development, with increases occurring on basilar dendrites in both cases [107]. So, similar to early exposure, adult animals housed in complex environments show increases in occipital and sensory cortices, as well as subcortical structures such as the nucleus accumbens (NAc) [50,76]. One exception may be spine density. Kolb and colleagues [74], compared the effects of differential housing in animals placed in complex environments at different ages. The researchers found age-related variations in the brains' response to the environment (juvenile enrichment produced decreases in spine density versus and increases with adult enrichment).

Interestingly, only one study to date has shown effects of differential housing on neuron morphology of the prefrontal cortex and this was in adult primates [81].

### ***Training***

In comparison to numerous studies related to the effects of complex housing on brain morphology, there are relatively few studies that have investigated the effects of training in the laboratory animal. Admittedly, there is little doubt that learning occurs in complex housing conditions, and thus the results may provide some generalization to other paradigms of formal learning as implied by Rosenzweig and Bennett [98]. Nonetheless, experience-dependent variations are inherent in the contention that

structural changes can be linked to behaviour, and therefore predict that anatomical changes would vary across learning paradigms. It is prudent therefore to review these studies separately.

Altered neuronal morphology has been established in a number of learning paradigms. Greenough and colleagues were some of the first to demonstrate that formal training could induce alterations in brain structure at the cellular level. In a study that investigated the effects of training in a visual spatial maze task, Greenough and colleagues [46], found that training produced increases in dendritic branching of the occipital cortex (Oc). In a follow up study that was designed to control for incidental learning, Chang and Greenough [28] demonstrated that both the earlier and current increases in dendritic morphology in the visual cortex could attributed to visual learning itself. Using the knowledge that 95% of the visual pathway crossed to the contralateral hemisphere, Chang and Greenough occluded one eye of the test subjects and dissected the corpus collosum to ensure no transfer of information between hemispheres.

In a set of later studies Greenough and colleagues [47,118], demonstrated that training-induced alterations in dendritic morphology were not restricted to the visual cortex. Taking advantage of the high degree of unilateral motor control in the frontal cortex, the researchers were able to dissociate the effects of non-specific and specific training. The results demonstrated that training produced selective changes in the dendritic morphology of sensory –motor cortex in layer V neurons. Of interest was that the effects varied between layers of the same region. So, although training increased layer II and III dendritic length and branching, these effects were not training specific per se as they occurred in both the ‘trained’ and ‘untrained’ hemisphere. In contrast, changes in

LV neurons were specific to the trained hemisphere. Another form of motor-training – acrobatic training- has also been shown to prompt changes in dendritic morphology of the cerebellar cortex [17]. Using an ‘exercise’ control group, the researchers were able to demonstrate that the effects were not due to activity per se but rather the learning/enhancement of motor skills.

Additional evidence for training-induced changes in cortical neuron morphology comes from a recent study that investigated the morphological effects of odor discrimination learning in the piriform (olfactory) cortex of rats [64]. Knafo and colleagues found increased apical spine density, albeit transient, as a result of learning the task. Paramount in these and subsequent studies is that they suggest the existence of a direct relationship between structure and function.

Importantly, there is evidence that would indicate a similar mechanism of cellular change as a result of training occurs in the human brain as well. Understandably, the opportunities to investigate structural changes in dendritic morphology in humans are restricted and must rely on post-mortem studies that are plagued by possible confounding factors.

### Human studies

The advances in imaging techniques have provided a means for the study of experience-initiated changes in humans that have otherwise been restricted to post-mortem studies. For example, using PET imaging in human subjects, researchers have been able to determine a time dependent activation of the PFC during motor- sequence learning. The results revealed that the PFC and posterior parietal cortex (PPC) were activated by novel or new learning but once the task became well learned the PFC

activation no longer occurred during task performance [57]. Extensive motor training in humans has also been shown to produce alterations in the somatosensory map. Elbert et al., [39] found that musicians who played string instruments had increased left-hand representation in the cortical map, with the greatest amount of reorganization occurring in subjects who started musical training earliest. Interestingly, in a later follow up study [25] the researchers were able to show that behavioral modification therapy was effective in returning the somatosensory map to a near normal state. The implications are that once altered by experience the somatosensory cortex remains flexible and receptive to future experience.

### ***Psychostimulants***

Psychostimulant use produces a behavioural phenomenon referred to as sensitization, an augmented motoric response with repeated exposure. Behavioural sensitization has been linked to the development of aberrant behaviours, such as increased incentive and drug- taking behaviour associated with addiction (e.g.[96]). Moreover, these behaviours continue to be represented in neural circuitry long after drug use has discontinued, as can be inferred from the ease of reinstatement following withdrawal (see [55]). Behavioural sensitization has also been directly linked to alterations in the structural morphology of stellate cells in the NAc and correlated with changes in dendritic morphology of pyramidal neurons in the prefrontal cortex, as well. Furthermore, in a series of experiments Robinson, Kolb and colleagues (e.g. [2, 6]) have shown that structural changes in the dendritic morphology of neurons in the NAc and PFC as a consequence of either cocaine or amphetamine use persist long after drug use has ended.

Evidence that the alterations in dendritic morphology were indeed representative of altered circuitry was later provided by Berlanga and colleagues [15] using electron microscopy techniques (EM). The researchers found that subjects that displayed behavioural sensitization during a cocaine drug-treatment period also showed a 49% increase in synapse per neuron ratio in the NAc core after a 3 week drug wash-out period. More specifically, the changes were related to a specific type of spine that receives glutamatergic input from the PFC and limbic system.

Consequences of the structural changes in neuronal circuitry were not limited to the drug taking experience, however. In later experiments Kolb and Robinson [75] identified long-term consequences in learning-related changes associated with complex environments. The researchers found that previous drug experience inhibited learning-related plasticity, showing an inhibition in the morphological changes in response to the environment. Together these studies provide evidence for structural modifications linked to experience-induced behavioural changes and suggest experience, such as drug use, that produce persistent structural changes may alter subsequent experience-induced plasticity [75,97]. Moreover, it gives rise to concerns related to early psychostimulant use during the development years when the brain may be more susceptible to environmental influences.

These concerns extend to the use of caffeine, the most widely used stimulant, as well. Caffeine is considered an atypical psychostimulant in that it is believed to be one of the only psychostimulants that produces tolerance rather than sensitization to DA agonistic effects in the NAc [35]. Nonetheless, similar to other psychostimulants, chronic

caffeine exposure has been found to alter dendritic morphology in the cingulate region of the PFC [59].

### Methylphenidate

Amphetamine has been used for the treatment of childhood attention deficit hyperactivity disorder (ADHD) since the mid 1930's before being displaced as the treatment of choice by methylphenidate in the 1960's. Methylphenidate is the generic form of the brand name Ritalin®, now the most commonly prescribed therapeutic agent in the treatment of ADHD. One of the earliest clinical trials of Ritalin was not related to ADHD however, but rather the efficacy of Ritalin in the treatment of aged-related decline in cognitive and motor skills [38]. In a population of institutionalized patients that were not being treated for additional psychiatric disorders, Ritalin was found to attenuate cognitive, and to a lesser degree motor deficits in dementia. Thus, it has long been recognized that Ritalin may be beneficial for the enhancement of learning and memory, independent of its therapeutic effects in treating ADHD. In fact it was not until the 1960's that Ritalin began being marketed as a treatment for ADHD. Now, after almost 50 years of clinical use in children, there is growing concern related to the potential long-term effects of this drug [1,2,12,111]. There is little doubt that this concern comes in part from the fact that the mechanisms by which Ritalin exerts its therapeutic effects are still unclear and as such the long-term effects of Ritalin use are unknown. In addition there is evidence that, owing to ambiguous diagnostic criteria and the difficulties in diagnosing children, many children may be inappropriately exposed to psychostimulants during development [84].

Although MPD is similar to amphetamine in structure, the action by which it increases dopamine (DA) availability more closely resembles cocaine. As with most other psychostimulants, MPD acts predominantly on dopaminergic neurons whose cell bodies are located in the ventral tegmental area (VTA). The VTA, is part of the large midbrain dopaminergic system that sends projections to the cerebral cortex, particularly the prefrontal cortex (mesocortical pathway), and basal ganglia (mesolimbic pathway). So, although VTA dopaminergic neurons innervate both cortical and subcortical regions, separate pathways allow for differential activation of these regions. Indeed, Berridge and colleagues [16] found that low doses of MPD preferentially activates NE and DA neurotransmission in the PFC, while having little effect on DA or NET levels in the NAc.

In humans, clinical doses of MPD range between 0.25 and 1.0mg/kg/day. Based on these doses blood plasma levels would be between the range from 8 – 40 ng/ml [104]. Berridge and colleagues [16], have determined doses that result in comparable blood plasma levels in rats using different modes of administration. The researchers found that i.p. administration of 0.5mg/kg MPD produced blood plasma levels of  $36 \pm 3$  ng/ml, with peak concentrations occurring five minutes post-injection. Comparable levels with oral administration were found at 2.0 mg/kg (blood plasma levels  $22 \pm 7$  ng/ml), with peak concentrations occurring 15 minutes post-consumption.

### *Behavioural effects*

Research has shown a diverse range of behaviours that are influenced by Ritalin use in laboratory animals. Unfortunately there are many inconsistencies in the results, making it difficult to draw conclusions with any certainty. Many of the inconsistencies are likely related to difference in age at the time of first exposure (especially relevant in

early exposure), dose, duration of treatment, mode of administration, and time of testing (immediate or delayed behaviour tests) after last administration.

Early exposure.

Whereas some studies have found that chronic Ritalin use produced behavioural sensitization, as inferred from enhanced motor activity (e.g.[26,40], others have reported no influence of early Ritalin use on motor activity [119]. There are also discrepancies in the reported influence of early Ritalin exposure on the development of cross-sensitization (an increased response to subsequent exposure to psychostimulants other than Ritalin). For example, there are a number of reports of an increased behavioural response to cocaine following a period of chronic Ritalin use in adolescence [3,22,49]. Yet, again there are also those studies that dispute that this behaviour is a necessary outcome of early Ritalin exposure. Other inconsistencies in findings include the potential for a reduced latency to self-administer psychostimulants such as cocaine [13,22]. Untreated rats will also self-administer drugs with continued exposure, the measures used in self-administration studies is not just the amount that the rats will self-administer but also the length of the time to develop or learn the behaviour, with shorter latency and increased amount of self-administration indicating a propensity to drug addiction.

Behaviours other than those related to the potential risk for addiction, have also been investigated, but to a much lesser degree. Some of the general behavioural effects found include a decrease motivation to initiate copulation, decrease in response to natural rewards, and increased anxiety [20].

### Adult.

Similar to early Ritalin exposure, research on the behavioural effects of Ritalin use in adults also has inconsistencies related to whether or not chronic exposure produces behavioural sensitization. For the most part the differences are related to dose, with low doses (0.5mg/kg – 1.0mg/kg) resulting in increases motor activation [16,82] and higher doses (2.0 mg/kg – 20.0 mg/kg) either no effect or a decrease [16,85]. A similar dose-related effect is also found in cognitive tasks with doses of 0.5 mg/kg – 2.0 mg/kg enhancing performance on tasks such as the T-maze, novel object recognition tasks and at higher doses (8.0 mg/kg – 10mg/kg) producing deficits or no influence [10,16,31]. It would also appear that doses of Ritalin over 5.0mg/kg are needed to increase self-administration of cocaine with subsequent exposure [99].

### ***Implications of Experience-induced changes***

This review of experience-induced changes in the brain focused on structural changes in morphology arguing that permanent alterations would be needed to support long-term behavioural changes. It is important then to consider the consequences and application of these findings. Indeed Hebb, who found that complex rearing conditions enhanced cognitive performance in maze task into adulthood, provided the first clue that early experience could alter later cognitive functions [51]. Although Hebb's experiments did not include structural morphology, the implications from later experiments would suggest this was indeed the case. The findings of long-term structural changes following both training and complex housing have also been applied to studies on recovery from brain injury. As already noted, Kolb [72] showed that complex housing could enhance

functional outcome after early frontal lesions in rats. An earlier study by Whishaw and colleagues [115] also found that hemidecorticate rats could benefit from such experiences post-operatively, showing increased functioning relative to standard housed operates. Briones and colleagues [23] have also shown that complex housing following brain ischemia in adult rats facilitated recovery and performance on a maze task. Thus, the benefits of complex housing do not appear to be age dependent. Indeed, Winocur [117] found that prolonged housing in complex environments enhanced performance on cognitive tasks, whereas moving rats from a complex to impoverished environment produced a decline in performance.

With what seems like endless evidence of the benefits of complex housing both during development and at times of injury or age-related decline, it might be surprising to find that complex environments may not always be beneficial, and may in fact increase the risk for later abnormal behaviour. For example, both Bowling [21] and Hill [52] found that the effects of early rearing in complex housing enhanced the behavioural response to later psychostimulant exposure (amphetamine and cocaine, respectively), suggesting an increased risk for later drug addiction.

#### **IV. Procedural Considerations**

##### ***Golgi-Cox analyses, an antiquated technique for modern questions?***

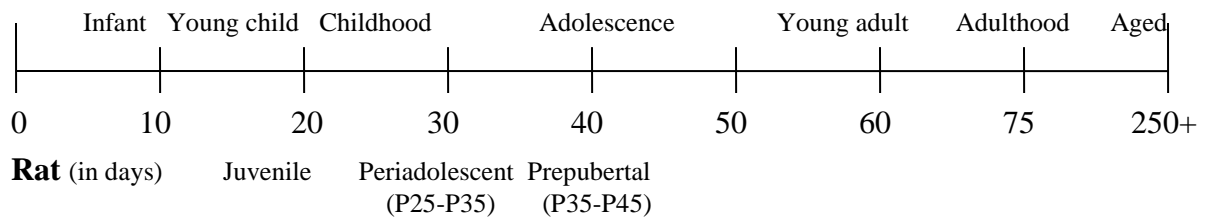
The studies that follow employ what some may regard as an antiquated technique for dendritic analyses. Although it is true that the original Golgi staining methodology was developed decades ago and more modern, technologically advanced alternatives are now available, the advantages that this technique offers have as yet not been met, or

surpassed [80]. First, Golgi-staining techniques offer random staining of between 1 to 4 % of neurons [93], a necessary component for unbiased analyses. Second, the staining technique allows for a one time staining of random cells throughout the brain. Thus, areas of interest may be expanded upon without the need for experiment replication, effectively conserving both time and animal use. This is especially beneficial when the research question is more general, with less specified areas of interest. Third, the reliability of the technique has been well established by decades of research. Fourth, the revised methodology outlined by Gibb and Kolb [41] provides a method for visualizing general dendritic morphology as well as dendritic spines, as well as being cost efficient.

### ***How old am I?***

The animal research is inundated with terms like “juvenile”, “adolescence”, “periadolescence”, “pre- and post- adolescence”, “adult”, “mature”, etc. The terms are intended to represent specific developmental periods in both human and non-human species. It is difficult however to draw parallels across species, as not only does the maturation rate differ significantly among species, but also developmental markers that signify the transition from one phase to the next (e.g. puberty in humans) are difficult to pin point. Below is a guide that can be used in translating the human and laboratory rat data into comparable time points of development. The chart provided represents an estimate of the developmental time lines, and has not been standardized, so must be used cautiously.

**Human**



modified from Anderson ([6] pg 428).

**Rationale**

The literature review highlights the structural and functional impact that experience can have on the brain. Owing to the fact that experience can continue to modify brain structure throughout life, purportedly as a support mechanism for continued learning and behavioural adaptations, the implication would be that early experiences that alter structural connectivity would have life-long influences on the brain's response to environmental factors. Variations in postnatal experience-induced changes in the structural morphology of the brain are found at different times during development, with periods of vulnerability correlating with maturation of specific regions. Thus, the late developing prefrontal cortex may be especially susceptible to early experience. Few studies however, have investigated experience-induced structural changes in the prefrontal cortex, a cortical region involved in various forms of cognitive and social behaviour, and none have investigated the relationship between early structural changes in the prefrontal cortex and later experience-induced plasticity and behaviour.

The experiments that follow were designed to answer a number of questions. The first question, "Are the effects of early experience imprinted in the pattern of connectivity in the prefrontal cortex?" A second related question was, "If so, how do these changes impact on the capacity of the brain to be shaped by future environmental factors, including learning?" The third question asked was "Are there functional correlates related to patterns of connectivity in the prefrontal cortex that can be observed in cognitive and social behaviour?"

Psychostimulant (Ritalin) use was chosen for the early experience as it represents a realistic model of human experience, and because psychostimulant use has been shown to alter connectivity patterns in the prefrontal cortex. The subsequent experiences (e.g.,

T-maze and social skills) were chosen because of their relationship with the prefrontal cortex. In a series of experiments (four) the behavioural effects of early, chronic exposure to low doses of Ritalin were assessed, with the behavioural results from one experiment motivating the design of each subsequent study. The behavioural analyses were then studied in relation to the anatomical data to elucidate functional and structural correlates. Two additional studies were conducted to determine if: 1) the results of early Ritalin exposure were unique to the early postnatal development period; and, 2) whether the cognitive tasks chosen indeed altered structural connectivity in the 'normal' adult brain. The dose of Ritalin used remained constant across all of the experiments, eliminating the potential for dose-related differences across studies and ages.

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# **I. Enduring effects of early methylphenidate use in rats: anatomical correlates of modified behavior**

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**Abstract**

Adolescence is a time of ongoing synaptic pruning and organization, and a time when the brain is especially vulnerable to environmental influences. Experiences that alter developing circuitry during this time have the potential to produce permanent changes in brain organization and behaviour. In a series of experiments we assessed the behavioural and anatomical consequences of early stimulant exposure in male rats. Rat pups were administered Ritalin (0.5 mg/kg s.c.) or vehicle (0.9% saline solution) twice daily in a novel environment for twelve consecutive days, beginning postnatal day (P) 22 or P24. Drug-response was recorded during the treatment phase, as well as the caffeine (0.015 mg/kg i.p.) and amphetamine (1.0 mg/kg) challenges to assess Ritalin-induced sensitivity to other stimulants. Short-term, anatomical effects were assessed in animals sacrificed immediately following the caffeine challenge (Exp.1), whereas the long-term anatomical analysis was conducted in animals following behavioural (cognitive, social, and drug sensitivity) evaluation of animals in a delayed non-match to sample T-maze task, play behaviour, and an amphetamine challenge. Behaviourally, early Ritalin exposure produced a very moderate increase in motor activity in juvenile rats during the initial treatment phase but no apparent long-term sensitivity to subsequent acute stimulant exposure. Treated rats also showed abnormal social behaviour, displaying reduced play initiation relevant to saline-treated playmates and impaired cognitive performance. Anatomically, dendritic morphology evaluated one week post-treatment, or in adulthood, revealed no changes in the NAc, but showed layer-specific alterations in dendritic fields that varied with timing of assessment. For example, increases in Cg3 LV neurons were found in rats sacrificed shortly after the 12 day treatment period, whereas rats exposed to further training and sacrificed in adulthood showed changes in Cg3 LIII neurons.

## **Introduction**

Experience-induced alterations in synaptic connectivity are believed to be an adaptive response to changing demands on neural networks within the central nervous system. The underlying mechanism(s) involved are unknown but appear to involve combinations of factors that include enhanced capillary formation, (e.g., [4]), induction of long-term potentiation (e.g., [34]), as well as altered protein production (e.g. [8,15,33]); all of which have the potential to increase the efficacy and/or strength of existing synaptic connections. As well, synaptic connectivity may be altered via enhanced or reduced dendritic fields, thereby creating changes in receptive dendritic area. For example, in a series of experiments Kolb and colleagues have shown that experiential factors such as housing in a complex environment [16], tactile stimulation [23], and cerebral injury [17,21,22], all influence synaptic connectivity as inferred from changes in dendritic arborization. Other experiences such as stress [27], task learning [4], and stimulant drugs [29,30], to name a few, have been associated with changes in dendritic arborization as well ( but see, [14] and [13] for review). Notably, experience-induced changes in dendritic morphology are areal-specific, producing changes in regions involved in processing the event-related stimuli. So for example, rats housed in enriched environments that are rich in visual and tactile stimuli show structural alterations in the visual and sensory cortices (e.g., parietal cortex [20]), but not the prefrontal cortex [10,20]. On the other hand stimulant drugs that have a predominant influence on receptors of neurons that ultimately project to, and influence prefrontal cortex and goal-directed behaviour produce structural alterations in the prefrontal cortex and striatal circuitry (e.g., [6,12,29,30]).

It follows that structural changes could also reflect a maladaptive process whereby adverse experiences promote alterations in neuronal connectivity that hinders development of appropriate responses to future experiences. Indeed, research by Kolb and colleagues [18] has shown that experience-induced changes in dendritic morphology can be blocked by previous exposure to psychostimulants such as amphetamine, cocaine or nicotine in adult rats. For example, whereas enriched housing normally increases dendritic length and spine density in the nucleus accumbens (NAc) and sensory cortex, this effect was blocked by prior exposure to psychomotor stimulants [27]. Curiously, the blockade of experience-dependent changes occurred in the sensory cortex even though the drug did not induce dendritic changes in sensory cortex. Importantly, the structural changes in the NAc were directly linked to the development of behavioural sensitization; a drug-induced increase in response that characterizes the initiation of drug-taking behaviour in addicts [5]. The functional significance of alterations in PFC circuitry is less well understood, but is thought to be related to the loss of inhibitory control that also characterizes a behavioural component of addiction; compulsive drug seeking (e.g.,[30]). The implications of these studies are useful for determining the neural basis for the persistent behavioural changes in drug addicts and lend insight into the resiliency of addictive behaviour.

Experience-dependent changes in the adolescent brain may be especially vulnerable to the influence of psychoactive drugs [1]. Moreover, the cerebral cortex may be especially affected as it continues to develop throughout the early postnatal period. In fact, in the case of the prefrontal cortex, which includes the latest developing cortical regions, maturation is not complete until early adulthood. The long-term consequences of

early drug exposure thus may interact with endogenous developmental changes and the effects of other forms of experience.

There is evidence that early amphetamine exposure in male rats [6] produces alterations in PFC dendritic morphology following early amphetamine exposure in male rats that are similar to those found following adult exposure, albeit at much higher doses. The long-term behavioural consequences of early stimulant exposure have not yet been determined, however. In a series of three experiments we used clinically relevant doses of methylphenidate (MPD;Ritalin), a widely prescribed stimulant for the treatment of adolescent attention deficit hyperactivity disorder (ADHD), to investigate the behavioural and anatomical consequences of early stimulant exposure in male rats. Indices of behaviour included motor activity as an assessment tool for both drug response and sensitivity to subsequent stimulant exposure, as well evaluation of play behaviour and performance in non-match to sample task to assess social and cognitive development. The latter behaviors were chosen because it is known that frontal lesions disturb both types of behavior so it might be expected that Ritalin-induced alterations in prefrontal neural networks could influence these types of behaviours.

## **General Materials and Methods**

**Subjects.** All subjects were male, Long-Evans rats that came from multiple litters. The pups were assigned to one of two groups (saline or Ritalin) in a pseudo-random fashion that matched body weights between groups. The pups were obtained at the time of weaning (postnatal day (P) 22) and grouped housed in standard laboratory hanging cages and maintained on a 12 hour light/dark cycle in a temperature controlled environment.

Each cage contained rats from both treatment groups. At the end of the experiment all rats were given an overdose of sodium pentobarbital and intracardially perfused with a 0.9% saline solution. The brains were harvested and post-fixed in a Golgi-Cox solution for two weeks before being transferred to a 30% sucrose solution where they remained until being sectioned. Brains were sectioned at 200  $\mu\text{m}$  on a Vibratome and mounted for staining. Mounted sections were stained using the Golgi-staining procedures set out by Gibb and Kolb [9].

### ***Drug administration***

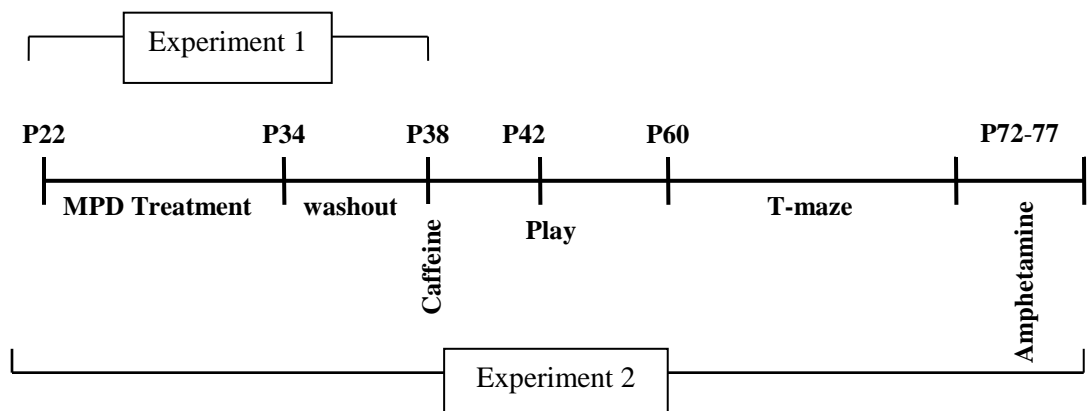
*Methylphenidate (Ritalin;MPD)*. Rats received subcutaneous (s.c.) injections of Ritalin (0.5mg/kg) or vehicle (0.9% saline solution) in the nape of the neck 2X daily (a.m. & p.m.), spaced 6 hours apart. Injections took place in a testing area (novel environment) away from the home environment. Once animals had been injected they remained in the testing room for approximately 10 minutes at which time they were returned to their home cages.

*Caffeine Challenge*. All rats received an acute dose of caffeine (0.015g/kg; i.p.), or vehicle (0.9% saline solution; i.p.) in the novel environment following a drug wash-out period of five days (see Figure 2.1.1). Five minutes post-injection, motor activity of individual subjects was monitored and recorded in an open field apparatus (described below) for 10 minutes.

*Amphetamine Challenge*. In Experiment Two only, animals received a single intraperitoneal (i.p.) injection of *d*-Amphetamine (0.5mg/kg) on the final day of the experiment and motor activity was recorded and animals sacrificed immediately after.

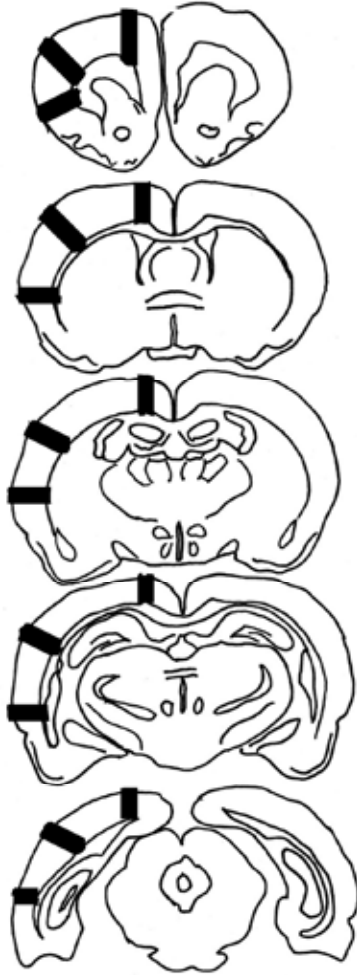
**Motor Activity.** Motor activity was monitored and recorded using an Omnitech Inc. Digiscan Animal Activity Monitor. The apparatus consisted of a clear, plexiglass box measuring 42cm x 41cm x 31cm. The box contained 6 separate photocells (4 lower, 2 upper) that measured activity as a product of breaks in the infrared beams of each photocell. Motor activity was automatically recorded by the attached Digiscan Analyzer. At the end of each test period the data was printed out, the analyzer reset, and the box cleaned to prepare for the next subject. Data collection included horizontal activity (number of beam breaks of lower photocells) and distance traveled (total cm between forward beam breaks).

Prior to testing, the rats were habituated in the activity boxes in groups and then individually over a three day period. For each testing day, motor activity was monitored in two, five minutes intervals, for a total of ten minutes.



**Figure 2.1.1.** An outlines the experimental time-line for Experiment One and Two. **Abbreviations:** P = postnatal day; MPD = methylphenidate (Ritalin); Caffeine and Amphetamine indicate acute drug challenges.

**Cortical Thickness.** Measurements were made by projecting the Nissl-stained sections on a Zeiss 2 POL projector set at a magnification of 20X. A metric ruler was used to measure three different cortical locations at each of 5 planes (Figure 2.1.2).

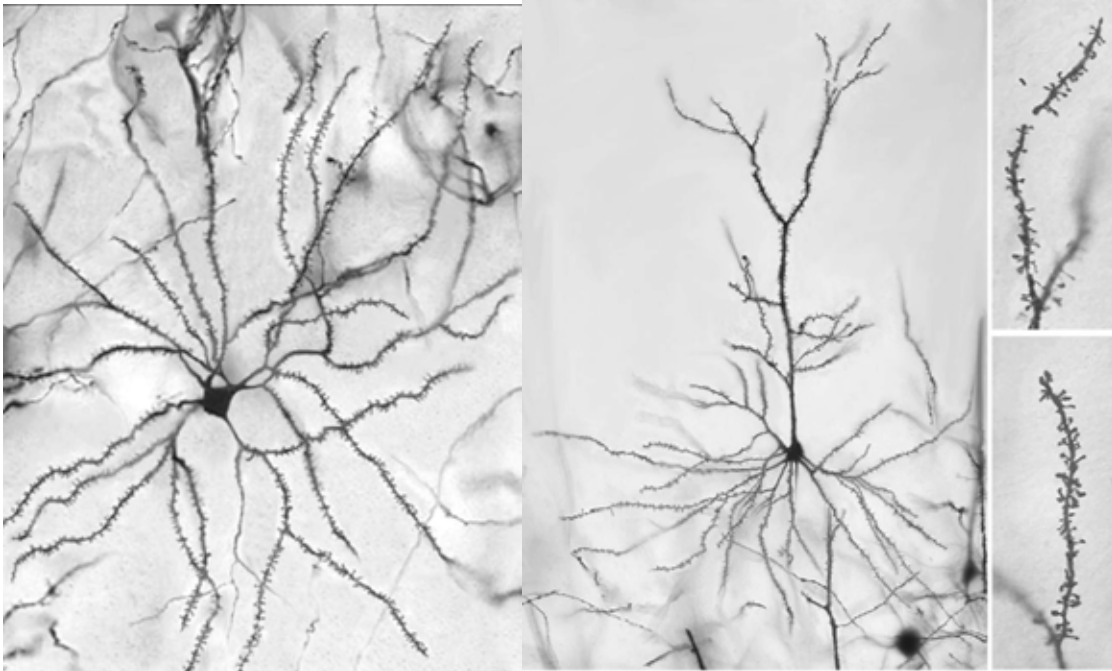


**Figure 2.1.2.** A cartoon illustration of sections used for the cortical thickness analysis. The bold lines indicate the ruler position and location that measures were taken from for each hemisphere (a total of six measurements for each of the sections. Means were later calculated for each of the five planes.

**Assessment of dendritic morphology.** After being submersed in the Golgi-Cox solution for 14 days, brains were transferred to a 30% sucrose solution for at least 3 days before being cut at 200  $\mu\text{m}$  on a vibratome and mounted on glass slides. Sections were stained

following the procedures described by Gibb and Kolb [9]. Pyramidal cells (Figure 2.1.3, center) of the anterior primary somatosensory cortex (Par 1, layer III), region three of the anterior cingulate cortex (Zilles Cg3, layer III and layer V), the dorsal agranular insular cortex (AID, layer III), and the CA1 subfield of the hippocampus Zilles [35], as well as medium spiny neurons (Figure 2.1.3, far left) of the NAc shell and core, as identified by Paxinos and Watson [25], were chosen for analysis based on previous reports of alterations in morphology as a consequence of stimulant use or housing experience.

Five cells from each hemisphere were chosen in each of areas to be analyzed. To be included in the analysis, cells had to be thoroughly impregnated by the solution so that the dendritic trees were clearly visible at 100X and not obscured by blood vessels or neighboring processes or debris (see Figure 2.1.3). Cells were drawn using camera lucida (250X) and the means of both apical and basilar dendritic branch order (number of branch bifurcations) and Sholl analysis (number of branches that crossed an overlay of concentric rings spaced 20  $\mu\text{m}$  apart) for each hemisphere were used for later comparisons. One exception was the CA1 where only basilar branches were drawn and included in analysis. Spine density calculations were conducted in apical dendritic trees of Cg3 LV and AID LIII only. Spine density was calculated by tracing a segment of a third-order dendrite at 1000X (see Figure 2.1.3, far right), including all spine protrusions that had a visible head. It was recognized that this is an underestimation of the total spine density as those spines on either the underside of the dendrite or on top of it were not visible to the drawer and therefore excluded (see [20] for additional details).



**Figure 2.1.3.** **Left:** Photograph of Golgi-Cox stained stellate cell from the NAc. **Center:** Photograph of a layer III pyramidal cell. **Right:** Photograph of dendritic spines on a segment of basilar dendrite.

### Experiment One

The short-term effects of chronic MPD exposure were assessed using motor activity as the behavioural index of drug response in a novel environment. Drug-induced sensitivity to a novel stimulant (caffeine) was assessed in a similar manner after a short drug-washout period. Animals were sacrificed immediately following the final behavioural assessment and the brains prepared for anatomical analyses. To determine the extent of low-dose psychostimulant use on dendritic morphology we extended the anatomical assessment to include regions that have previously been reported to be influenced by either the environment (e.g., [16,32]), or stimulant use in adult (e.g. [31]) and juvenile [7] rats.

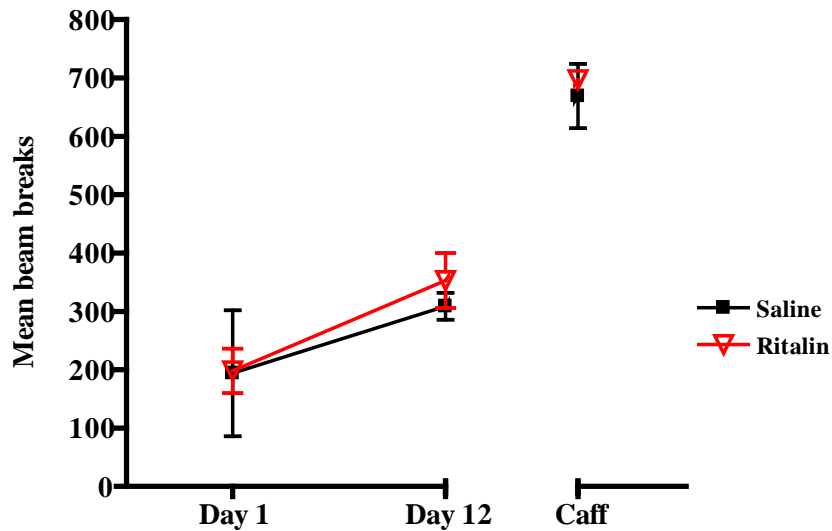
**Subjects.** A total of twelve male pups were used in the experiment. Pups were obtained from three separate litters on postnatal day (P) 22 and randomly assigned to one of two groups; saline or Ritalin treatment. Subcutaneous injections of MPD were administered twice-daily in the activity test room (morning and afternoon) for twelve consecutive days. Animals were given ad-lib food and water throughout the experiment. Five days after the last injection (drug-washout period) rats were given a caffeine challenge (single i.p. injection)

## **Results**

### ***Behavioral Results***

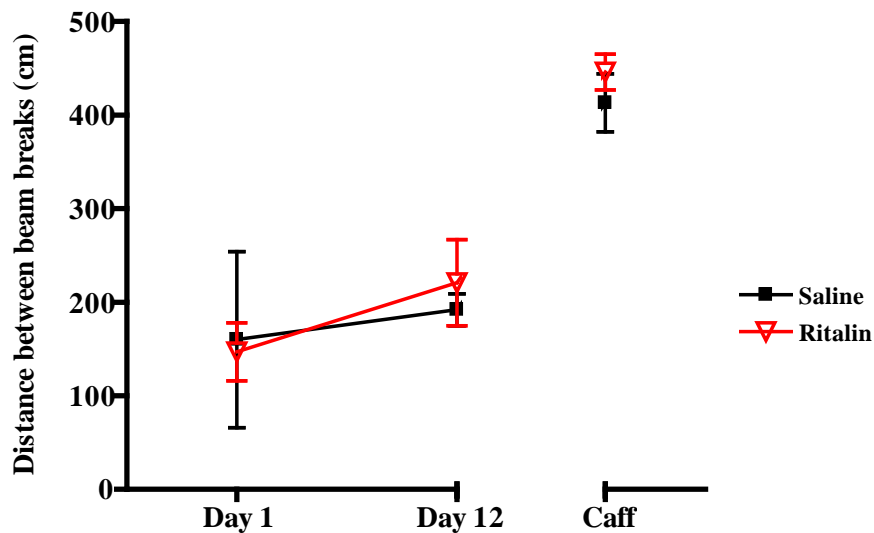
***Locomotor activity.*** Ritalin produced an increase in horizontal activity (Figure 2.1.4a) and distance traveled (Figure 2.1.4b) between Day 1 and Day 12 that was not found in rats treated with vehicle alone. Yet, both groups showed an increased motor response to caffeine when compared to their activity levels on Day 12 (Figure 2.1.4a & 4b). Paired-sample t-tests of horizontal activity on Day 1 vs Day 12 for each treatment condition showed a significant increase in horizontal activity in the Ritalin-treated, [ $t(3.24) = 0.023$ ], but not the saline-treated [ $t(1.15) = 0.33$ ], animals. Paired-sample t-tests showed that both the Ritalin and saline groups had a significant increase in horizontal activity when Day 12 was compared with the activity level of the caffeine challenge.

### Experiment 1. Horizontal activity



**Figure 2.1.4a.** The line graph illustrates the average number of beam breaks (horizontal activity) on day 1, 12 and during the caffeine challenge. Although no group differences were found, only the Ritalin-treated rats showed a significant escalation in activity over the treatment period (Day 1 -12), albeit a small one.

### Experiment 1. Distance traveled



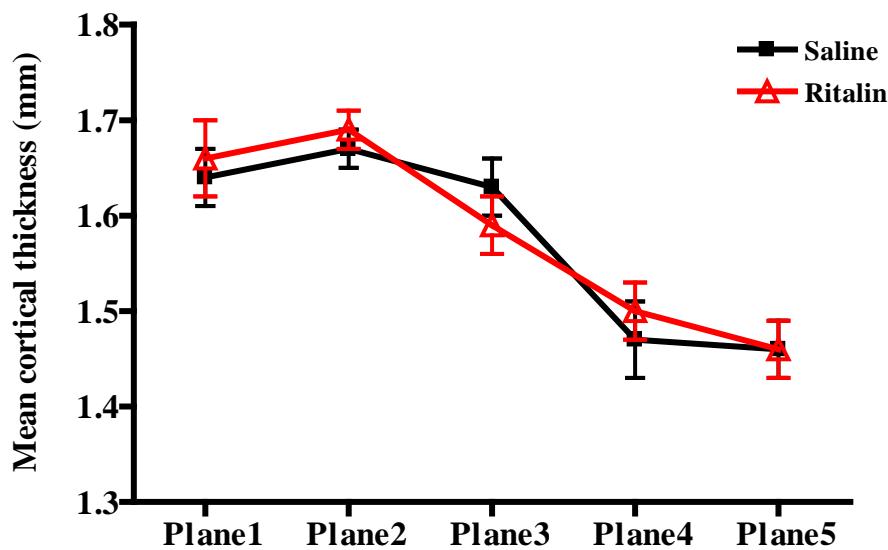
**Figure 2.1.4b.** The graph illustrates the average distance between beam breaks (distance traveled) during the same testing periods.

### **Anatomical Results**

After 12 days of Ritalin injections (2X daily), treated rats looked healthy, were playful, and in general were not distinguishable from controls in appearance or behavior.

**Cortical Thickness.** Early Ritalin exposure also did not appear to influence cortical thickness in adolescent rats (Figure 2.1.5). A repeated-measures ANOVA of cortical planes showed no overall significant main effect of treatment [ $F(1,22) = 0.03, p = 0.86$ ], and no differences on any of the five planes measured ( $p$ 's > 0.3).

### **Experiment 1. Cortical Thickness**



**Figure 2.1.5.** The graph illustrates the means and standard errors of each of the five planes. A repeated-measures ANOVA revealed no significant differences between the treatment groups on any of the planes measured ( $p$ 's < 0.5).

**Dendritic analyses.** Early Ritalin exposure reduced the dendritic field of layer III Par 1 and Cg3 neurons. In contrast, layer V neurons in the Cg3 showed enhanced apical and basilar dendritic fields. No other alterations in connectivity were uncovered.

**NAc core and shell.** Low-dose Ritalin treatment did not alter dendritic morphology in either the NAc core or shell (Table 2.1.1.). ANOVA's showed comparable branch order

numbers [ $F(1,20) = 0.08, p = 0.78$ ], and dendritic length [ $F(1,21) = 0.56, p = 0.47$ ], between the saline and Ritalin treated rats in the NAc core. The results were similar in the NAc shell, with ANOVA's showing no main effect of treatment in dendritic branch order [ $F(1,19) = 0.33, p = 0.57$ ], nor dendritic length [ $F(1,19) = 0.93, p = 0.35$ ].

**Par1.** Ritalin treatment produced a decrease in the basilar and apical branches of Par 1 (layer III) pyramidal neurons (Table 2.1.1.). ANOVA's showed a significant treatment effect on apical [ $F(1,23) = 5.50, p = 0.03$ ], and basilar [ $F(1,23) = 9.37, p = 0.006$ ] branch order but no changes in dendritic length on either [ $F(1,23) = 0.24, p = 0.63$  and  $F(1,23) = 2.39, p = 0.14$ , respectively].

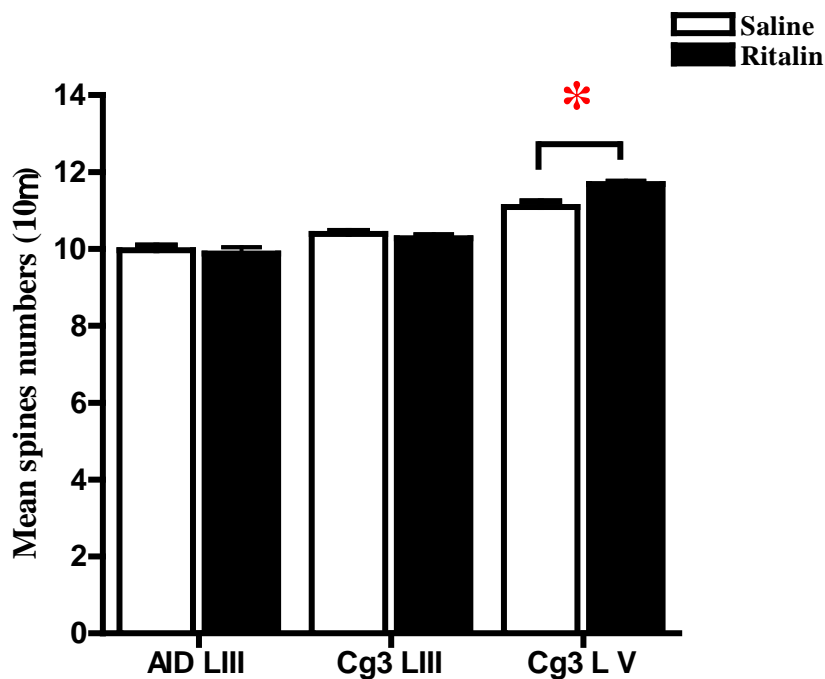
**CA1.** The basilar dendrites of CA1 neurons in the hippocampus showed no effect of Ritalin treatment (Table 2.1.1.). ANOVA's revealed no treatment effect on either branch order [ $F(1,21) = 0.29, p = 0.60$ ], or length [ $F(1,21) = 0.44, p = 0.52$ ].

**AID.** There were also no treatment-induced effects on the dendritic arbor of layer three pyramidal cells in the AID (Table 2.1.2.). ANOVA's revealed no effect of treatment on either apical or basilar branch order, [ $F(1,20) = 0.29, p = 0.87$ ] and [ $F(1,20) = 1.45, p = 0.24$ ], respectively. ANOVA's of apical and basilar dendritic length (Table 2.1.2.) also showed no main effect of treatment [ $F(1,20) = 0.02, p = 0.89$ ] and [ $F(1,20) = 0.71, p = 0.41$ ], respectively. Finally, an ANOVA of AID spine density (Figure 2.1.6) revealed no significant effect of treatment [ $F(1,22) = 0.22, p = 0.64$ ].

**Cg3 layer V.** In contrast to the AID, early Ritalin treatment produced an increase in basilar branch order (Table 2.1.2.), apical dendritic length (Table 2.1.2.), and basilar spine density (Figure 2.1.6) in layer V pyramidal neurons of the anterior Cg3. ANOVA's of layer V basilar and apical branch order showed a significant main effect of treatment

on basilar [ $F(1,21) = 9.24, p = 0.006$ ], but not apical dendrites [ $F(1,21) = 0.49, p = 0.49$ ]. The Sholl analysis revealed a significant effect of treatment on apical dendrite length [ $F(1,21) = 5.34, p = 0.03$ ], but only a trend for basilar dendritic length [ $F(1,21) = 3.81, p = 0.065$ ]. Basilar spine density of Cg3 layer V neurons also showed a significant effect of Ritalin treatment [ $F(1,21) = 14.46, p = 0.001$ ].

### Experiment 1. Spine density



**Figure 2.1.6.** The bar graph illustrates the mean number of apical spines for each of the regions indicated on the X-axis. Ritalin treatment resulted in an increased spine density in layer V of the Cg3 but no effect of treatment in layer III of either the AID or Cg3.

**Cg3 layer III.** The only change found in layer III Cg3 pyramidal neurons, however, was a decrease in basilar branch order (Table 2.1.2.). An ANOVA of branch number showed a main effect of treatment on the proximal basilar branches [ $F(1,22) = 7.75, p = 0.01$ ], but no other effects ( $p$ 's > 0.10).

## **Experiment Two**

The following experiment was conducted to investigate the long-term consequences of early Ritalin exposure on stimulant sensitivity, cognitive and social behaviour, as well as the possible anatomical correlates of behaviour.

**Subjects.** Twelve male pups were obtained from two separate litters on postnatal day (P) 22/23 and randomly assigned to one of two groups; saline or Ritalin treatment. Pups received either 0.5 mg/kg of Ritalin or equal amounts of 0.9% saline vehicle for twelve consecutive days as in Experiment One. Five days after the last injection (P38) rats were given an acute dose of caffeine (0.015g/kg in a single i.p. injection) and motor activity recorded. Following the caffeine challenge, rats were given an additional five day drug wash-out period (see Figure 2.1.1a) before individual play behavior was evaluated (P42). T-maze training began two weeks after the last challenge. As individual subjects reached criterion in the T-maze task, they were removed from further testing and given an amphetamine (1 mg/kg) challenge. Immediately, following the final drug challenge the animals were sacrificed and the brains harvested for later analyses.

### ***Behavioural Tests***

**Motor Activity.** In addition to the assessment of motor response to Ritalin and the caffeine challenge in adolescence, the effect of early drug experience on long-term sensitivity to naïve stimulants was assessed in adulthood using a *d*-amphetamine (1.0 mg/kg) challenge at the end of the experiment. Activity was assessed as described previously.

**Play Behavior.** Play behavior in rodents can be used to evaluate social behavior as a result of changes in prefrontal cortical functioning, such as might be expected with early

drug experience. Young rats use a particular set of motor sequences that characterize adolescent play and distinguishes it from the adult play repertoire (for review see,[26]). In the current study we quantified a set of these behaviors that included the number of: 1) attacks (initiated play with snout on nape of mate's neck); 2) complete rotation defense (subordinate response); 3) partial rotation defense (dominant response); and, 4) evasion defense (avoidance). Twelve 'novel playmates' that were size and age -matched were used to avoid the possible confound of any pre-existing relationships between experimental animals.

Play behavior was filmed within a play enclosure/arena that was located in a novel testing room to dissociate the play-environment from the drug-environment. The play arena was a square (50 cm X 50 cm), open-top box. The walls (52 cm height) consisted of plywood sides, a mirrored back wall and a clear-plexi-glass front. The floor was also plexi-glass but was covered with corncob bedding. All rats (including the playmates) were habituated to the new environment in groups (2 – 3) for 20 – 30 minutes per day for a period of three days. At the end of the third day of habituation all rats were isolated (single housed) for a 24 hour period prior to filming to motivate play behavior, thereby increasing the amount of collectable data for later quantification. On the test day rats were individually transported to the play area in pairs, each rat was coded with a livestock marker (black or yellow) for easy identification, and then both animals were placed into the play arena. Behavior was filmed for 10 minutes, beginning with the first interaction. At the end of each period of data collection rats were removed and returned to group housing.

***T-Maze task.*** The T-maze apparatus consisted of a T-shaped configuration where the entrance alley (80 X 10cm) was the base of the 'T' and two alleys ('arms') extend in either direction from the far end of the entrance alley (80 cm X 10cm). Food wells were located at the far end of each arm. The wooden maze walls were 48 cm high, as were the removable, sliding doors located at the entrance of each arm and the hinged door at the T-maze entrance.

The T-maze procedure consisted of two phases. In the first, the training phase, animals were introduced to the T-maze apparatus and trained to navigate to the end of the maze 'arms' to retrieve a food reward from the food wells. The training phase of this task took an average of 4 days at which time all of the rats eagerly 'ran' to the food wells to retrieve the fruit-loop reward. In phase two, the testing phase, the rats were placed into the maze, allowed to retrieve a reward in one arm before being removed and reintroduced into the maze after a 10 sec interval, at which time they were required to choose the arm opposite of the previously rewarded arm to obtain an additional food reward.

The testing phase was comprised of 10 trials with each trial consisting of a 'sample' and 'non-match to sample' component. At the beginning of each trial, the arms of the maze were wiped with a 30% alcohol solution and the food wells at the end of both arms were baited. A solid gate was then placed across the entrance of one arm of the maze (chosen pseudo-randomly in advance). Thus, the open arm was the 'sample' component and the blocked arm would later become the 'correct' choice for the 'non-match to sample' portion of each trial. To start the trial, the rat was placed into the entrance alley and the door closed to block the exit. The animal ran down the open arm and retrieved/consumed the food reward (sample phase). The rat was returned to a holding cage outside the maze

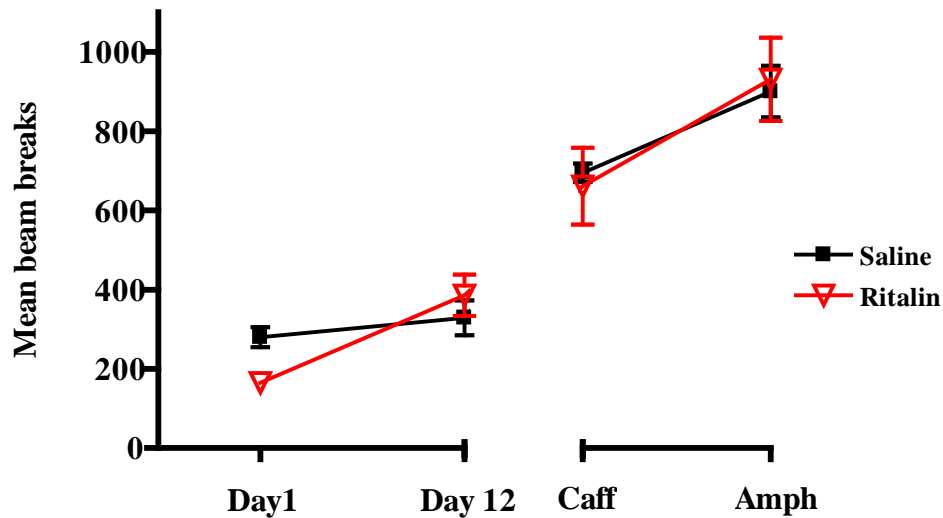
by the experimenter, the divider was removed, the maze quickly wiped with alcohol solution, and the rat returned to the maze entrance (~ 10 sec). At this point only the 'non-match to sample' arm held a food reward. The rat was allowed to choose to enter one of the arms and removed after either consuming the reward if a correct choice was made, or not, if an incorrect choice was made. These procedures were repeated for 10 consecutive trials for each subject. The total correct score/errors out of 10 were calculated and recorded. To reach criterion and complete the task the rats had to receive a score of at least 8/10 on four consecutive days.

## **Results**

### ***Behavioral Results***

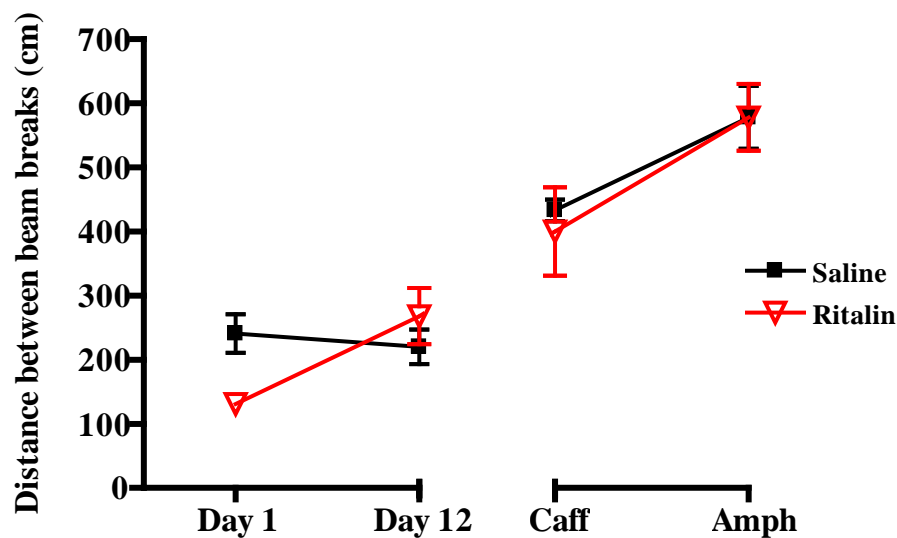
***Locomotor Activity.*** Owing to differences in horizontal activity and distance traveled between groups on the first day of injections ( $p = 0.006$ ;  $p = 0.008$ , respectively), we used Day 1 as the baseline to compare subsequent activity for each group using paired-samples  $t$ -tests (Figure 2.1.7a & b). Ritalin- rats showed a moderate but significant increase in motor activity between Day 1 and Day 12 for both horizontal activity ( $p = 0.005$ ), and distance traveled ( $p = 0.012$ ), that was not present in saline-treated rats ( $p = 0.47$  &  $p = 0.41$ , respectively). In contrast, on the caffeine and amphetamine challenges, animals in both the Ritalin and saline groups showed an increase in horizontal activity (caffeine:  $p = 0.002$ ;  $p < 0.01$ ; amphetamine:  $p = 0.004$ ,  $p < 0.01$ , respectively) and distance traveled (caffeine:  $p = 0.009$ ;  $p < 0.01$ ; amphetamine:  $p = 0.005$ ;  $p < 0.01$ , respectively).

## Experiment 2. Horizontal activity



**Figure 2.1.7a.** The graph depicts a moderate increase in motor response (horizontal activity) during the initial Ritalin treatment phase (Day 1 -12), but no differences in response to subsequent stimulants caffeine (4 days) or amphetamine (~28 days) post-treatment.

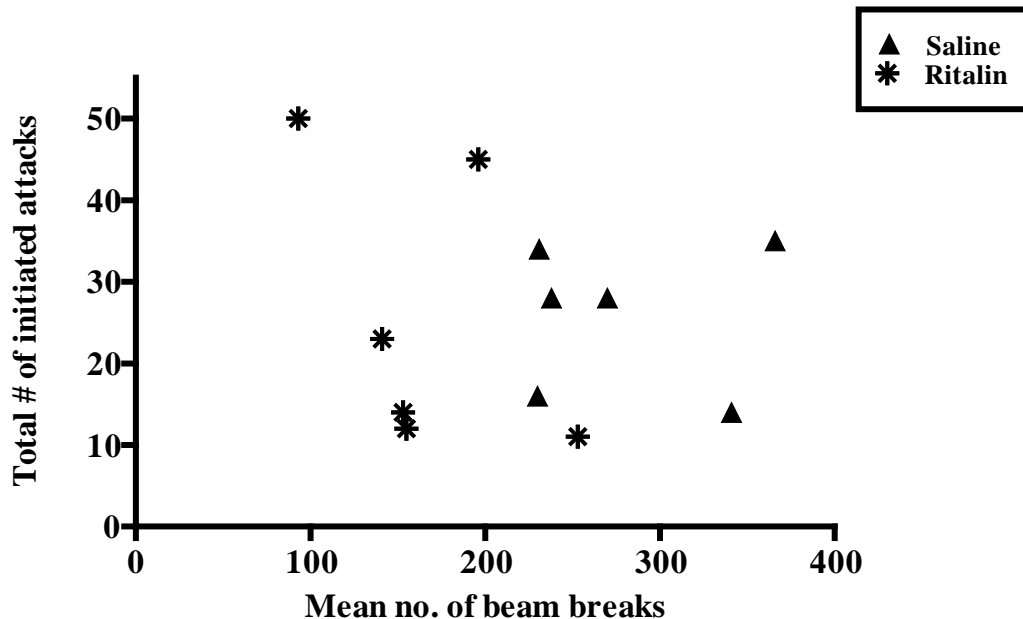
## Experiment 2. Distance traveled



**Figure 2.1.7b.** The graph depicts a moderate increase in motor response (distance traveled) during the initial Ritalin treatment phase (Day 1 -12), but no differences in response to subsequent stimulants caffeine (4 days) or amphetamine (~28 days) post-treatment.

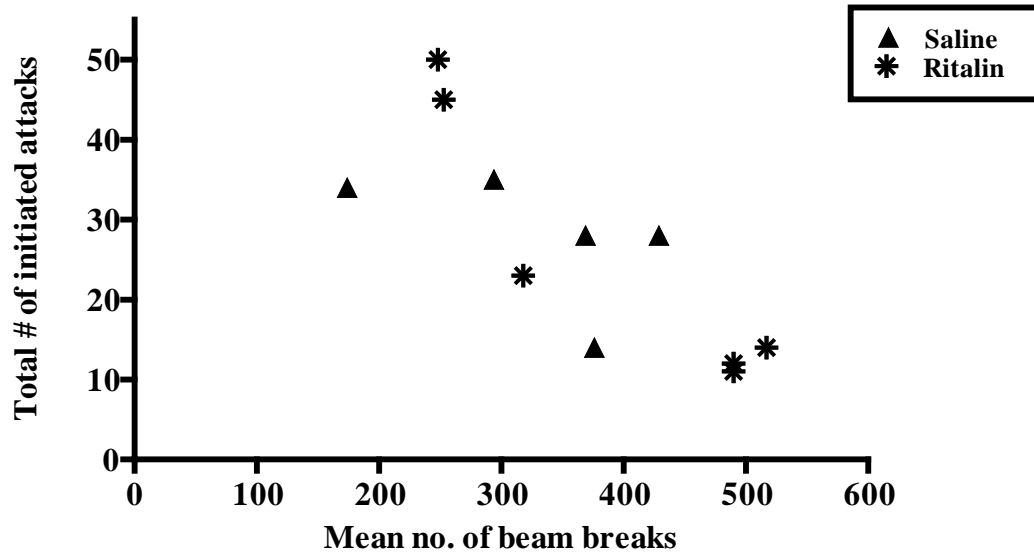
**Play behavior.** Play behavior was analyzed to take into account individual difference in drug response as determined in the analyses of motor activity. In summary, animals that exhibited a ‘high’ motor response to Ritalin were less willing to initiate play as shown by the total number of ‘attacks’ for each rat. Bivariate correlations of play behavior and activity on Day 1 (see Fig. 2.1.8a) and Day 12 (see Fig. 2.1.8b) showed no correlation between activity and play behaviour in Saline rats ( $r = 0.01$ ,  $p = 0.98$ ;  $r = -0.55$ ,  $p = 0.32$ ). In contrast, although Ritalin treated rats also showed no correlation between Day 1 activity and play behaviour ( $r = -0.44$ ,  $p = 0.38$ ), there was a strong negative correlation between activity on Day 12 and the number of initiated attacks during play behaviour ( $r = -0.93$ ,  $p = 0.007$ ), suggesting the effect was caused by chronic Ritalin treatment and not a reflection of activity levels per se.

**Experiment Two - Day 1 activity/Attacks scatterplot**



**Figure 2.1.8a.** A scatterplot of Day 1 horizontal activity and the number of initiated attacks during play assessment showed no correlation in either saline nor the Ritalin ( $r = 0.01$ ;  $r = -0.44$ , respectively).

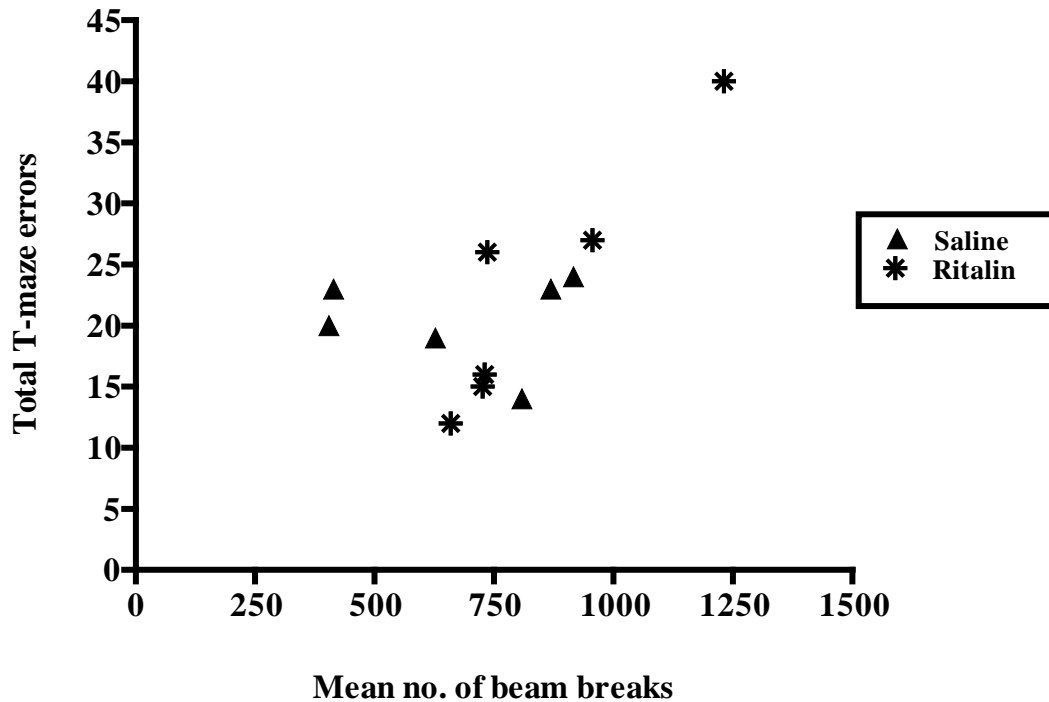
## Experiment Two - Day 12 activity/Attacks scatterplot



**Figure 2.1.8b.** A scatterplot of Day 12 horizontal activity and the number of initiated attacks during play assessment showed no correlation in Saline rats ( $r = -0.55$ ), but a strong negative correlation in Ritalin rats ( $r = -0.93$ ).

**T-maze activity.** Owing to the individual differences in response to Ritalin we ran a bivariate correlation test to determine whether or not a relationship existed between activity levels during the 12 day injection period and the number of errors in the T-maze task using the last five minutes of each test session. We found a strong, positive correlation in Ritalin-treated rats between horizontal motor activity and T-maze errors ( $r = 0.92$ ,  $p = 0.009$ ), as well as between T-maze errors and distance traveled ( $r = .85$ ,  $p = 0.03$ ) on Day 12 (see figure 2.1.9 for horizontal data). No correlations were found between Day 12 horizontal activity or distance traveled and T-maze errors in the Saline-treated rats ( $r = 0.004$ ,  $p = 0.99$ ;  $r = 0.04$ ,  $p = 0.95$ , respectively).

## Experiment 2. Day 12 Horizontal activity/T-maze scatterplot



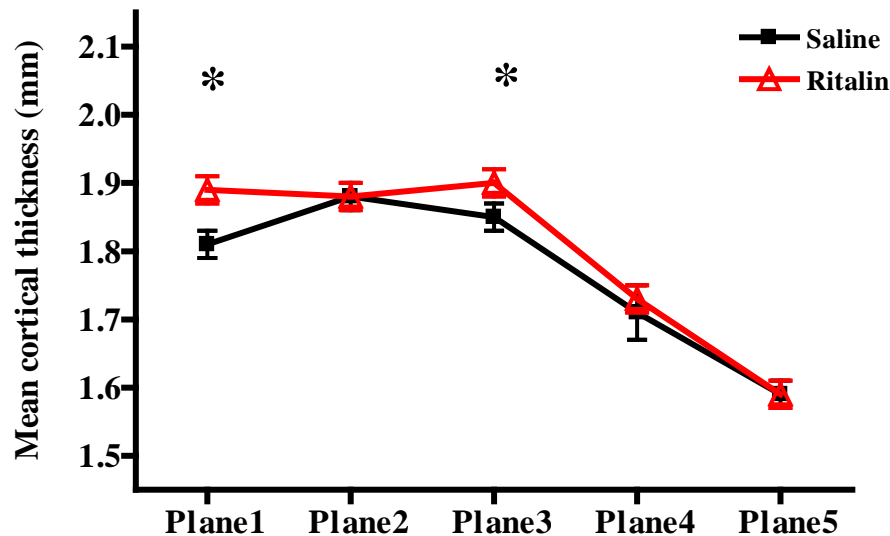
**Figure 2.1.9.** The scatterplot illustrates that the strength of the motor response was predictive of later cognitive performance in Ritalin but not Saline-treated rats ( $r = 0.92$ ;  $r = 0.004$ , respectively).

### *Anatomical results*

**Cortical Thickness.** Early Ritalin exposure produced a thicker anterior cortex (Plane 1 and Plane 3) in adulthood but had no effect on the other planes measured (Figure 2.1.10).

A repeated-measures ANOVA of cortical planes showed no significant main effect of treatment [ $F(1,22) = 3.03$ ,  $p = 0.096$ ]. The pairwise comparison revealed, however, that rats pretreated with Ritalin had a significantly thicker cortical mantle on planes one and three ( $p = 0.01$ ,  $p = 0.04$ , respectively) with the other planes showing no effect of treatment ( $p$ 's  $> 0.5$ ).

## Experiment 2. Cortical Thickness



**Figure 2.1.10.** The line graph demonstrates the mean measures of cortical thickness taken from each of the five planes. The asterisks indicate a significant increase in cortical thickness on planes one and three as a result of early Ritalin exposure.

### *Dendritic analyses.*

Ritalin pretreatment increased the dendritic field of layer III Par 1 and Cg3 neurons, though differently, whereas neurons in layer III of the AID and layer V of the Cg3 showed either a decrease in dendritic field or no effect of treatment at all. The NAc and CA1 also failed to show any treatment effect (See summary Table 2.1.1.3...).

**NAc shell.** As in Experiment One, administration of Ritalin during adolescence produced no changes in the dendritic morphology of spiny neurons in the NAc shell. The NAc core was included in the present experiment. ANOVA's showed no effect of treatment on branch order [ $F(1,21) = 0.68, p = 0.46$ ], nor dendritic length [ $F(1,21) = 1.08, p = 0.31$ ] (Table 2.1.1.).

**Par1.** In contrast to the decrease in branch order found in Experiment One, there was a Ritalin-induced increase in dendritic length in the dendritic field of layer III pyramidal

neurons (Table 2.1.1.). ANOVA's showed a significant effect of Ritalin on apical but not basilar dendritic length,  $F(1,23) = 4.44$ ,  $p = 0.047$  and  $F(1,23) = 1.99$ ,  $p = 0.17$ , respectively. No significant changes were shown in apical or basilar dendritic branching of Ritalin-treated rats,  $F(1,23) = 0.60$ ,  $p = 0.45$  and  $F(1,23) = 2.19$ ,  $p = 0.15$ , respectively. **CA1.** As in Experiment One, Ritalin produced no changes in the dendritic field of CA1 hippocampal neurons (Table 2.1.1.). ANOVA's showed no effect of treatment on dendritic branching nor on length,  $F(1,23) = 3.29$ ,  $p = 0.08$  and  $F(1,23) = 0.91$ ,  $p = 0.35$ , respectively.

Ritalin	NAc core	NAc core	NAc shell	NAc shell	PAR 1 LIII	PAR 1 LIII	PAR 1 LIII	PAR 1 LIII	CA1	CA1
	B(br)	B(sh)	B(br)	B(sh)	B(br)	A(br)	B(sh)	A(sh)	B(br)	B(sh)
Exp.1	○	○	○	○			○	○	○	○
Exp.2	N/A	N/A	○	○	○	○	○	Ÿ	○	○

**Table 2.1.1.** Illustrates changes found in Ritalin-treated animals when compared to Saline-treated controls in Experiment 1 and 2. **Abbreviations:** NAc = nucleus accumbens; PAR 1 = primary somatosensory cortex; CA1 = regions of hippocampus; LIII = layer three; LV = layer five; B = basilar; A = apical; br = branch order; sh = Sholl analysis.

**AID.** Pyramidal neurons in the OFC showed a decrease in basilar branch order as a result of early Ritalin exposure, although no changes were found in dendritic length (Table 2.1.2.) or spine density. An ANOVA of branch order showed a significant effect of treatment on distal basilar branching [ $F(1,21) = 7.50$ ,  $p = 0.01$ ], but only a trend for an effect of treatment on overall basilar dendritic branching [ $F(1,21) = 3.72$ ,  $p = 0.068$ ]. The analyses of dendritic length showed no effect of treatment on either apical [ $F(1,21) < 0.001$ ,  $p = 0.98$ ], or basilar [ $F(1,21) = 0.46$ ,  $p = 0.51$ ], dendritic branching, nor a treatment effect of spine density [ $F(1,22) = 1.61$ ,  $p = 0.22$ ].

**Cg3 layer III.** Early Ritalin exposure produced an overall increase in length of both apical and basilar dendrites in Layer III pyramidal neurons that was not present in Experiment One, although there was a similar decrease in basilar branch order (Table 2.1.2.). An ANOVA of branch order showed an effect of treatment on basilar [ $F(1,17) = 8.56, p = 0.01$ ], but not apical [ $F(1,17) = 0.88, p = 0.36$ ] branch order. Yet, analysis of dendritic length (Sholl) showed a significant increase in both apical [ $F(1,17) = 4.76, p = 0.04$ ], and basilar [ $F(1,17) = 6.91, p = 0.02$ ], dendrites of Ritalin-treated rats.

**Cg3 layers V.** Contrary to the findings of Experiment One, branch order in Cg V neurons was unaffected by early Ritalin exposure, as was the overall dendritic length of basilar and apical branches (Table 2.1.2.) and spine density. ANOVA's showed no significant main effect of treatment on spine density [ $F(1,22) = 0.07, p = 0.79$ ], but no differences between Ritalin and saline treatment in apical or basilar dendritic length [ $F(1,20) = 0.18, p = 0.67$ ], and [ $F(1,20) = 0.37, p = 0.55$ ], respectively. Nor did we find differences in apical and basilar branching, [ $F(1,20) = 0.29, p = 0.60$  and  $F(1,20) = 0.18, p = 0.68$ ], respectively.

Ritalin	AID	AID	AID	AID	Cg3	Cg3	Cg3	Cg3	Cg3	Cg3	Cg3	Cg3
	LIII	LIII	LIII	LIII	LIII	LIII	LIII	LIII	LIII	LV	LV	LV
	B(br)	A(br)	B(sh)	A(sh)	B(br)	A(br)	B(sh)	A(sh)	B(br)	A(br)	B(sh)	A(sh)
Exp.1	○	○	○	○		○	○	○	Ÿ	○	○	Ÿ
Exp.2	d	○	○	○		○	Ÿ	Ÿd	○	○	○	○

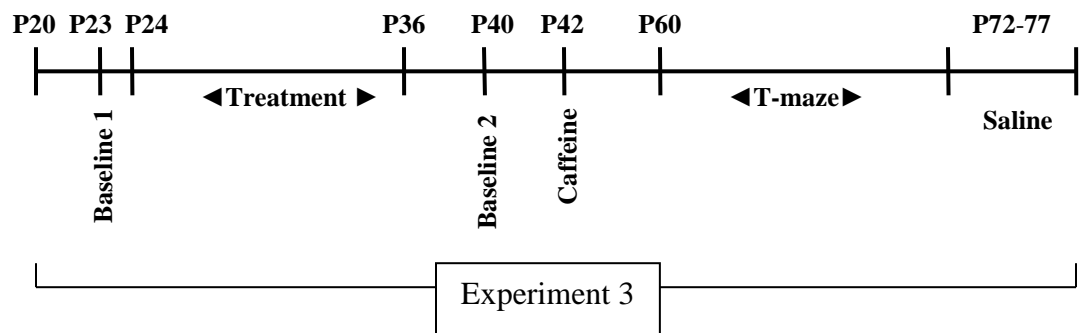
**Table 2.1.2.** An illustration of changes found in Ritalin-treated animals when compared to Saline-treated controls in each experiment. **Abbreviations:** AID = dorsal anterior insular cortex; Cg3 = region 3 of the cingulate cortex; LIII = layer three; LV = layer five; B = basilar; A = apical; br = branch order; sh = Sholl analysis.

### **Experiment Three**

The following experiment was designed to address some of the questions that arose from the results in Experiment Two. First, in Experiment two we reported a significant difference between Ritalin and saline groups on the first day of drug administration. The initial differences in activity raised the question of whether or not the ensuing divergence between the groups occurred as a result of a pre-existing characteristic(s), such as activity level. To address this question, baseline activity measures were collected prior to the first treatment in the current experiment. A second question raised was whether the correlation between Day 12 and the caffeine challenge in Experiment Two was related to an increased activity level that persisted in the Ritalin but not Saline-treated rats over the four day wash-out period. To address this question, base measures of activity were collected after a four day washout period but prior to the caffeine challenge. We also took this opportunity to explore the possibility that the moderate increases in motor activity were related to an enhanced 'sensitivity' to the novel environment (test area). To address this question, all rats received placebo injection for three concurrent days at the end of the T-maze task rather than the amphetamine challenge used in Experiment Two. The changes and protocol for Experiment Three are detailed in Figure 2.1.11.

**Subjects.** To investigate, eighteen male pups were obtained from six separate litters on postnatal day (P) 22 and randomly assigned to one of two groups; saline or Ritalin treatment. Prior to treatment, each rat was habituated to the test area on two consecutive days and on the third day motor activity was recorded (Figure 2.1.1b). Ritalin (0.5mg/kg) or equal amounts of saline (0.9% solution) injections began the following day (P26). T-maze training began about two weeks after the last challenge (see Figure 2.1.11).

Baseline motor activity was re-assessed after a four day drug-washout period and prior to caffeine administration. The caffeine challenge was conducted two days later, after an additional ‘washout’ phase. As before, the T-maze training began after an additional two week drug-washout period. As a final assessment of behavior to the drug environment, all rats received saline injections for a three day period in the drug test environment at the completion of the T-maze task. Immediately, following the final drug challenge the animals were sacrificed and the brains harvested for later analysis.



**Figure 2.1.11.** An outline of the experimental time-line for Experiment Three.  
**Abbreviations:** P = postnatal day; MPD = methylphenidate (Ritalin); Caffeine and amphetamine indicate acute drug challenges.

## Results

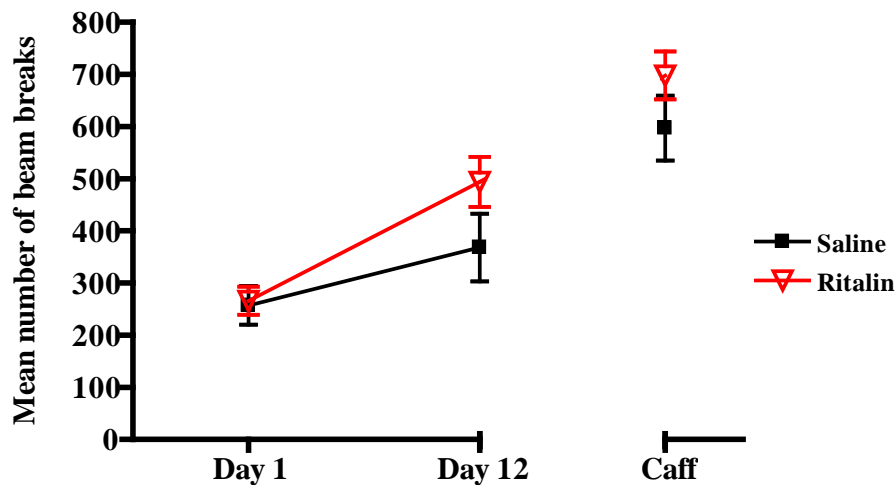
### *Behavioral Results*

**Locomotor Activity.** Pre-treatment activity measures showed no initial differences between treatment groups [ $F(1,16) = 0.66, p = 0.43$ ], nor were there any differences in activity levels between Ritalin and Saline treated rats after a four day washout [ $F(1,16) = 0.02, p = 0.89$ ]. The final activity measures at the end of the experiment also revealed no

residual changes in activity levels between the groups and no differences in response to the novel environment [ $F(1,15) = 0.74, p = 0.43$ ].

Nonetheless, only Ritalin-treated rats showed a significant increase in behavior between Day 1 and Day 12 of the treatment period (Figure 2.1.12). Analysis of horizontal and distance measures revealed very similar findings, thus only the horizontal results are presented here. A repeated-measures ANOVA of horizontal activity on Day 1, Day 12 and the caffeine challenge revealed no significant effect of treatment [ $F(1,15) = 2.70, p = 0.12$ ], but there was a significant within-subject effect of activity [ $F(1,15) = 79.84, p < 0.001$ ], in which both groups showed a significant increase in response to caffeine relative to Day 12 activity measures ( $p$ 's  $< 0.005$ ). Yet, the Ritalin-treated showed a significant increase in activity between Day 1 and Day 12 ( $p = 0.001$ ), whereas Saline-treated rats did not ( $p = 0.15$ ).

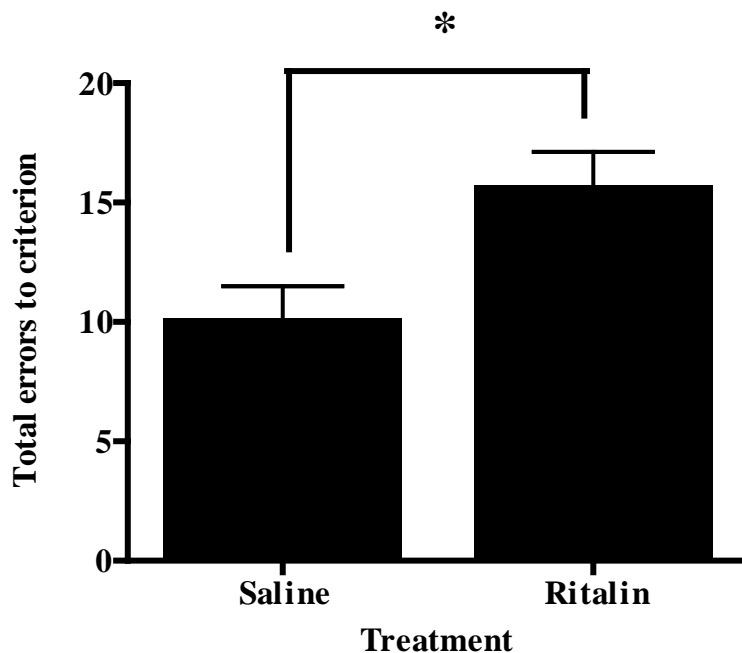
### Experiment 3. Horizontal Activity



**Figure 2.1.12.** A line graph of horizontal activity during the treatment period (Day 1 – 12) and during caffeine challenge. Early Ritalin exposure produced an escalating behavioural response over the treatment period that was not present in Saline-treated rats. Yet, both groups showed a similar response to subsequent exposure to a novel stimulant (caffeine).

**T-maze task.** Early Ritalin exposure produced a decrease in cognitive performance as demonstrated by the increased number of errors in the T-maze task (Figure 2.1.13). In contrast to Exp. 2, we found no correlation between Day 12 activity and T-maze errors in either Saline or Ritalin-treated rats ( $p$ 's  $< 0.05$ ), although there was a weak correlation between pre-caffeine activity and errors in Ritalin-treated rats that was not found in saline group ( $p = 0.01$ ;  $p = 0.07$ , respectively). Nonetheless, Ritalin treatment produced a 55% increase in the total number of errors to reach criterion relative to the Saline-treated animals. An ANOVA of total errors with treatment as the variable showed a significant effect of treatment [ $F(1,16) = 6.58$ ,  $p = 0.02$ ]. Unlike Experiment 2, however, activity was not correlated with errors in either saline or Ritalin-treated rats ( $p$ 's  $> 0.05$ ).

### Experiment 3. T-maze task



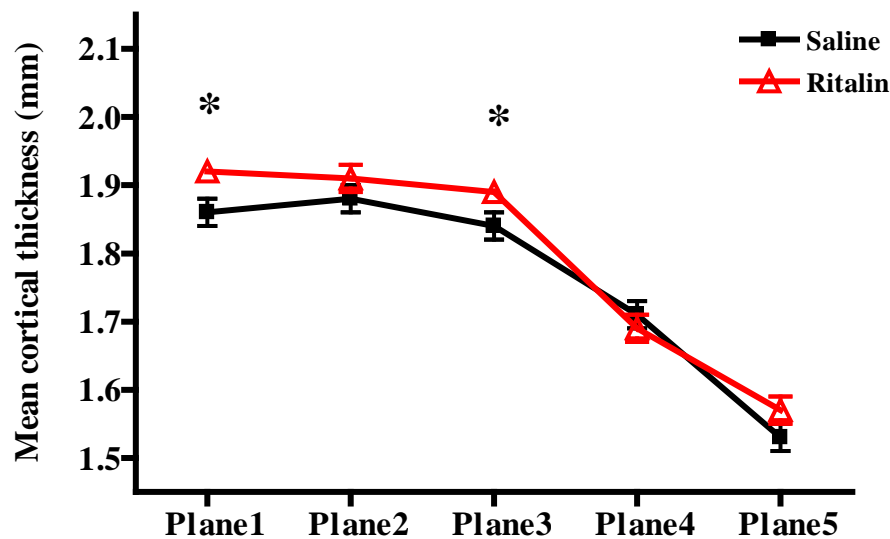
**Figure 2.1.13.** The bar graph demonstrates that early exposure to Ritalin produces an enduring effect on cognitive performance in latter (adult) life as shown by the increased number of total errors to reach criterion on the T-maze task.

## *Anatomical Results*

### *Cortical Thickness*

Ritalin exposure during the first few weeks post-weaning again produced a thicker anterior cortical mantle (Plane one and Plane three) in adulthood (Figure 2.1.14). The remaining planes appeared unaffected by the treatment. A repeated-measures ANOVA of cortical planes showed a trend towards a significant main effect of treatment,  $F(1,34) = 3.50$ ,  $p = 0.07$ . The pairwise comparison revealed a significant increase in the cortex of Ritalin-treated rats on planes one and three ( $p = 0.025$ ;  $p = 0.030$ , respectively) but no differences in cortical thickness on the remaining three planes ( $p$ 's  $> 0.15$ ).

### Experiment 3 Cortical Thickness



**Figure 2.1.14.** A line graph illustrating the means and standard errors for each of the five cortical planes measured. Early Ritalin treatment produced a significant increase in cortical thickness on planes one and three, as in Experiment Two.

**Summary of Experiments in Study One**

	Basilar							Apical				Spine (basilar)	
	Cg3		OFC	Par1	CA1	NAc		Cg3	OFC	Par1	Cg3	OFC	
<b>Ritalin</b>	LIII	LV	LIII	LIII		Core	Shell	LIII	LV	LIII	LIII	LV	LIII
<b>Experiment 1</b>	<b>i</b>	<b>h</b>	∅	<b>i</b>	∅	∅	∅	∅	<b>h</b>	∅	<b>i</b>	<b>h</b>	∅
<b>Experiment 2</b>	<b>i</b>	<b>h</b>	∅	<b>i</b>	∅	N/A	∅	<b>h</b>	∅	∅	<b>h</b>	∅	∅

**Table 2.1.3.** Arrows indicate direction of dendritic changes relative to Saline-treated controls in each experiment. **Blue arrows** = branch order; **Red arrows** = Sholl (length); **Spine** = spine density; ∅ = no change; Cg3 = region three of the cingulate gyrus; OFC (AID) = orbital prefrontal cortex; Par 1 = primary somatosensory cortex; CA1 = subfield of the hippocampus; NAc = nucleus accumbens core or shell; LV = layer five neurons and LIII = layer three neurons.

## **Discussion**

We investigated the influence of early Ritalin use on behaviour and neuronal morphology in male rats. Experiment 1 looked only at the neuronal effects of juvenile Ritalin exposure on the periadolescent brain. Experiments 2 and 3 looked both at the effect of juvenile Ritalin exposure on prefrontal cortical-dependent behaviors and on neuronal morphology in adulthood. It was predicted that 1) Ritalin would produce structural changes in prefrontal regions and that the effects might be different in young and adult rats; 2) that the drug experience might influence later frontal-dependent behavior; and, 3) that the training in the prefrontal-related tasks might alter neuronal morphology differently in the saline and drugged animals. Because psychomotor stimulants are known to alter neuronal morphology in nucleus accumbens and hippocampus but not in parietal cortex [2,24,29,30], we also analyzed cells in these regions. Thus the behavioural analyses were followed by an extensive examination of dendritic morphology in pyramidal neurons in the PFC (AID and Cg3), Par1, and CA1, as well as medium spiny neurons in the NAc. The motor response produced by low dose Ritalin was not sufficient to promote behavioural sensitization in any of the current experiments. The overall increase in motor activity found was very modest in comparison to the striking behavioral effect that is characteristic of behavioral sensitization (see [28] for review). Alone, these findings would suggest that early exposure to low doses of Ritalin is sufficient to alter developing circuitry in such a manner as to produce impaired social and cognitive function in adulthood, without necessarily producing sensitization. The current results also demonstrate that the effects of low-dose Ritalin are quite different in the developing brain compared to the mature brain, as it has previously been

reported that low doses of Ritalin improve cognitive function in adult rats (e.g.[3]). Aside from age, the benefits reported by Berridge and colleagues were assessed in adult males following acute exposure, unlike the long-term use in the current experiments.

The absence of stimulant-induced alterations in the dendritic morphology of medium-spiny neurons of the NAc may at first seem quite unusual. Robinson and Kolb, as well as a host of other researchers convincingly and repeatedly have correlated changes in NAc cell morphology with psychostimulant use, but these studies also reported the induction of behavioral sensitization (e.g.,[2,11,24]) that was not found in the current studies. Therefore, owing to the subtle increase in motor behavior in the current experiments, the results reported here are in fact in keeping with the studies of Robinson and others and support the contention that behavioral sensitization, rather than drug use alone, is associated with altered NAc connectivity. Furthermore, Berridge et al.,[3] reported that low doses of intraperitoneal Ritalin (0.25 & 0.5 mg/kg), although sufficient to produce significant changes in catecholamine activity and cognitive function in the PFC, produces no to moderate changes in catecholaminergic activity of the NAc and no had effect on locomotor activity.

The increases in apical and basilar dendritic fields of Cg3 layer V neurons in animals sacrificed shortly after Ritalin treatment (Exp.1) are very similar to those reported following the same dose (0.5mg/kg) of amphetamine in adolescent rats [7] that were also sacrificed shortly after a 12 day treatment regimen (~ 2 weeks). These changes were not persistent, however, as animals that were sacrificed in adulthood (Exp. 2) displayed a different pattern of altered connectivity. In the latter case, alterations in PFC dendritic fields were restricted to layer III Cg3 neurons, and included a reduction in the

basilar dendritic field of the orbital prefrontal cortex (e.g., AID). It would appear then that the initial drug-induced alterations were not static and that the initial changes in layer V neurons may have altered connectivity in such a way as to influence the ongoing developmental pattern of connectivity, associated with, but not directly influenced by Ritalin itself.

Alterations in the pattern of connectivity in the sensory cortex (Par1) as a result of early Ritalin use also changed with time, showing opposite effects between adolescence and adulthood. The behavioural implications of these changes are not clear, but may reflect a stimulant-induced blockade of experience-induced plasticity similar to what has been reported in adult rats pre-exposed to amphetamine [19]. If this is indeed the case, then the reduction in dendritic morphology in layer III Par 1 neurons in Exp.1, but not in Exp.2, would suggest that the blockade is relatively transient in the adolescent brain, a finding that is contrary to adult amphetamine or cocaine exposure as reported by Kolb and colleagues. An alternative, however, is that the transient effect is related to the low dose used in the current experiments rather than the timing of the drug experience itself.

In summary, the behavioural abnormalities found following early, chronic Ritalin exposure may reflect a vulnerability that is unique to the developing adolescent brain. Altered dendritic morphology in the PFC, but not subcortical regions (NAc), suggest that Ritalin use during this vulnerable period promotes aberrant connectivity that is associated with alterations in cognitive profile in adulthood. The behavioural tests in the current study were limited to a single measure of play and cognitive behavior but provide a basis for a more thorough behavioural investigation in the future.

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## **II. Early Methylphenidate exposure inhibits experience-induced plasticity of complex housing in rats**

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## **Abstract**

We investigated the influence of early methylphenidate (Ritalin) exposure in adolescence on subsequent experience-induced plasticity and behaviour. Male rat pups were given subcutaneous injections of either saline (0.9%) vehicle or Ritalin (0.5mg/kg) twice daily for twelve consecutive days during the adolescent period (P25 – P37). Following a 4 day drug-free washout phase, animals from each treatment group were housed in either a standard lab cage or a large ‘enriched’ enclosure in groups of 6 – 8. Animals remained in the perspective housing condition for either 4 or 30 days. Following the prolonged housing (30 days), saline and Ritalin-treated animals were given an acute amphetamine challenge (1.0 mg/kg) and motor activity was recorded. The brains from all groups were harvested and prepared for Golgi-Cox analysis. Dendritic measures of length and branch order were obtained for pyramidal neurons in regions known to be affected by psychostimulant use (Zilles Cg3 and AID) or enriched housing (Zilles Par1). Short (4 day) and prolonged (30 day) housing produced structural changes in pyramidal dendrites, differentially increasing dendritic fields in regions of the prefrontal cortex and Par 1. Ritalin pretreatment completely blocked the enrichment effects. Behaviourally, enrichment diminished the motor response to an acute amphetamine challenge, irrespective of earlier drug experience. Early drug and housing experiences thus influence the later responsiveness of the brain to other experiences.

## **Introduction**

Enriched-housing, defined by Krech (in Diamond [3]) as an experimental environment that includes both “friends” and “toys”, has been used as a scientific model of experience-induced plasticity in rats since the 1960’s. In early studies by Rosenzweig et al., [16] and Diamond, Krech and Rosenzweig [4], it became evident that the thickness of the cortex could be increased in rats housed for prolonged periods in an enriched environment. Follow-up studies by these and other researchers (for review see [3]) have since revealed that the experience-induced increase in cortical thickness is in a large part related to changes in neuronal morphology, such as increased soma size and dendritic arborization that ‘push’ the neurons apart, thereby increasing the depth of a given cortical layer. Changes in dendritic morphology that include increases in dendrite length and spine density are now believed to be a primary mechanism of experience-induced plasticity that supports learning and memory formation (see [8]). The premise is that changes in dendritic morphology alter the size of the dendritic field and in doing so increase the connectivity capacity, allowing for new synaptic input and modifications in cortical circuitry. Importantly, such a mechanism of learning would have to be available throughout the life span, allowing for continued learning and modifications to existing circuitry. Indeed, this is the case, as even aged animals show experience-induced alterations in connectivity as a consequence of enriched housing [9], albeit at a reduced level [6] (see also [3]). Furthermore, cognitive impairments as a result of isolated housing can be at least partially reversed by placement in an enriched environment in both young [7] and aged [17] rats, which implies that brain is somewhat malleable throughout life.

One surprising result from enrichment studies is that although enriched housing in adulthood produces widespread changes across the cortical mantle and striatum, there are no changes in the prefrontal cortex [12]. In contrast, dendritic organization in the prefrontal areas, but not other cortical areas, is consistently altered by psychoactive drugs including stimulants (amphetamine, cocaine, nicotine, [2,13,14], cannabinoids [11], and morphine [15]. Thus, whereas enriched housing has no effect on dendritic morphology in prefrontal regions, psychoactive drugs selectively affect the prefrontal cortex and not other cortical regions. What is especially interesting, however, is that prior exposure to stimulants (at least amphetamine, cocaine, nicotine) blocks enriched-housing dependent changes in the parietal cortex even though the drugs do not directly alter dendritic morphology in the parietal cortex. The drug and complex housing studies suggest that certain types of experience may interfere with subsequent experience-induced change.

Given that the developing brain is especially responsive to experiences such as enriched housing, we wondered how early drug exposure might influence the synaptic changes normally associated with enriched housing. We decided to manipulate the duration of enriched housing because we hypothesized that the effects of enriched housing and/or housing-drug interactions might vary over time. Finally, we examined whether or not the previous drug and/or housing experience(s) would alter the response to an acute amphetamine challenge in adulthood. This latter measure gave us a second measure of the effects of early experiences on later behavioral measures.

## **Methods and Procedures**

**Subjects.** Thirty-four male rats from six litters were obtained at the time of weaning (postnatal day (P) 21). The rat pups were weighed, ear marked for identification purposes, and housed in large plexi-glass hanging tubs in groups of eight. All animals were maintained on a 12 hour light/dark schedule in a large colony room and given ad lib access to food and water.

### ***Drug Administration***

***Methylphenidate (Ritalin;MPD).*** The drug-treatment phase began shortly after weaning (P22-25) at which time the pups were pseudo-randomly assigned to either a saline vehicle (0.9%) or Ritalin (0.5mg/kg) group in a cross-litter design. Twice a day (a.m. & p.m.) the pups were transported to a testing room (naïve environment) where they received subcutaneous (s.c) injections of either saline (0.9%) or Ritalin (0.5mg/kg) for 12 consecutive days. Motor activity was monitored periodically throughout the treatment phase. At the end of the drug-treatment period daily injections were discontinued and subjects remained drug-free (washout) and undisturbed for a period of four days (~P40) prior to being placed in the enriched housing conditions.

***Amphetamine Challenge.*** On the final day of the experiment all animals in the 30 day housing condition were given a single i.p. injection of *d*-Amphetamine (1.0 mg/kg). Five minutes after the injection, motor activity was recorded for a period of 20 minutes to assess the naïve drug response following early drug exposure and housing environment. The animals in the 4 day housing condition were not included in this portion of the experiment owing to the proximity of time between last Ritalin injection and time-frame

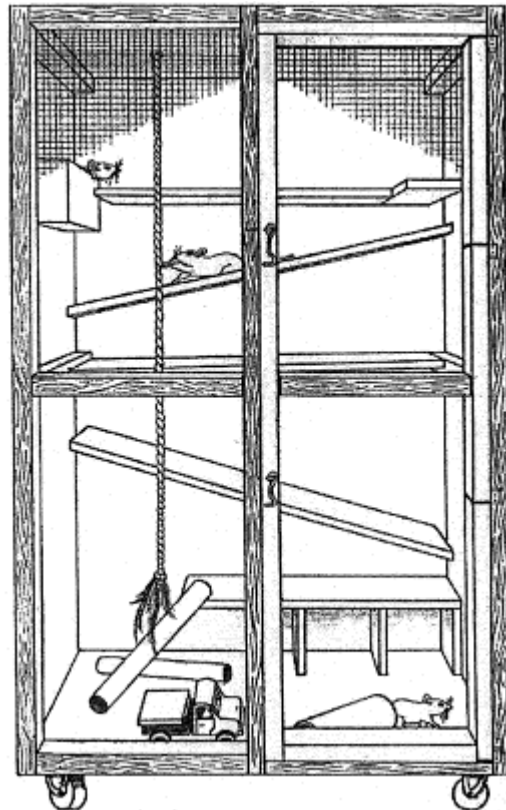
for the amphetamine challenge which was to immediately follow removal from the housing condition.

***Housing Conditions.*** Following the drug-washout period (P38-41), the subjects from each treatment group were randomly assigned to standard ‘lab’ cages or an ‘enriched’-environment for a period of either 4 or 30 days. Lab cages were large plexi-glass hanging tubs (39 X 57 X 21 cm) with corn-cob bedding covering the floor, but otherwise barren. The complex environment used for the enriched-housing group consisted of a large (61 X 122 X 183 cm) ‘condo’-like structure (Figure 2.2.1). The sides and front of the ‘condo’ were made of heavy wire mesh and the back vertical wall was galvanized steel. Three platforms were attached to the back wall at various heights with wooden ramps that provided access to each level. The floor was covered with corn-cob bedding. Objects, that included plastic ‘toys’, paper, cardboard boxes, and PVC pipe were strewn over the floor and the platforms to encourage exploration. The ‘condos’ were cleaned weekly and the objects replaced each time. As with the lab-caged rats, the enriched –housed rats were given ad libitum food and water.

### ***Behavioral Analysis***

***Activity Apparatus.*** Animals were individually tested in the AccuScan Instruments Inc., Versamax animal activity monitoring system (open field) to measure motor activity. The activity apparatus consisted of a clear plexi-glass cage measuring 42cm x 42cm x 30cm with a removable plexi-glass lid. Each cage was equipped with horizontal and vertical sensors (infra red beams) that monitored activity in selected intervals for a total of 20 minutes. Recorded measures of horizontal activity (number of beam breaks on the lower bar in each sample period), and total distance (path traveled in centimeters) were

retrieved and used for analysis. Once the testing period was complete, the VersaDat function scanned the files for errors to verify the integrity of data collected. The files were then converted for import to an excel spread sheet.



**Figure 2.2.1.** Schematic illustration of enrichment condo used to house animals in the enriched housing condition (picture from Kolb, Gibb & Gorny,[9])

### *Anatomical Procedures*

**Tissue Preparation.** Subjects were given an overdose of sodium pentobarbital at the end of the experimental procedures and intracardially perfused with a 0.9% saline solution.

The brains were harvested and post-fixed in a Golgi-Cox solution for two weeks at which time the brains were transferred to a 30% sucrose solution where they remained until being sectioned. Brains were sectioned at 200  $\mu\text{m}$  on a Vibratome and mounted on gelatin

coated slides for staining. Mounted sections were stained using the modified Golgi-Cox staining procedures set out by Gibb and Kolb [5].

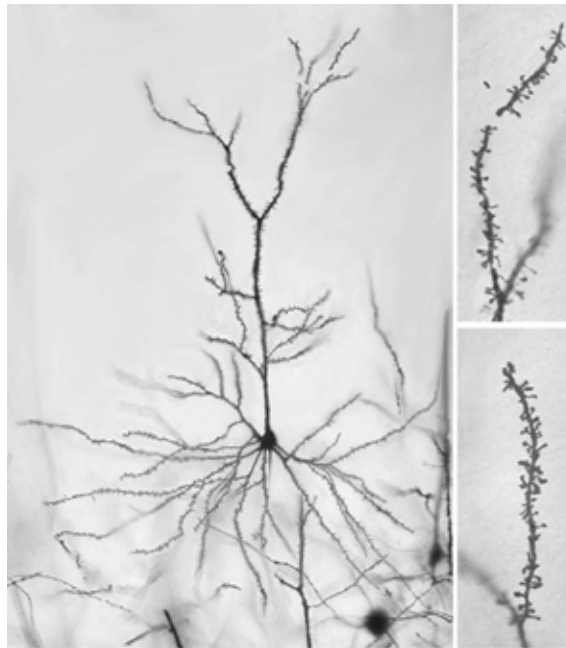
*Assessment of dendritic morphology.* Neurons from select brain regions known to be affected by psychostimulant use and/or enriched housing were reconstructed (see below) and the dendritic morphology quantified for analysis. The cells included in the current study were pyramidal cells (e.g., Figure 2.2.2, left) of the primary somatosensory cortex (Par 1, layer III), region three of the anterior cingulate cortex (Zilles Cg3, layer III and layer V) as well as pyramidal neurons of the dorsal agranular insular cortex (AID, layer III) described by Zilles [18].

To be included in the analysis, cells had to be; 1) thoroughly impregnated by the Golgi-Cox solution so that the dendritic trees were clearly visible at 100X and not obscured by blood vessels, neighboring processes or debris, and 2) relatively intact with minimal breakage. The cells were drawn using camera lucida (200X) and quantified by means of; 1) dendritic branch order (number of branch bifurcations), and 2) Sholl analysis (number of branches that crossed an overlay of concentric rings spaced 20  $\mu\text{m}$  apart). The means from five neurons in each hemisphere were calculated and used for later comparisons.

Spine density was also determined for fourth-order terminals of basilar dendritic trees in Cg3 LV and AID LIII. As with dendritic branch order and Sholl, spine density for each hemisphere was determined by calculating the mean of five terminals (10  $\mu\text{m}$  in length). Spines were drawn at 1000X (see Figure 2.2.2, center) and included all spine protrusions that had a visible head. It was recognized that this method of evaluation is an

underestimation of the total spine density as those spines on both the bottom and top of the dendrite would not be visible to the experimenter (see[12] for additional details).

Cells within each region were by one of three different experimenters (WC, KK, & CC) blind to the groups until analysis.



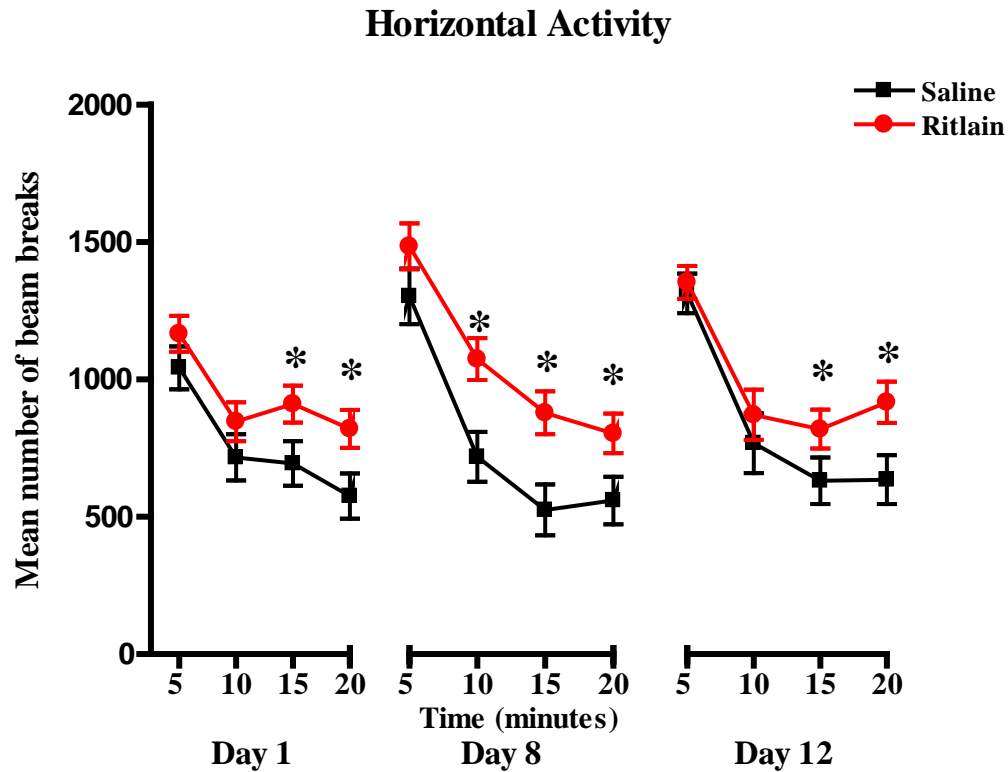
**Figure 2.2.2. Left:** Photograph of a layer III pyramidal cell. **Right:** Photograph of dendritic spines on a segment of basilar dendrite.

## Results

### *Motor Activity*

*Treatment Phase.* Daily Ritalin treatment produced an increase in horizontal activity relative to the saline-treated littermates (Figure 2.2.3). The response, however, was a stable drug effect, rather than behavioral sensitization as the effect remained relatively stable from day 1 to day 12 of the treatment phase. A repeated-measure ANOVA of horizontal activity in five minute increments showed a significant effect of drug treatment,  $F(1,32) = 9.25$ ,  $p = 0.005$ . Pairwise comparisons revealed no significant

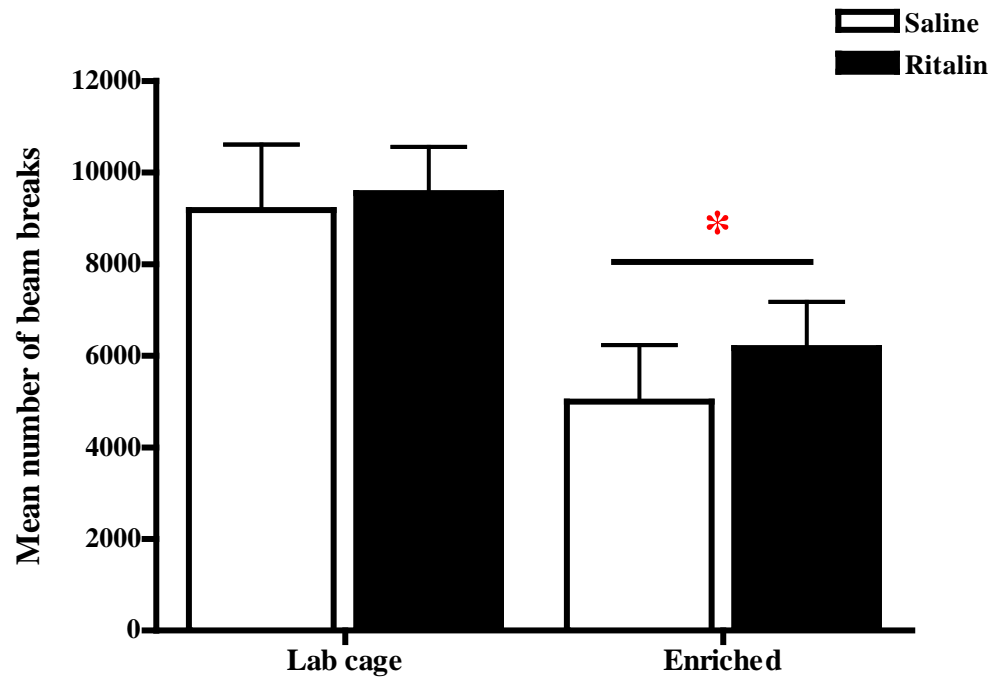
differences between the last five minutes of Day1, Day 8 and Day 12 in either Ritalin-treated (all  $p$ 's > 0.20) or Saline-treated groups (all  $p$ 's > 0.40).



**Figure 2.2.3..** A summary of mean horizontal activity in 5 minute increments over the 20 minute test period on days 1, 8 and 12. The asterisks show that Ritalin treatment produced an enhanced response on each of the days although there were no significant changes between Day 1 and Day 12 in either treatment group.

**Amphetamine challenge.** Owing to the potential for group differences as a consequence of housing, motor activity was analyzed by treatment and housing condition in the amphetamine challenge (Figure 2.2.4.). Indeed, the results indicate that housing condition rather than treatment influenced the response to subsequent psychostimulant use, in this case amphetamine. An ANOVA of horizontal activity with treatment and housing as variables showed a main effect of housing,  $F(1,15) = 10.24$ ,  $p = 0.006$ , but not drug treatment,  $F(1,15) = 0.42$ ,  $p = 0.53$ , nor the interaction,  $F(1,15) = 0.11$ ,  $p = 0.74$ .

### Amphetamine challenge (horizontal activity)



**Figure 2.2.4..** A summary of total mean horizontal activity of pretreated Ritalin or saline rats that were later housed in either lab or enriched environments for 30 days prior to the amphetamine challenge. The results show an effect of housing on activity but no effect of drug treatment.

#### *Dendritic Morphology*

The effects of enriched housing on dendritic morphology varied with the duration of the experience. Early Ritalin exposure invariably blocked the effect of enriched housing and further altered synaptic connectivity. Long-term (30 day) enriched produced increases in basilar, but not apical, dendritic length and/or branch order in the Par 1 and Cg3 LV but had no effect on AID neurons. In contrast, short-term (4 day) enrichment produced increases in apical length of Par 1 neurons, an increase in AID spine density, but had no effect on Cg3 neurons.

Although Ritalin pretreatment itself had only a limited influence on dendritic morphology; increasing AID spine density and decreasing spine density in the Par 1 as

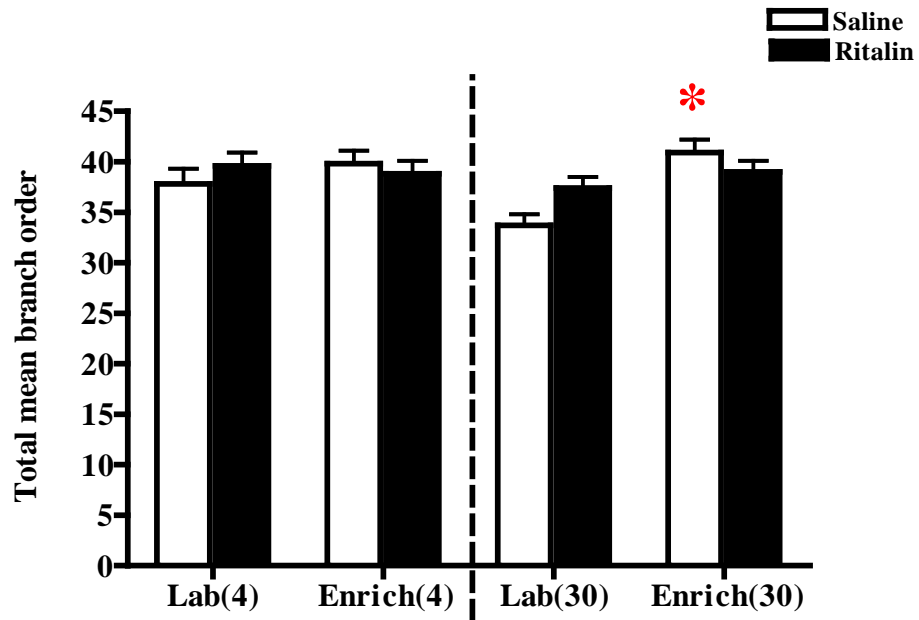
well as dendritic length of Cg3 LIII apical dendrites; pre-exposure to Ritalin blocked all effects of enriched housing (both short and long-term) on Par 1 and Cg3 LV neurons. As well, in combination with enrichment, Ritalin-pretreatment produced a decrease in Par 1 apical branch order, AID apical length, but an increase in Cg3 LV spine density.

***Par1 LIII Branch order.*** Prolonged (30 day), but not short-term (4 day), enriched housing produced an increase in basilar branch order (Figure 2.2.5a). In contrast, Ritalin + Enrich rats showed a decrease in apical branch order that was restricted to short-term enriched housing, but no effects of treatment in animals housed in enriched environment for an extended period of time (Figure 2.2.5b).

An ANOVA of basilar branch order with treatment and housing as variables showed a significant main effect of housing [ $F(3,58) = 3.72, p = 0.02$ ], but not drug treatment [ $F(1,58) = 0.46, p = 0.50$ ], nor interaction [ $F(3,58) = 1.67, p = 0.18$ ]. The pairwise comparisons showed saline rats housed in condos for 30 days had an increase in basilar branching relative to saline rats housed in lab cages for 30 days ( $p = 0.003$ ), but no differences were found between saline groups housed in lab and enriched conditions for 4 days ( $p \geq 0.35$ ). There were no differences between Ritalin-treated groups housed in either condition ( $p$ 's  $> 0.20$ ).

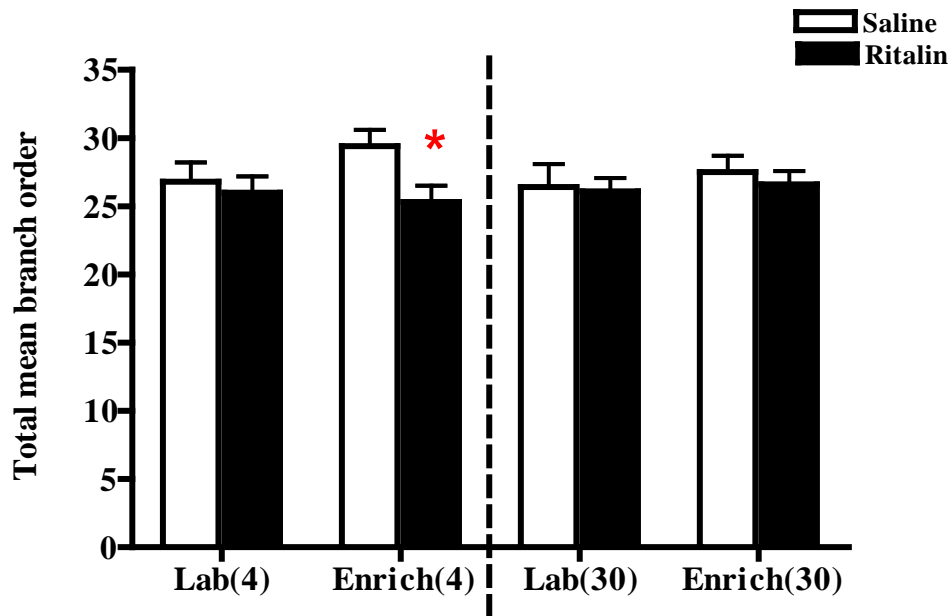
In contrast, analysis of apical branches revealed no main effect of housing [ $F(3,58) = 0.36, p = 0.78$ ], treatment [ $F(1,58) = 2.96, p = 0.09$ ], nor interaction [ $F(3,58) = 1.02, p = 0.39$ ] on branch order. The pairwise comparison revealed only that basilar branch order was significantly reduced in Ritalin-treated rats placed in the condo for 4 days relative to their untreated counterparts ( $p = 0.02$ ). No other differences among the groups were found ( $p$ 's  $> 0.10$ ).

**A. Par1 LIII Basilar Branch order**



**Figure 2.2.5a.** An illustration of the mean basilar branch order that depicts an enrichment effect after prolonged housing but no effect of short-term housing nor Ritalin pretreatment.

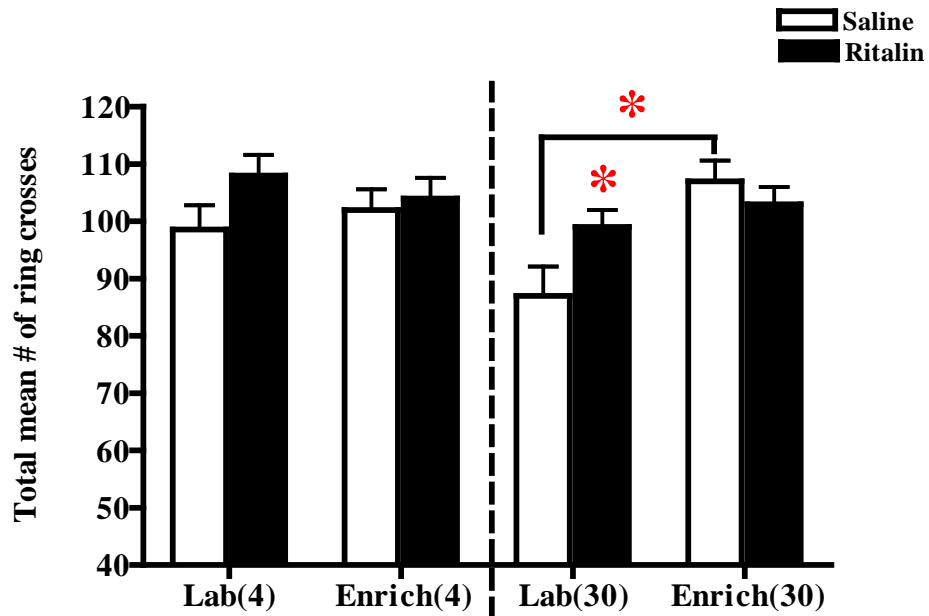
**B. Par1 LIII Apical Branch order**



**Figure 2.2.5b.** An illustration of the mean branch order of apical dendrites. The graph demonstrates that branch order was significantly decreased in Ritalin + Enrich rats in the 4 day condition only. There was no effect of enrichment alone.

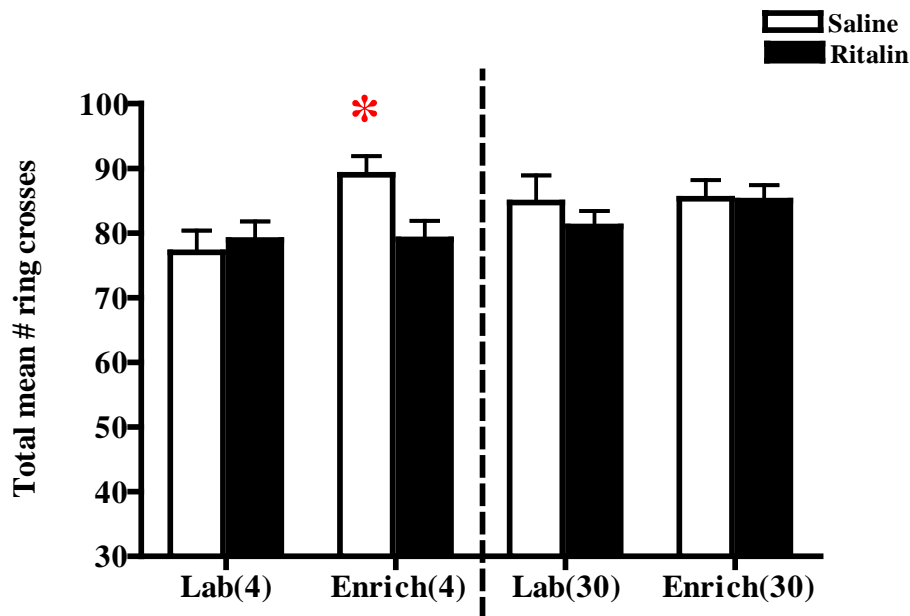
**Sholl.** Similar to branch order, rats housed in the enriched condition showed increases in basilar dendritic length (Sholl), but only after 30 days (Figure 2.2.6a), whereas apical dendritic length was increased in rats housed in enriched environments for 4 days but not 30 days (Figure 2.2.6b). In both cases, however, the increase was blocked in rats pretreated with Ritalin. An ANOVA of basilar length with housing and drug treatment as variables showed a main effect of housing [ $F(3,58) = 3.56, p = 0.02$ ], but not drug treatment [ $F(1,58) = 3.21, p = 0.08$ ], and no interaction [ $F(3,58) = 1.93, p = 0.14$ ]. The pairwise comparison however, revealed that animals housed for 30 days in enriched environment had significantly longer basilar dendritic length relative to those housed in lab cages ( $p = 0.003$ ). No differences were found among the Ritalin-treated groups ( $p$ 's  $> 0.05$ ). An ANOVA of apical dendrites showed no significant main effect of housing [ $F(3,58) = 2.17, p = 0.10$ ], drug treatment [ $F(1,58) = 1.94, p = 0.17$ ], or interaction [ $F(3,58) = 1.49, p = 0.23$ ]. The pairwise comparison revealed a significant increase in apical length in saline animals housed for 4 days in the enriched environment relative to the 4-day lab-housed rats ( $p = 0.01$ ), an effect that was absent in the Ritalin-pretreated groups ( $p = 0.99$ ). No other group differences were found ( $p$ 's  $> 0.05$ ).

### A. Par1 LIII Basilar length



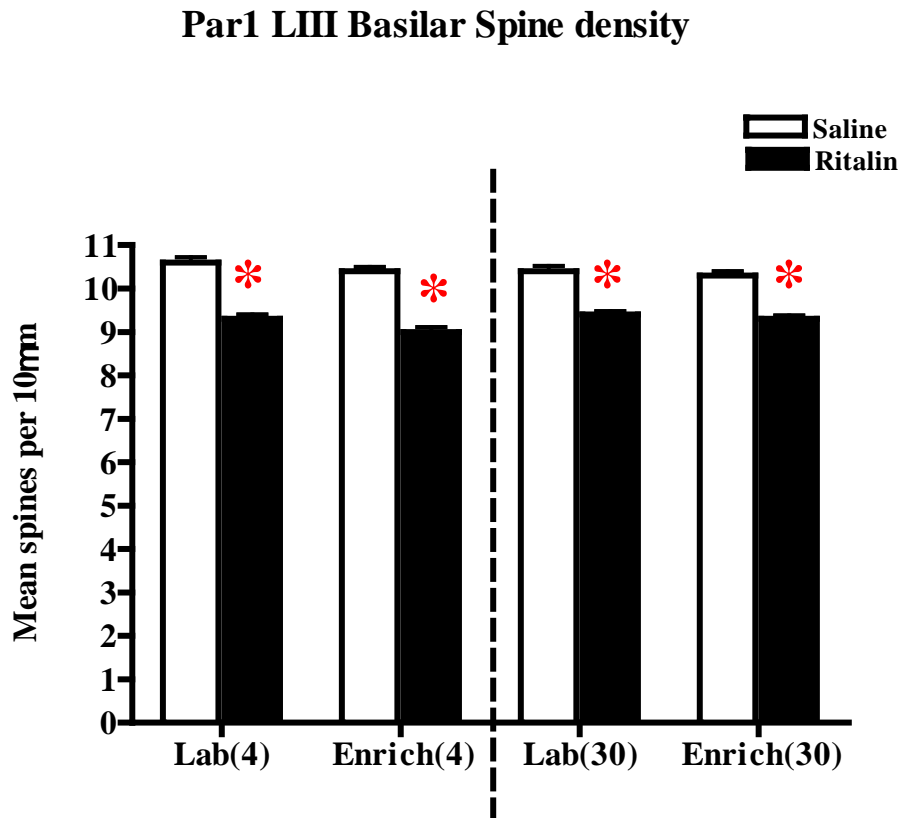
**Figure 2.2.6a.** The graph depicts the effect of housing and treatment on basilar dendritic length. The results show that enrichment (30 day) enhances dendritic length, but the housing effect is not evident in rats pretreated with Ritalin.

### B. Par1 LIII Apical length



**Figure 2.2.6b.** An illustration of the effect of housing and drug treatment on apical dendritic length. The results show that short term (4 day) enriched housing increases apical dendritic length, but the effect is blocked by pre-exposure to Ritalin.

**Par 1 layer III spine density.** Ritalin pretreatment reduced spine density in basilar dendrites, but we found no effect of housing (Figure 2.2.7). An ANOVA of spine density with drug treatment and housing as variables showed a significant main effect of drug treatment [ $F(1,59) = 267.0, p \leq 0.001$ ], but no main effect of housing [ $F(3,59) = 1.64, p = 0.19$ ], nor the interaction [ $F(3,59) = 2.18, p = 0.10$ ].



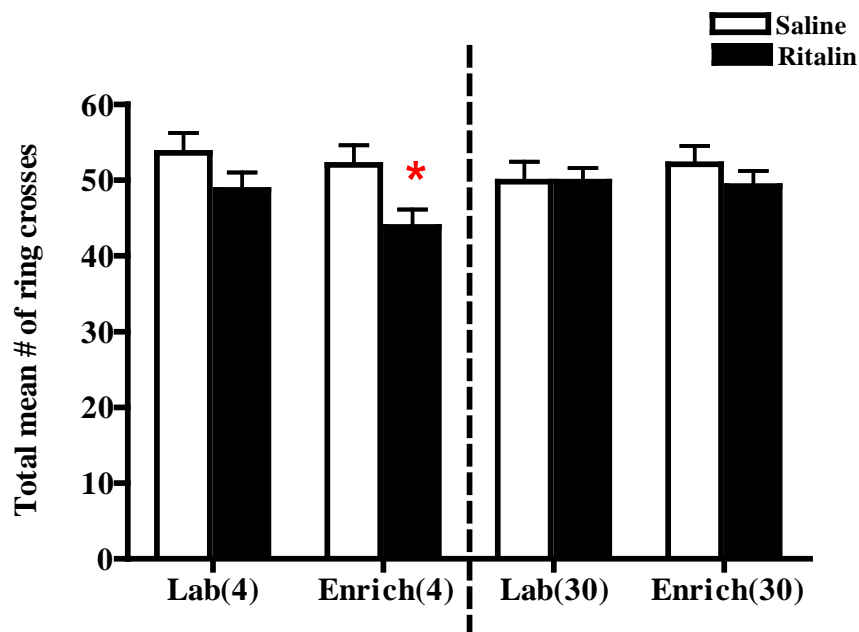
**Figure 2.2.7.** Summary of the effect of early Ritalin exposure and enrichment on spine density of the Par 1. Ritalin pretreatment produced a decrease in density irrespective of housing condition.

**AID LIII.** Although Ritalin alone did not influence apical dendritic length, the combination of short-term enriched housing and drug treatment decreased apical dendritic length (Figure 2.2.8) but had no effect on basilar length. In contrast, Saline-treated rats

showed no effect of housing on either apical or basilar length, irrespective of duration. Branch order in apical and basilar dendrites showed no influence of experience or drug.

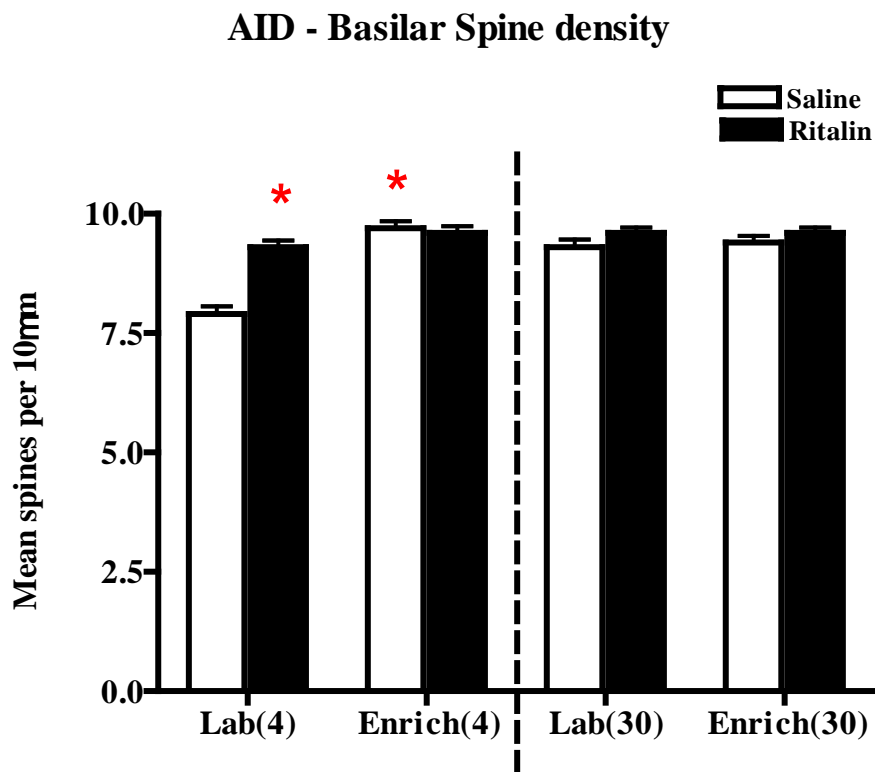
An ANOVA of apical length with housing and treatment as variables showed a significant main effect of drug treatment  $F(1,54) = 5.85, p = 0.02$ , but not housing,  $F(3,54) = 0.71, p = 0.55$ , nor interaction,  $F(3,54) = 1.08, p = 0.37$ . The pairwise comparisons revealed that Ritalin + Enriched for 4 days significantly reduced apical dendritic length ( $p = 0.02$ ) relative to the saline rats in the same conditions. An ANOVA of basilar length showed no effect of either drug treatment  $F(1,55) = 0.56, p = 0.46$ , or housing  $F(3,55) = 0.37, p = 0.77$ , nor the interaction,  $F(3,55) = 0.93, p = 0.43$ . ANOVA's of apical and basilar branch order showed no effect of drug treatment or housing ( $p$ 's > 0.07).

### AID - Apical Sholl analysis



**Figure 2.2.8.** An illustration of dendritic length of layer III apical neurons of the AID. The graph illustrates an effect of Ritalin+ enrichment, but only when housed in the environment short-term (4 days).

**AID Spine density.** The number of spines was increased in rats with either pretreatment with Ritalin or enriched-housing for 4 days (Figure 2.2.9). The combination of both experiences had no additive effect, however. An ANOVA of basilar spine density with housing and drug treatment as variables showed a significant main effect of housing [ $F(3,60) = 4.93, p = 0.004$ ] and drug treatment [ $F(1,60) = 3.96, p = 0.05$ ], but no interaction [ $F(3,60) = 1.13, p = 0.35$ ].

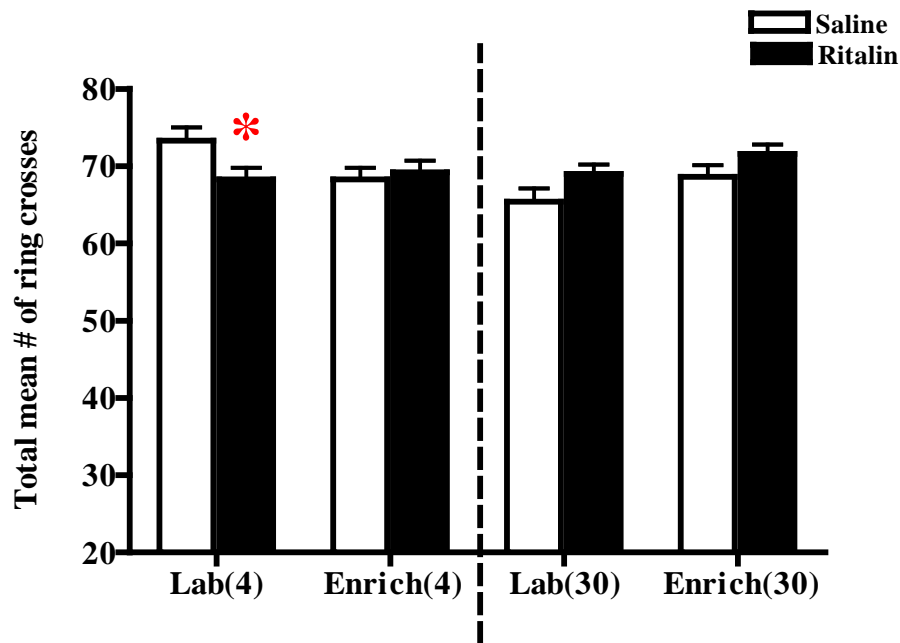


**Figure 2.2.9.** A summary of the mean number of spines over a 10 µm section of basilar dendrites. The results show an increased spine density in rats pretreated with Ritalin, as well as in rats housed in enriched conditions for 4 days. There were no apparent additional effects of the combined experiences.

**Cg3 LIII.** The apical Sholl analysis showed that dendritic length was reduced in animals pretreated with Ritalin, or housed in enriched conditions. The combination of Ritalin + enriched, however, appeared to ‘block’ the decline (Figure 2.2.10). An ANOVA of apical

dendritic length with housing and drug treatment as variables showed a significant interaction,  $F(3,60) = 3.14$ ,  $p = 0.03$ , but no main effect of drug treatment,  $F(1,60) = 0.34$ ,  $p = 0.56$ , nor housing,  $F(3,60) = 2.18$ ,  $p = 0.10$ . The pairwise comparison revealed time and housing produced a decline in dendritic length in Saline rats (all  $p$ 's  $< 0.05$ ), whereas Ritalin treatment produced only an initial decrease ( $p = 0.03$ ) that was not changed by housing condition or duration of manipulation ( $p$ 's  $\geq 0.10$ ). Neither basilar dendritic length, nor apical or basilar branch orders were influenced by either housing or treatment (all  $p$ 's  $> 0.05$ ).

### Cg3 (LIII) apical sholl analysis

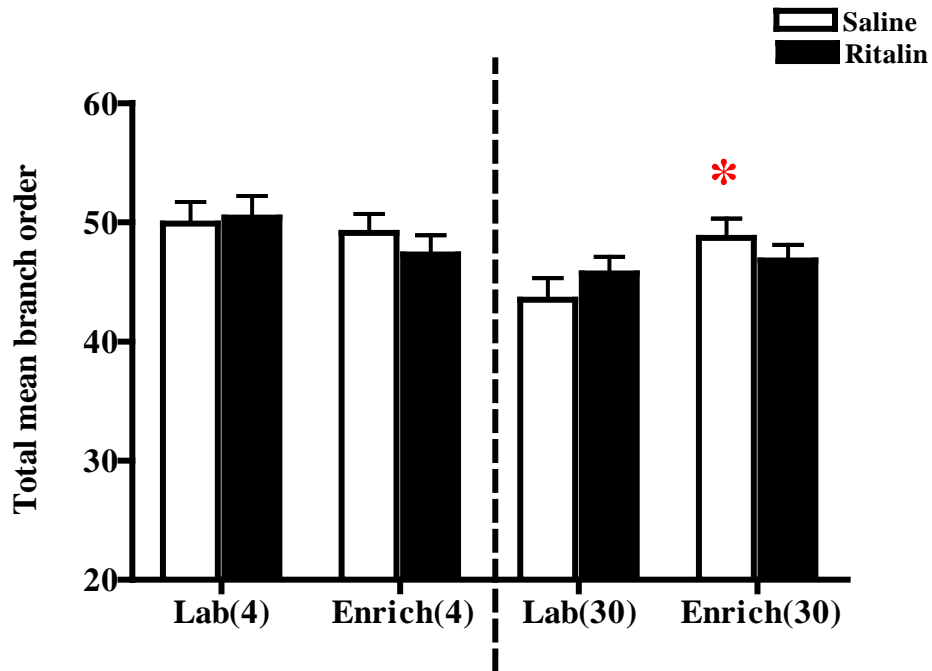


**Figure 2.2.10.** An illustration of changes in dendritic length as a consequence of time, housing, and treatment. Saline-treated rats show a decline in dendritic length over the duration of the experiment, irrespective of housing condition. In contrast, Ritalin pretreatment produced an initial decrease in dendritic length that was not modified by housing condition or time.

***Cg3 layer V.***

***Cg3 LV Branch order.*** Although there were no immediate effects of either housing (4 days) or drug treatment, prolonged enriched housing produced an increase in basilar branch order of Saline, but not Ritalin, treated rats (Figure 2.2.11). An ANOVA of branch order with drug treatment and housing as variables showed a significant main effect of housing,  $F(3,56) = 3.77$ ,  $p = 0.015$ , but not drug treatment,  $F(1,56) = 0.05$ ,  $p = 0.83$ , or interaction,  $F(3,56) = 0.79$ ,  $p = 0.51$ . In Saline-treated rats the pairwise comparison revealed a significant decrease in branch order in animals housed in lab cages for 30 days relative to all other housing conditions ( $p$ 's  $< 0.04$ ). In contrast, Ritalin-treated rats housed in lab cages for 30 days showed a decline in branch order relative to rats housed in lab cages for 4 days ( $p = 0.04$ ) but no differences when compared to enriched housing groups, regardless of the duration of the housing condition. Apical branch order appeared unaffected by either drug treatment or housing condition. An ANOVA of branch order showed no main effect of housing,  $F(3,56) = 0.84$ ,  $p = 0.48$ , treatment,  $F(1,56) = 0.46$ ,  $p = 0.50$ , nor an interaction,  $F(3,56) = 0.76$ ,  $p = 0.52$ .

### Cg3 (LV) basilar branch order

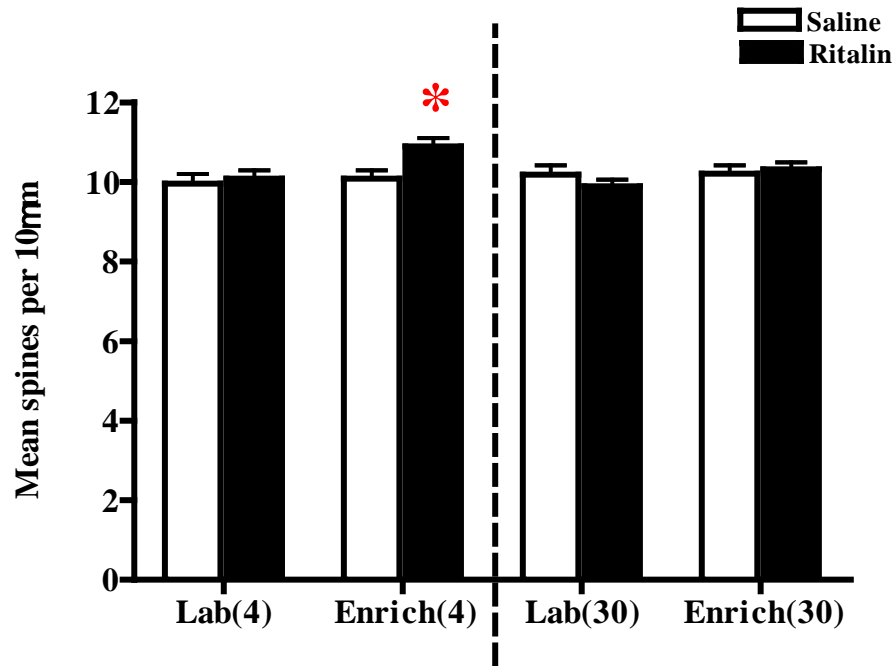


**Figure 2.2.11.** An illustration of the effects of time, housing, and treatment on basilar branch order. Saline animals show a decrease in branch order over time that is attenuated with enriched housing. In contrast, although Ritalin-treated rats showed the same decrease over time, the enriched-housing effect was blocked by previous drug experience.

**Cg3 LV Sholl.** Neither pretreatment with Ritalin nor housing condition influenced dendritic length (Sholl) in layer V Cg3 pyramidal neurons. Apical and basilar Sholl analyses showed that all groups were comparable. ANOVA's of apical and basilar dendritic length with drug treatment and housing as variables showed no main effect of drug treatment [ $F(1,56) = 3.49, p = 0.07$ ], housing [ $F(3,56) = 1.21, p = 0.32$ ], or interaction [ $F(3,56) = 0.20, p = 0.90$ ] on apical length, nor was there a significant main effect of drug treatment [ $F(1,56) = 0.73, p = 0.40$ ], housing [ $F(3,56) = 1.92, p = 0.13$ ], or the interaction [ $F(3,56) = 0.51, p = 0.68$ ] on basilar dendritic length.

***Cg 3 LV Spine density.*** Although Ritalin alone had no effect on spine density, the combination of Ritalin and enriched-housing produced a significant increase in spine density after only 4 days in the environment. The drug treatment effect waned by day 30, producing only a moderate increase relative to lab-housed counterparts (Figure 2.2.12). In contrast, enriched-housing had little effect on the spine density of layer V Cg3 neurons in untreated rats, regardless of whether they were left in the environment for 4 or 30 days. An ANOVA of spine density with housing and drug treatment as variables showed a trend towards a significant interaction,  $F(3,60) = 2.51$ ,  $p = 0.07$ , but no main effect of housing,  $F(3,60) = 2.20$ ,  $p = 0.10$ , nor drug treatment,  $F(1,60) = 1.66$ ,  $p = 0.20$ . The pairwise comparisons revealed that Ritalin-treated rats exposed to enriched housing for 4 days had a significantly higher spine density relative to lab-housed or saline treated rats over the same period of time ( $p$ 's = 0.007). The effect was transient however, as both Ritalin and Saline-treated animals in lab or enriched housing conditions for 30 days showed no significant differences ( $p$ 's > 0.05).

### Cg3 (LV) Spine density



**Figure 2.2.12.** An illustration of mean basilar spine density on fourth-order dendritic branches. The graph shows that Ritalin+enriched (4 days) had a significantly higher spine density but the effect appeared to be transient as there were no differences among the groups in housing conditions for prolonged periods of time (30 days).

## Summary of Experiments in Study Two

	Basilar			Apical			Spine (basilar)				
	Cg3	OFC	Par1	Cg3	OFC	Par1	Cg3	OFC	Par1		
<b>Ritalin vs Saline</b>	LIII	LV	LIII	LIII	LIII	LV	LIII	LIII	LV	LIII	LIII
Lab(4 day)	∅	∅	∅	<b>h</b>	<b>i</b>	∅	∅	∅	∅	<b>h</b>	<b>i</b>
Lab (30 day)	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	<b>i</b>
<b>Ritalin vs Saline</b>											
Complex (4)	∅	∅	∅	∅	∅	∅	<b>i</b>	<b>i</b>	<b>h</b>	∅	<b>i</b>
Complex (30)	∅	∅	∅	<b>h</b>	∅	∅	∅	∅	∅	∅	<b>i</b>
<b>Complex vs Lab(Saline)</b>											
Complex (4)	∅	∅	∅	∅	<b>i</b>	∅	∅	<b>h</b>	∅	<b>h</b>	∅
Complex (30)	∅	<b>h</b>	∅	<b>h</b>	∅	∅	∅	∅	∅	∅	∅

**Table 2.2.1.** Arrows indicate direction of dendritic changes relative to rats housed in standard lab cages/ or treated with saline. **Blue arrows = branch order; Red arrows = Sholl (length); Spine = spine density; ∅ = no change; Cg3 = region three of the cingulate gyrus; OFC (AID) = orbital prefrontal cortex; Par 1 = primary somatosensory cortex; LV = layer five neurons and LIII = layer three neurons.**

## Discussion

The most notable findings in the current experiment are that 1) enriched housing in adolescence influences dendritic morphology in both prefrontal and sensory cortical pyramidal neurons, but varies with the duration, 2) early Ritalin experience blocks the anatomical effects of enrichment, and in combination with enriched housing produce a unique pattern of connectivity; and 3) enriched housing in periadolescence alters later response to amphetamine. These findings will be discussed separately.

### *Duration of enriched housing differentially influences prefrontal and parietal cortices*

In contrast to a previous study of enriched housing in adult rats [17], in we found increased spine density in both prefrontal regions after 4 days enrichment and increased branching in basilar dendrites of Cg3 LV neurons after 30 days the current study.

Although only a few studies have examined the effect of enrichment on the prefrontal

cortex, the current results may be explained by either by the duration of enriched housing or the age at enriched housing. The previous study only examined neuronal morphology following 3 months of enrichment, which contrasts with the 4 and 30 days in the current study. It may be that any effect of enrichment in the PFC is transient and related to ‘novelty’ and learning. Once learned, the information is stored elsewhere and no longer requires prefrontal cortical input.

Our finding that enriched housing produced an experience-dependent increase in dendritic morphology of parietal cortex (Par1) neurons is consistent with earlier studies that have reported enrichment-induced increases in Par 1 dendritic arborization. A novel finding, however, was that spine density in these neurons was unaffected by the housing condition. Previously, Kolb and colleagues [9] have reported a decrease in Par 1 spine density following juvenile enriched housing, whereas animals housed in enriched environments in adulthood showed an increase in spine density in the same population of neurons. The decrease following juvenile enrichment was in contrast with a previous study that had reported an increase in Par1 as a consequence of juvenile enrichment. The researchers had speculated that the duration of housing condition may have been a factor in the discrepancy (90 days vs 30 days), a suggestion that is supported in a review by Diamond, in which it was pointed out that the 30 day enrichment protocol came about because the effects were larger than those found after 80 days of enriched housing. Duration may in part account for the novel finding in the current experiment but it is also likely that age was a factor, considering animals in the current study did not enter into the perspective housing conditions until postnatal day 38 compared to just after weaning in the previous studies. Another possible factor of course is that the previous experience

during the drug-testing phase may have resulted in an increase in both the lab and enriched animals, producing a ceiling effect.

Finally, we were surprised to find that the effects of 4 and 30 days of enrichment were qualitatively different. For example, whereas parietal apical dendritic length was increased after 4 days, but not 30 days of enriched housing; basilar length was increased after 30 but not 4 days of exposure. Similarly, Cg 3 dendritic branching was increased only after 30 days of enrichment whereas AID spine density was increased only after 4 days of enrichment. Experience is clearly differentially interacting with different cortical regions and although we do not know how this relates to later behavior or plasticity, it is a good guess that it must.

***Early Ritalin exposure stimulated activity and produced chronic changes in dendritic organization.***

Daily Ritalin use in adolescent rats produced an overall increase in activity at each test day. The effect was moderate, however, and failed to escalate as would be expected with the induction of behavioural sensitization. This finding is consistent with a previous study in which chronic Ritalin use also failed to produce sensitization (Comeau et al., in submission) in adolescent male rats. The absence of behavioral sensitization is further shown by the fact that Ritalin-treated subjects showed no differences in response to an acute amphetamine challenge compared to drug naïve animals in adulthood. A similar finding has been reported by Carlezon, Mague and Andersen [1], as well as Bolanos [12], although in both of these earlier studies it was shown that previous Ritalin use *diminished* the response to psychostimulants in later in life. The slight discrepancy here is likely

related to dose however, as few studies have used such a low dose in their investigation of the neurobiological effects of Ritalin.

One of the most important findings in the current study is that early Ritalin pretreatment blocked all anatomical effects of enrichment studied here, with the exception of AID spine density. Thus, anatomical changes that likely would be advantageous to the developing and injured brain alike appear to be completely inhibited by the early drug experience. What is so unexpected is that these results are more reminiscent of the effects of much larger doses of amphetamine or cocaine used to investigate the effects of on enriched housing in the adult brain [10]. It would seem then that drug experience may have larger effects in the immature than adult brain. We can speculate that the early drug exposure will have a greater effect on later experience-induced plasticity, perhaps because the developing brain develops an aberrant pattern of connectivity that may not be adaptive to future experiences.

#### ***Enriched housing reduces the response to a subsequent amphetamine challenge***

Housing animals in complex environments significantly reduced the later response to a single challenge dose of amphetamine, independent of Ritalin experience. This result suggests that early experiences will influence the later effect of psychomotor stimulant drugs and we can speculate that the experiences may affect the likelihood of addiction to psychomotor stimulants in adulthood. Rats housed in enriched environments are proposed to be less anxious and this could potentially interact with later stimulant challenges [1-4]. We did not assess anxiety-like behaviour in the present study so this remains speculation at this point.

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### **III. The effects of prolonged methylphenidate use in adolescent rats are moderated by training**

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**Abstract.**

Clinical use of methylphenidate (Ritalin) often extends from the pre-pubertal to post-pubertal developmental periods and beyond. Psychostimulants are known to influence neuronal functioning in the prefrontal cortex, which is still developing during the time that Ritalin is most prescribed. Yet, there is a lack of research investigating the effects that prolonged Ritalin use may have on cognitive and social development, behaviours that are subserved by the prefrontal cortex. In the current study the drug treatment period was extended to include both the pre and post-pubertal stages of development. Injections of 0.5mg/kg of Ritalin (2X daily) began immediately following weaning (postnatal day (P) 22) and continued until P60. A number of behaviors and experiences were introduced during and/or after the treatment period, including open field testing, play behavior, and a non-match to sample T-maze task. A subset of rats did not participate in the T-maze training paradigm, but they were handled and transported to the testing area along with the animals that were included in behavioural testing. The results showed that prolonged, early Ritalin exposure enhances motor activity and negatively impacts on cognitive skills, producing a decline in performance on the T-maze task in adulthood. Social behaviour was also altered during Ritalin treatment with saline-treated rats showing a distinct preference for the same when given a choice between saline or Ritalin-treated playmates. Further, the anatomical results revealed that both training and Ritalin altered the dendritic fields of layer III prefrontal cortical regions, whereas the combination of Ritalin + training increased the dendritic field in layer V prefrontal neurons only. There were no drug or training effects on neurons of the parietal, hippocampus, or nucleus accumbens.

## Introduction

Chronic psychostimulant exposure has the potential to disrupt normal brain functioning, perhaps even permanently. The implications of permanent alterations in brain circuitry are especially pertinent when considering the use of stimulants to treat childhood disorders such as attention deficit hyperactivity disorder (ADHD). Although one of the most widely prescribed treatments for ADHD for over 50 years [24], methylphenidate (MPD; Ritalin) has gained considerable attention in the past couple of decades, with growing concerns as to the possible long term consequences of its use [1, 28, 55].

One concern that has received much attention is the potential for an increased risk of developing addictive-like behaviour as a consequence of early Ritalin exposure [14, 36]. Similar to drugs of abuse such as cocaine and *d*-amphetamine in property and action [20, 34], Ritalin is typically prescribed at doses lower than those required for the development of addiction, however. Indeed, the link between altered connectivity in the nucleus accumbens (NAc) and behavioural sensitization, a marker of addictive behaviour [47, 48], has been reliably established with neuroanatomical measures such as dendritic morphology and connectivity [11, 49], but has not been replicated in animal studies using a clinically relevant dose of *d*-amphetamine [21] nor Ritalin [19]. These findings would suggest that low-dose Ritalin may not directly affect addiction-related circuitry. Furthermore, the behavioural results from a number of studies that have investigated the effects of early psychostimulant use have been inconsistent with the hypothesis that early Ritalin exposure may increase the risk for later addiction [8, 25, 35, 39, 41]. It should be noted here that there are studies that provide contrary behavioural evidence [4, 14, 42, 51]. These contradictions are likely due to the variations in dose, treatment duration, and

age of initial exposure across studies, making direct comparisons impossible.

Nonetheless, the conflicting evidence indicates that the potential for Ritalin to alter later responses to experiential events, including illicit drug use, needs further study.

In addition, the ability of both cocaine and amphetamine to block enrichment-induced changes in the cortex, as well as the nucleus accumbens (NAc), as reported by Kolb et al [31] indicates that there is also a need to examine the ability of early Ritalin use to alter later experience-induced changes. To date, research into the effects of early Ritalin exposure on social and cognitive development is lacking although there is undoubtedly the potential to influence subsequent experience-induced plasticity.

The main action of many psychostimulants, including Ritalin, is the blockade of the dopamine and norepinephrine transporters (DAT & NET, respectively) with the secondary effect being an increase in extracellular DA levels [7, 56, 57]. Although low-dose Ritalin use has not been shown to permanently alter NAc circuitry as determined by the absence of significant alterations in dendritic morphology, the prefrontal cortex (PFC) also receives extensive dopaminergic input from the ventral tegmentum area (VTA), and is therefore a site of action for catecholamine agonists [6], including Ritalin [10, 40]. Indeed, psychostimulant use has been shown to alter the functioning and structure of the prefrontal cortex (PFC) even in adulthood. Further, owing to the fact that the PFC is a late developing cortical region with maturation continuing into early adulthood [2, 3], even low-dose Ritalin use during this period may have an increased potential to influence PFC circuitry. Importantly, the full effect of early stimulant experience may not be fully realized, however, until late developing brain regions and the behaviours they support mature.

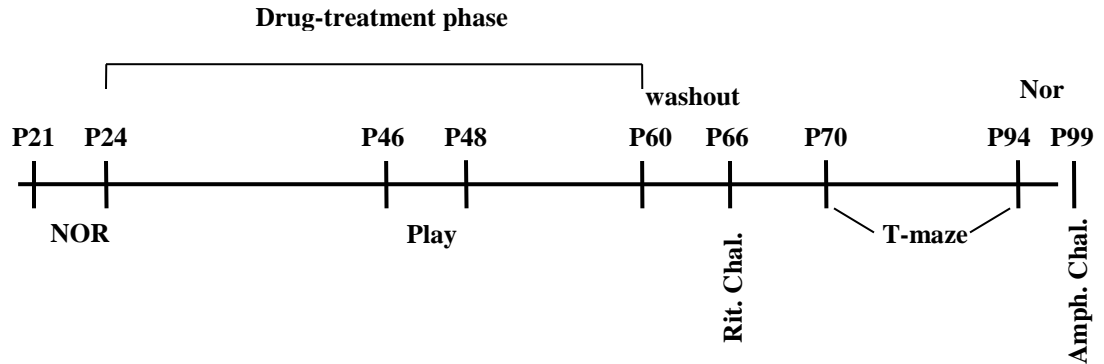
The continued development of the PFC into early adulthood and the fact Ritalin use in clinical populations is often extended from early childhood to adolescence and beyond, warrants a more thorough examination of prolonged Ritalin use. The few studies that have investigated the effects of extended Ritalin use have implemented doses that are more in line with the investigation of sensitization (e.g., [23] ) and therefore may not have clinical relevance to early childhood exposure for treatment purposes.

We examined the behavioural and anatomical effects of low-dose Ritalin use in rats following the prolonged exposure that would occur in clinical populations, taking into account that treatment duration, age, dose, and experiential influences are all important considerations. In the current study we used a treatment regiment that transcended across the preadolescent and adolescent period. A broad range of behavioural measures were used to determine not only the possible sensitizing effects of long-term stimulant use, but also the effects on cognitive and social development as well as the potential risk for later addictive behaviour. To aide in the determination of the potential influence of Ritalin on experience-induced plasticity, we also included ‘training’ and ‘no-training’ groups. In keeping with the notion that long-term use may produce alterations in the NAc as well as the PFC, we used Golgi-Cox analyses to determine structural changes that would in effect indicate altered circuitry in these regions.

## **Materials and Methods**

**Subjects.** Twenty one Long-Evans male rats were used in the current study. Rats were obtained from four different litters on the day prior to weaning (postnatal day (P) 21). Pups were randomly assigned to receive one of two treatments; 1) Ritalin (0.5mg/kg) or,

2) saline (0.9% NaCl dissolved in dH<sub>2</sub>O solution). Testing began at P21 and continued to about P100 (Figure 2.3.1).



**Figure 2.3.1.** An illustration of the experimental design and test time line. Abbrev: P = postnatal day; NOR = novel object recognition task; washout = drug-free period; Chal. = acute drug administration.

Once weaned, all animals were housed in large, plexi-glass hanging tubs (39 X 57 X 21 cm) in mixed groups (Ritalin and saline) of five or six and maintained on a 12 hour light/dark cycle throughout the experiment. Animals were given ad lib food and water except during the T-maze training period at which time they were on a restricted food schedule. During the restricted food schedule, each animal received 20 – 25 grams of rat chow at the end of daily testing. The following morning (at least two hours prior to testing), uneaten food pellets were removed. At the end of the experiment all rats were given an overdose of sodium pentobarbital and intracardially perfused with a 0.9% saline solution. The brains were harvested and post-fixed in a Golgi-Cox solution for two weeks at which time the brains were transferred to a 30% sucrose solution where they remained until being sectioned. Brains were sectioned at 200  $\mu$ m on a Vibratome and mounted for staining. Mounted sections were stained using the Golgi staining procedures set out by Gibb and Kolb [46].

## **Drug Administration**

***Methylphenidate (Ritalin;MPD).*** The initial drug-treatment phase began when pups weighed about 60 grams on average (~P23). Rats received subcutaneous (s.c.) injections of Ritalin (0.5mg/kg) or vehicle (0.9% saline in dH<sub>2</sub>O vehicle solution) in the nape of the neck twice a day (a.m. & p.m.), spaced 6 hours apart for 37 consecutive days (~P60). Prior to each injection, all rats were removed from their home cage and taken to the testing area where they received the injections. Thus, all injections occurred in an ‘away’ or novel environment. At the end of the drug-treatment period daily injections were discontinued and subjects remained drug-free (washout period) for a period of five days (~65), at which time all animals received a single acute Ritalin challenge (0.5mg/kg) to assess drug sensitivity.

***Amphetamine Challenge.*** On the final day of the experiment (~ P100), all rats were given a single i.p. injection of *d*-Amphetamine (1.0 mg/kg) and activity monitored to test for any residual drug-sensitivity. The rats were individually placed into the activity apparatus five minutes post-injection and motor activity was recorded for 20 minutes. Rats were sacrificed immediately after testing and the brains harvested.

## **Behavioural Analyses**

### ***Activity Apparatus***

Animals were individually tested in the AccuScan Instruments Inc., Versamax animal activity monitoring system (open field) to measure motor response. The activity apparatus consisted of a clear plexi-glass cage measuring 42cm x 42cm x 30cm with a removable plexi-glass lid. The cage(s) was equipped with horizontal and vertical sensors (infra red beams) that monitored activity in selected intervals for a total of 20 minutes.

Recorded measures of horizontal activity (number of beam breaks on the lower bar in each sample period), total distance (path traveled in centimeters), and the number of movements (number of separate horizontal movements identified as such by a one second break in ambulatory activity) were retrieved for analysis. Once the testing period was complete, the VersaDat function scanned the files for errors to verify the integrity of data collected. The files were then converted for import to an excel spread sheet.

### ***Play Behaviour***

Play behaviour analysis was used to; 1) detect obscure peculiarities in social behaviour, and 2) observe possible changes in the typical pattern of play behaviour (for review see, [18]) in Ritalin-treated subjects. To accomplish this, the animals were analyzed in groups of three during play. Group triads consisted of either two Saline and one Ritalin-treated animal or two Ritalin and one Saline –treated pup. Ritalin-induced oddities in behaviour were assessed by quantifying the willingness of Saline-treated rats to initiate and participate in play with Ritalin-treated rats if given a choice. At the same time, initiation and play response were analyzed in Ritalin-treated animals. Subjects began habituation in the play arena at P42 (prior to day 21 a.m. injections). Animals were introduced to the area in groups of about 5-6 and left to acclimate to the new surroundings for 20-30 minutes each day for three consecutive days. Following the third habituation period, the rats were individually housed (isolated) for a 24 hour period. At the end of the 24 hour period rats were taken to the play arena *prior* to the a.m. injection and placed inside the enclosure with play-mates. The behaviour was video-taped for a 10 minute period with the experimenter out of the room. As subjects completed the task, they were removed

from the enclosure and returned to their home environment where they were once again group housed for the remainder of the experiment.

### ***T-maze Task***

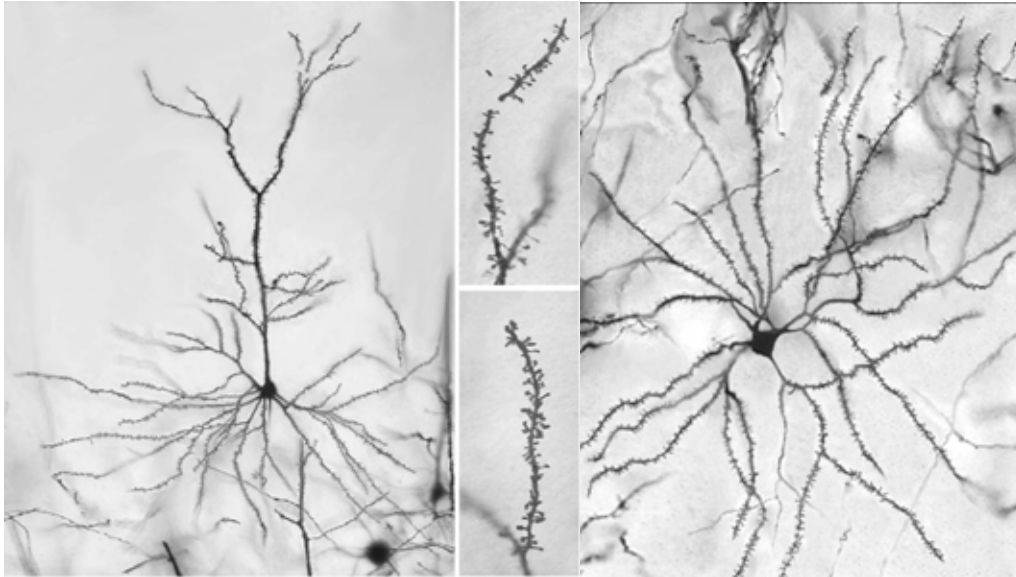
Rats were habituated to the T-maze apparatus prior to the actual training phase. Once habituated, rats were trained to run to the end of the arms of the apparatus to obtain a food reward (piece of fruit loop). Subjects were rewarded regardless of the arm they chose during the training phase. The testing phase began once all of the rats readily entered the arms to retrieve a reward. In the testing phase, rats were required to learn a non-match to sample paradigm of the T-maze task (similar to that used by Bartolini and colleagues [9]). In short, each trial consisted of two components. In the first, one arm was blocked forcing the subject into the open arm. In the second component the block was removed and the rat was reintroduced to the maze (10 sec. delay). To retrieve a reward, the rat had to choose the arm opposite of where the reward had just been obtained (component one). Subsequent trials began about 30 seconds after the end of the second component. Each subject was given 10 consecutive trials per day. A correct response on at least 8 out of 10 trials for four consecutive days was required to reach criterion.

### ***Anatomical Analysis***

Neurons from select brain regions known to be affected by psychostimulant use were reconstructed using a camera lucida (Figure 2.3.2) and dendritic morphology were quantified for analyses. The cells included in the current study were pyramidal cells (e.g., Figure 2, left) of the primary somatosensory cortex (Par 1, layer III), region three of the anterior cingulate cortex (Zilles Cg3, layer III and layer V) as well as pyramidal neurons of the dorsal agranular insular cortex (AID, layer III) described by Zilles [32].

To be included in the analysis, cells had to be; 1) thoroughly impregnated by the Golgi-Cox solution so that the dendritic trees were clearly visible at 100X and not obscured by blood vessels, neighboring processes or debris, and 2) relatively intact with minimal breakage. The cells were drawn using camera lucida (200X) and quantified by means of; 1) dendritic branch order; a quantification of the number of bifurcations off each dendritic process that extends from the soma, is a useful means of determining alterations in dendritic length, and 2) Sholl analysis; a quantification of the number of processes that cross the rings (spaced 20  $\mu\text{m}$  apart) of a concentric circle placed over the cell representation, is another useful method of quantifying dendritic length. The means from five neurons in each hemisphere were calculated and used for later comparisons.

Spine density was also determined for fourth-order terminals of basilar dendritic trees in Cg3 LV and AID LIII. As with dendritic branch order and Sholl analysis, spine density for each hemisphere was determined by calculating the mean of five terminals (10  $\mu\text{m}$  in length). Spines were drawn at 1000X (see Figure 2.3.2, center) and included all spine protrusions that had a visible head. It was recognized that this method of evaluation is an underestimation of the total spine density as those spines on both the bottom and top of the dendrite would not be visible to the experimenter (see [17] for additional details). Cells within each region were drawn by one of three experimenters blind to the groups until analysis.



**Figure 2.3.2. Left:** Photograph of a layer III pyramidal cell. **Center:** Photograph of dendritic spines on a segment of basilar dendrite. **Right:** Photograph of Golgi-Cox stained stellate cell from the NAc.

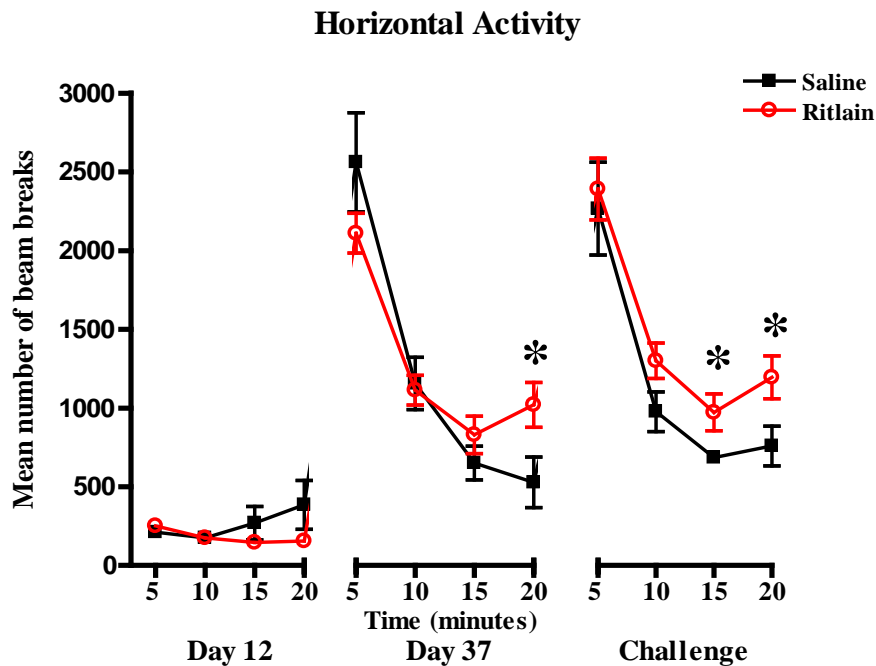
## Results

### *Behavioural Results*

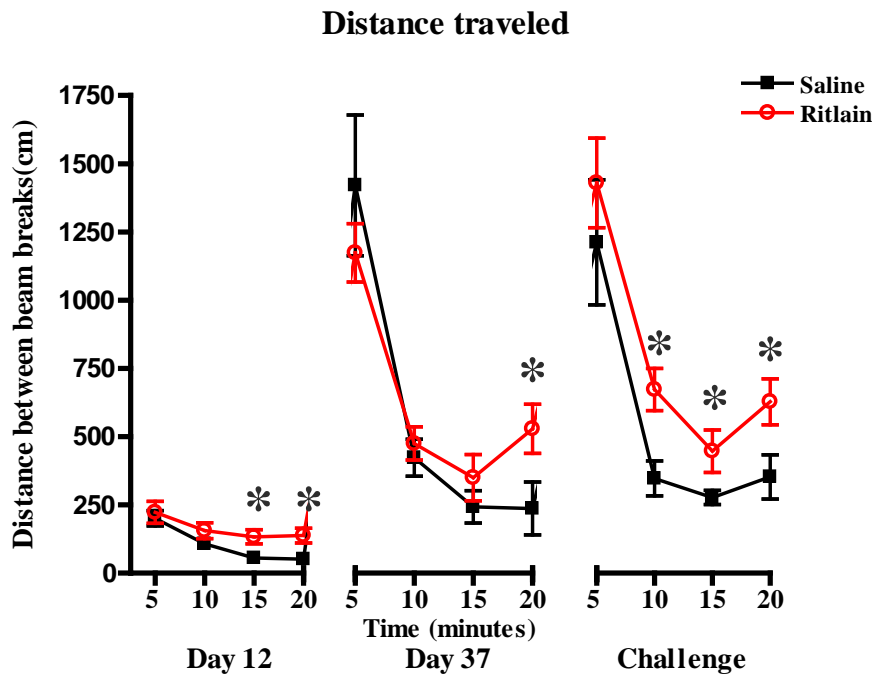
**Motor activity.** Horizontal activity (Figure 2.3.3a) and distance traveled (Figure 2.3.3b) as measures of motor activity illustrate the Ritalin-induced increase of motor activity that began on about day 12 of treatment and increased over the course of the 37 day treatment period. The Ritalin challenge administered after a 5 day drug washout period, demonstrates an increased behavioural sensitivity in pre-treated Ritalin rats. Repeated-measures ANOVA's of the four 5 minute intervals showed that 12 days of treatment was insufficient to produce increased motor activity in overall horizontal activity [ $F(1,19) = 1.51, p = 0.23$ ], or distance traveled [ $F(1,19) = 2.69, p = 0.12$ ]. The pairwise comparisons did reveal a significant increase in motor response by the third and fourth - 5 minute interval ( $p = 0.05$ ;  $p = 0.03$ , respectively) for distance traveled but not for horizontal activity ( $p = 0.18$ ;  $p = 0.08$ , respectively). On the last day of the treatment regimen (Day

37), treated rats showed an increased motor response on the fourth - 5 minute interval for horizontal activity ( $p = 0.04$ ) and distance traveled ( $p = 0.05$ ). Repeated-measures ANOVA's of activity on Day 37 of treatment showed no overall effect of treatment on horizontal activity [ $F(1,19) = 0.08, p = 0.79$ ], or distance traveled [ $F(1,19) = 0.20, p = 0.66$ ] on between- subject analysis, although the pairwise comparisons revealed a significant increase in both by the fourth - 5 minute interval.

**Ritalin Challenge.** The challenge demonstrates an increased sensitivity as a result of previous drug exposure (Figure 2.3.3a & 3b). Repeated-measures ANOVA's of activity with treatment as the variable showed a significant effect of treatment on horizontal activity [ $F(1,19) = 5.41, p = 0.03$ ], and distance traveled [ $F(1,19) = 7.75, p = 0.012$ ].



**Figure 2.3.3a.** A summary of horizontal activity on days 12, 37 and during the Ritalin challenge. The graphs shows that by Day 37 the Ritalin-treated animals were showing a drug-induced increase in motor activity and exhibited a drug “sensitivity” five days after the daily injections had ended.

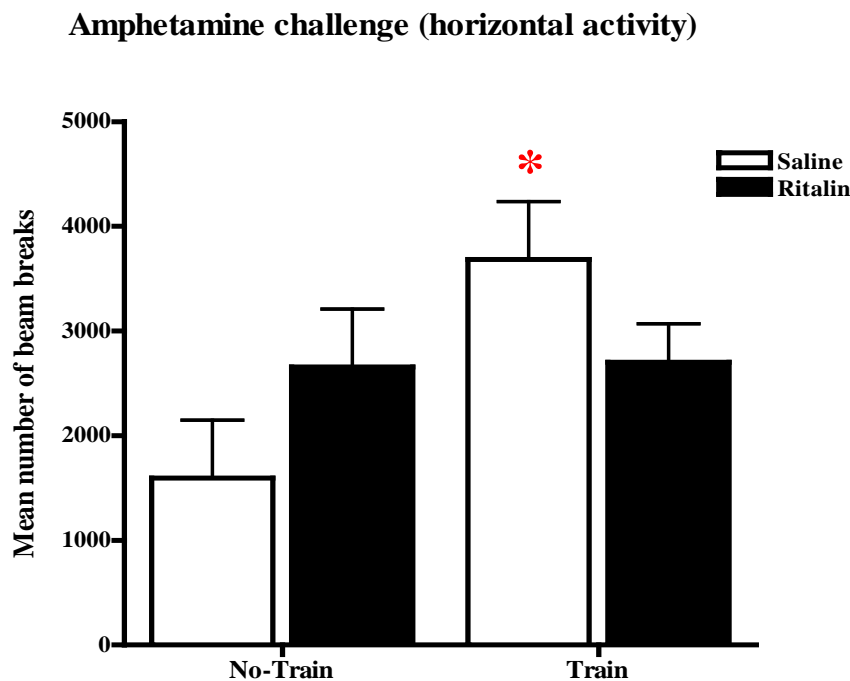


**Figure 2.3.3b.** A summary of distance traveled that again demonstrates a drug-induced increase in activity even five days after the daily injections had ended.

**Adult Amphetamine Challenge.** An amphetamine challenge was administered to all animals at the end of the experiment to assess the persistence of Ritalin-induced changes in motor activity. The animals used in the behavioural studies were separated from those animals that had been excluded from the training tests for this analysis in order to assess drug and training effects separately. Previous training enhanced the motor response to amphetamine in ‘drug naïve’ rats. In contrast, whereas there were no differences between the untrained saline and Ritalin treated rats, previous Ritalin use blocked the training-induced drug sensitivity exhibited by saline-treated animals (Figure 2.3.4a & 4b). An ANOVA of activity with treatment and training as variables showed a significant main effect of training on horizontal activity [ $F(1,17) = 4.34, p = 0.05$ ] and a marginal effect on distance traveled [ $F(1,17) = 4.14, p = 0.058$ ], but no significant main effect of

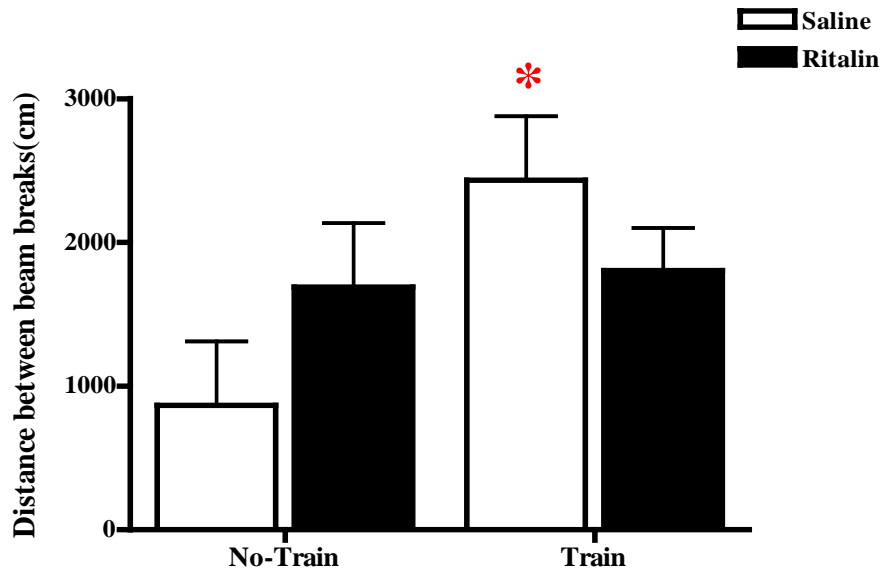
treatment on either horizontal activity [ $F(1,17) = 0.006, p = 0.94$ ], or distance traveled [ $F(1,17) = 0.06, p = 0.82$ ], but a marginal interaction on horizontal activity [ $F(1,17) = 3.97, p = 0.063$ ], although not on distance traveled [ $F(1,17) = 3.09, p = 0.097$ ]. Thus, training significantly elevated the behavioural response to amphetamine in untreated rats for both horizontal ( $p = 0.016$ ) and distance traveled ( $p = 0.023$ ) but not in Ritalin pretreated rats ( $p$ 's  $> 0.81$ ).

**T-maze task.** Rats exposed to Ritalin as juveniles appeared to be at a disadvantage in the T-maze task, making almost twice as many errors as the untreated group ( $M = 21, M = 12$ , respectively) before reaching criterion (Figure 2.3.5). A one-tailed  $t$ -test of errors with treatment as the variable,  $t_{(11)} = -1.76, p = 0.05$ .



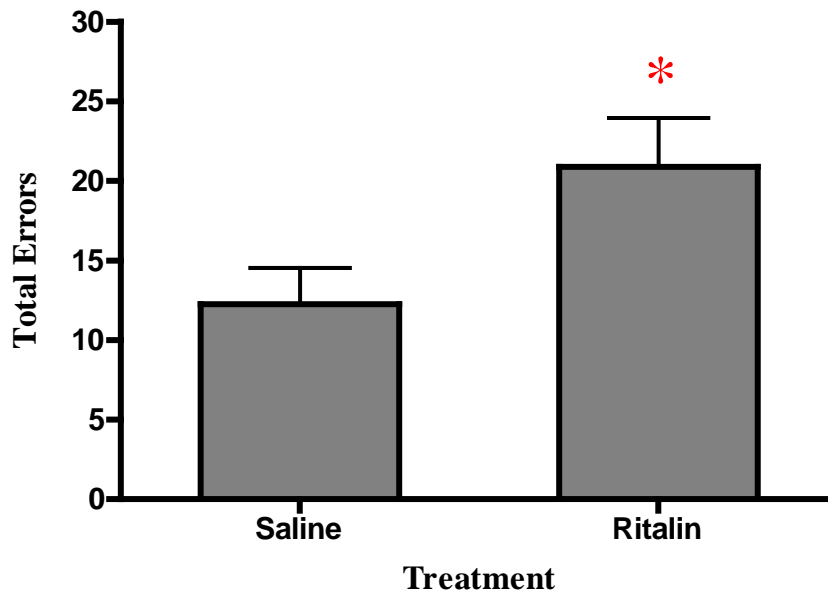
**Figure 2.3.4a.** A summary of the mean horizontal activity during the amphetamine challenge. The graphs show that training significantly influenced motor response to the amphetamine challenge, whereas pre-exposure to Ritalin blocked the training effect.

### Amphetamine challenge (distance)



**Figure 2.3.4b.** A summary of the distance traveled during the amphetamine challenge. The graphs show that training significantly influenced motor response to the amphetamine challenge, whereas pre-exposure to Ritalin blocked the training effect.

### T-maze errors

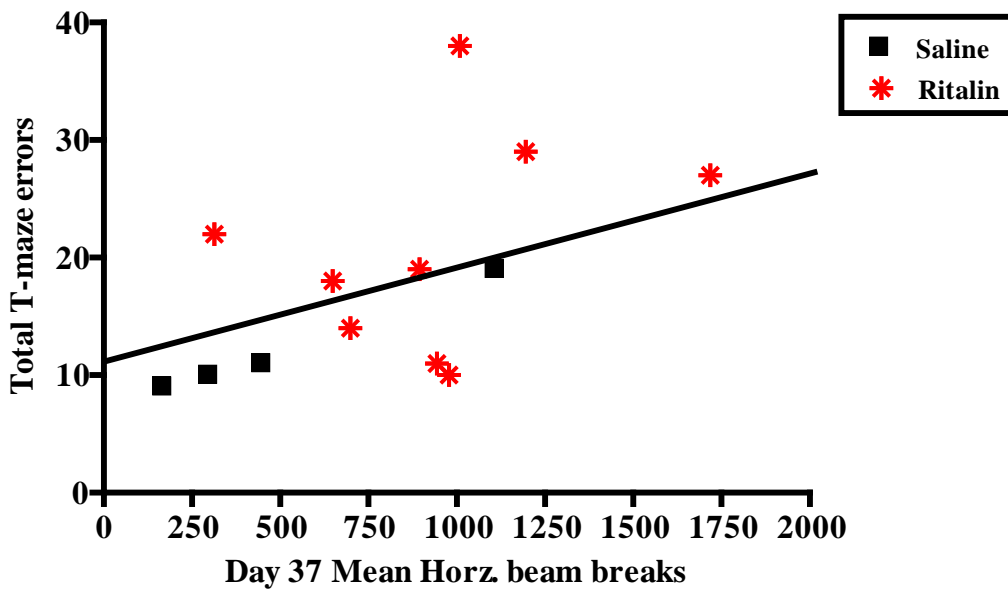


**Figure 2.3.5.** Illustrates the total number of errors to reach criterion. Ritalin-treated rats made twice as many errors as the saline-treated rats.

### Correlational Analysis

Interestingly, performance in the T-maze task could be predicted using motor activity on the final day of the initial treatment regimen (Figure 2.3.6). A bivariate correlation test showed a strong correlation between T-maze errors and all three measure of motor activity; horizontal, ( $r = .995$ ,  $p = 0.005$ ), distance traveled, ( $r = .996$ ,  $p = 0.004$ ) and, number of movements, ( $r = .997$ ,  $p = 0.003$ ).

#### T-maze/Horizontal activity correlations

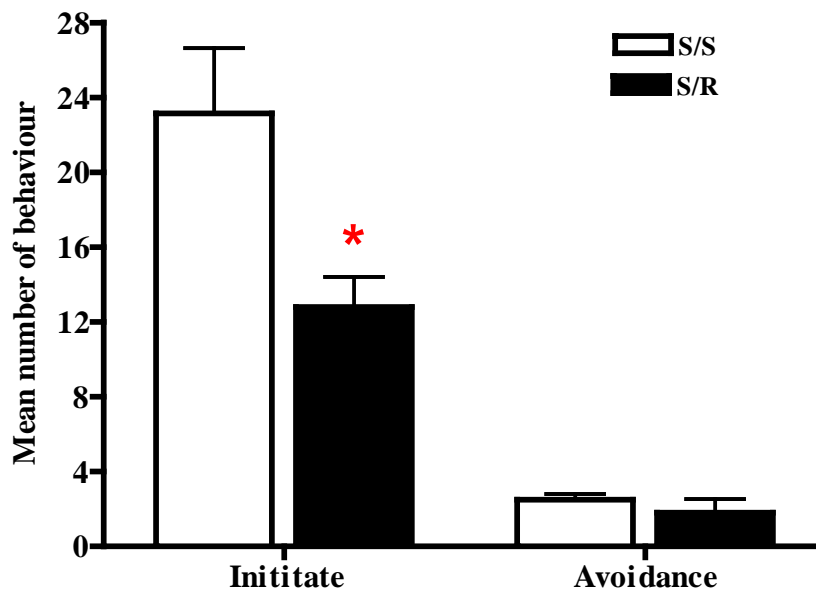


**Figure 2.3.6.** A representation of T-maze and horizontal activity showing the correlation between total number of T-maze errors and activity level on the final day of the treatment period. The graph demonstrates a strong correlation exists between the two measures.

**Play behaviour.** Analysis of play behaviour was conducted using triads of Saline and Ritalin-treated rat, or two Ritalin and one Saline-treated rat. The results showed that Saline-treated rats were discriminative regarding their play partners (Figure 2.3.7), choosing to initiate play significantly more often with saline, rather than Ritalin-treated

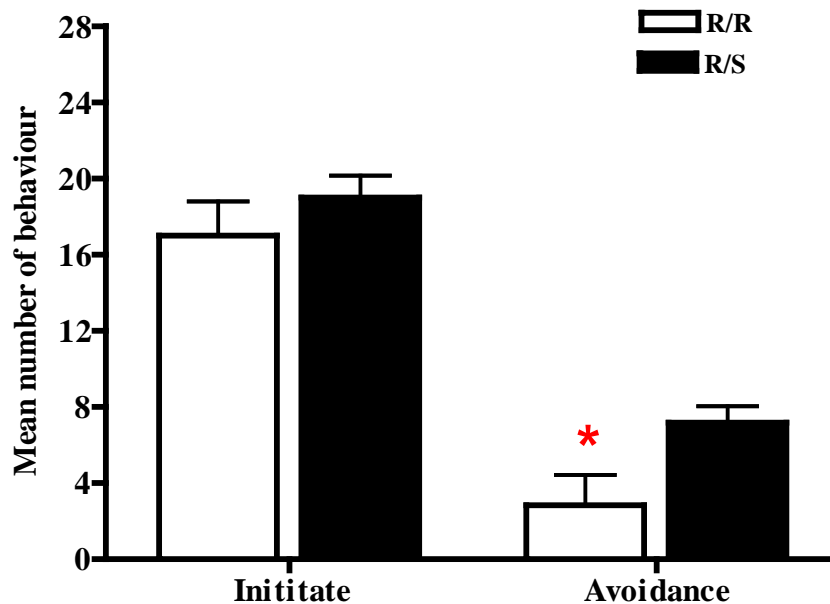
animals [ $F(1,10) = 5.52, p = 0.04$ ], although they showed no discrepancy in avoidance behaviour [ $F(1,10) = 0.79, p = 0.39$ ]. In contrast, Ritalin-treated rats did not show a playmate preference, initiating play equally between Ritalin and Saline-treated animals [ $F(1,10) = 0.87, p = 0.38$ ]. Interestingly, they did however, tend to use ‘avoidance’ behaviour more often when Saline animals were initiating the play [ $F(1,10) = 5.54, p = 0.04$ ].

### Saline play behaviour



**Figure 2.3.7a.** A summary of the number of times Saline rats initiated play or used avoidance behaviour in response to both saline (S/S) or Ritalin (S/R).

## Ritalin play behaviour



**Figure 2.3.7b.** A summary of the number of times Ritalin rats initiated play or used avoidance behaviour in response to Ritalin (R/R) or Saline (R/S) rats.

### *Anatomical Results*

Analysis of dendritic morphology revealed that early Ritalin exposure as well as later training altered connectivity in the prefrontal cortex, albeit in different ways. The combination of early drug exposure and training also produced unique alterations in circuitry prefrontal cortical regions. In summary, Ritalin produced a decrease in spine density of the AID, but increased the apical and basilar dendritic field of Cg3 LIII, but not LV, neurons. On the other hand, training produced the opposite effect in the AID, increasing basilar spine density. Training, as with Ritalin, however, increased the dendritic field of LIII Cg3 neurons, but only in the basilar tree. Interestingly, the only significant alterations in Cg3 LV neuronal circuitry were found in the Ritalin + training group. The combination of experiences produced increases in basilar spine density as

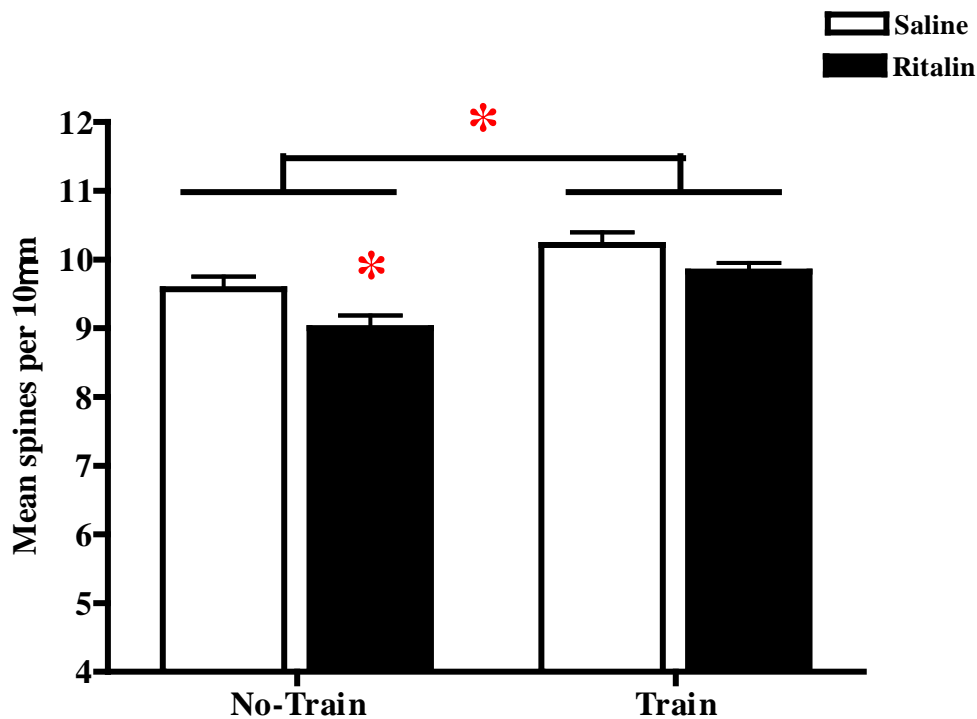
well as apical and basilar dendritic length. In contrast, the Cg3 LIII showed a decrease in the dendritic field as a result of the combined experience, an effect that was opposite of either Ritalin or training alone (see table 2.3.1 for summary).

***AID layer III.***

***Spine density.*** Prolonged Ritalin use in early life produced a *reduction* in basilar spine density of layer III AID pyramidal neurons of the prefrontal cortex. The effect was opposite of that of training, which *increased* the spine density neurons in this region in both Saline and Ritalin-pretreated rats (Figure 2.3.8). The training-induced increase was robust; reversing the Ritalin-induced decrease found in animals that had not participated in the training. An ANOVA of spine density with treatment and training as variables showed a significant main effect of treatment [ $F(1, 38) = 7.75, p = 0.008$ ], training [ $F(1, 38) = 18.48, p < 0.001$ ], but no interaction [ $F(1, 38) = 0.28, p = 0.60$ ]. The pairwise comparison revealed that the treatment effect was significant in the No-training group ( $p = 0.036$ ), not in the Training group ( $p = 0.09$ ).

***Sholl and branch order.*** Measures of dendritic length (Sholl) and branch order revealed no other obvious changes ( $p$ 's all  $< 0.10$ ) in the dendritic field of AID pyramidal cells (data not shown).

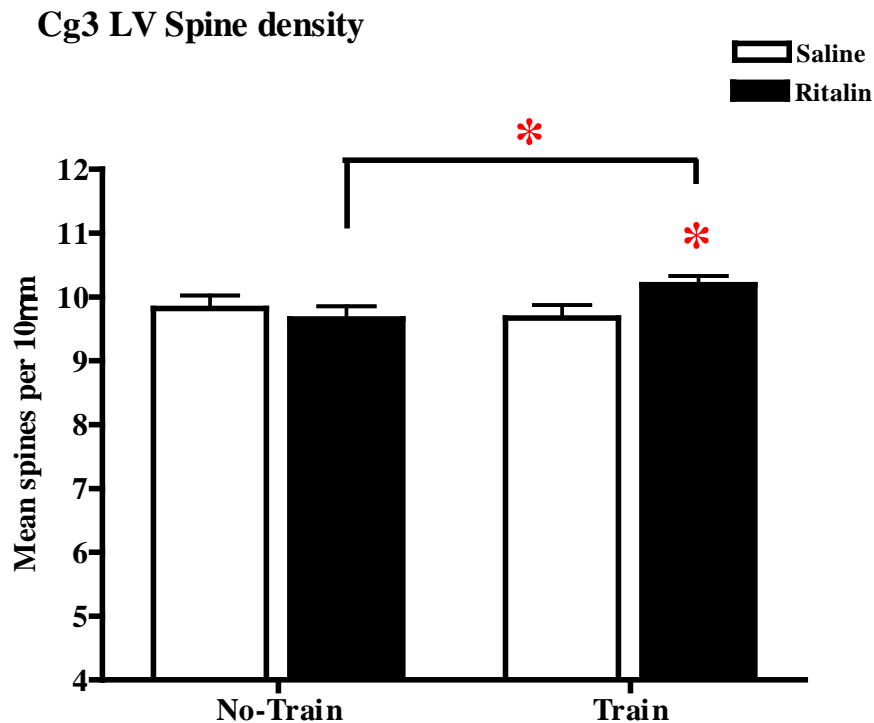
### AID LIII Spine density



**Figure 2.3.8.** A summary of mean spine density in basilar dendrites of layer III AID neurons. Ritalin alone produced a decrease in spine density, whereas training increased spine density.

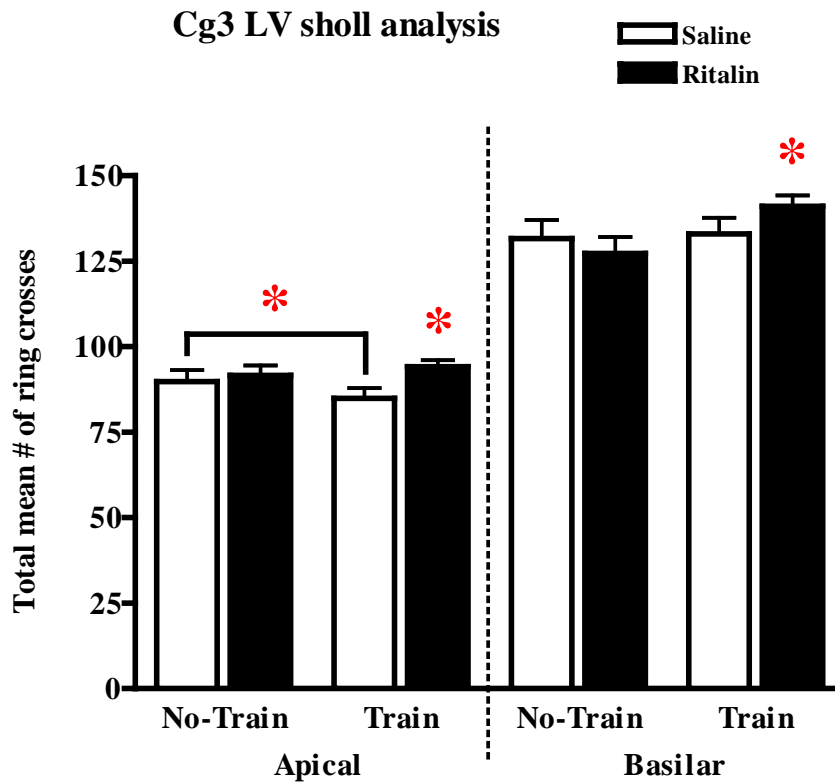
#### *Cg3 LV.*

**Spine density.** Although neither Ritalin nor training alone affected spine density in layer V pyramidal cells, the combination of Ritalin + Training increased spine density relative to either experience alone (Figure 2.3.9). An ANOVA of spine density with treatment and training as variables showed no significant main effect of treatment, [ $F(1,38) = 0.90, p = 0.35$ ], or training [ $F(1,38) = 1.08, p = 0.31$ ], but a trend for the interaction [ $F(1,38) = 3.34, p = 0.08$ ]. The pairwise comparison revealed a significant increase in the spine density of rats that had received Ritalin *and* training relative to Ritalin treatment ( $p = 0.03$ ) or training ( $p = 0.04$ ) alone.



**Figure 2.3.9.** A summary of the mean spine density of Cg3 LV basilar dendrites. The graph illustrates that Ritalin + training significantly increased the number of spines relative to the untrained Ritalin rats, but otherwise neither training nor Ritalin had any effect.

**Sholl and branch order.** The combination of Ritalin + training increased apical dendritic length over training alone and increased the length of basilar dendrites over Ritalin alone (Figure 2.3.10). As with spine density, neither Ritalin nor training alone affected dendritic length or branch order. Branch order was unaffected by treatment. An ANOVA of apical dendritic length (Sholl analysis) with treatment and training as variables showed a trend towards a significant main effect of treatment [ $F(1,36) = 3.68, p = 0.06$ ], but not training [ $F(1,36) = 0.17, p = 0.69$ ], nor an interaction [ $F(1,36) = 1.66, p = 0.21$ ]. Basilar dendritic length also showed no main effect of treatment [ $F(1,36) = 0.17, p = 0.69$ ], training [ $F(1,36) = 2.65, p = 0.11$ ], or interaction [ $F(1,36) = 1.79, p = 0.19$ ]. Analysis of branch order revealed no other significant changes ( $p$ 's all  $< 0.10$ ).



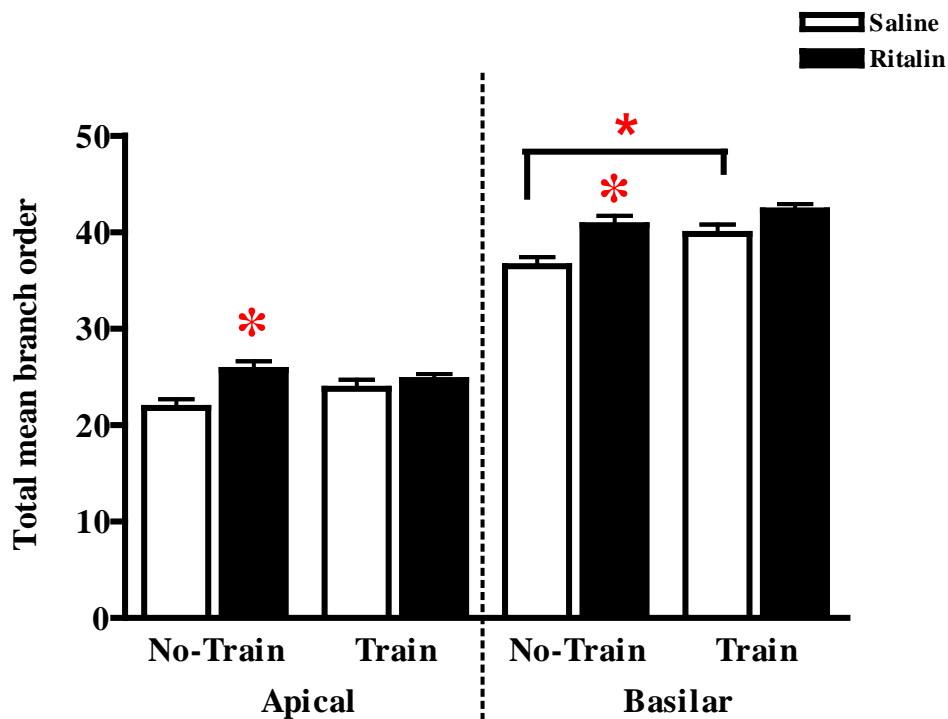
**Figure 2.3.10.** A summary of dendritic length in Cg3 layer V pyramidal cells. The graph demonstrates a significant decrease in apical dendrite length as a result of training that is blocked by previous Ritalin use. In contrast, basilar dendritic length is increased by the combination of Ritalin + training.

### *Cg3 LIII.*

**Sholl and branch order.** Both Ritalin and training produced changes in layer III of the Cg3. In summary, although Ritalin increased branch order of apical dendrites, subsequent training reversed the effect (Figure 2.3.11a). Basilar branch order was also increased by both Ritalin and training alone but the combination of the two had no additional impact. The Sholl analyses also highlighted the effect of both training and Ritalin (Figure 2.3.11b). Whereas training created a decrease in apical dendritic length in rats pretreated with Ritalin, the basilar dendrites showed a Ritalin-induced increase in length that was unaffected by subsequent training.

An ANOVA of apical branch order showed a significant main effect of treatment,  $[F(1,38) = 8.13, p = 0.007]$ , but not training,  $[F(1,38) = 0.34, p = 0.57]$ , or interaction  $[F(1,38) = 3.19, p = 0.08]$ . In the basilar dendritic field, an ANOVA of dendritic branch order showed a significant main effect of treatment  $[F(1,38) = 14.08, p = 0.001]$ , and training,  $[F(1,38) = 7.51, p = 0.009]$ , but no interaction  $[F(1,38) = 1.04, p = 0.32]$ .

### Cg3 III Branch Order

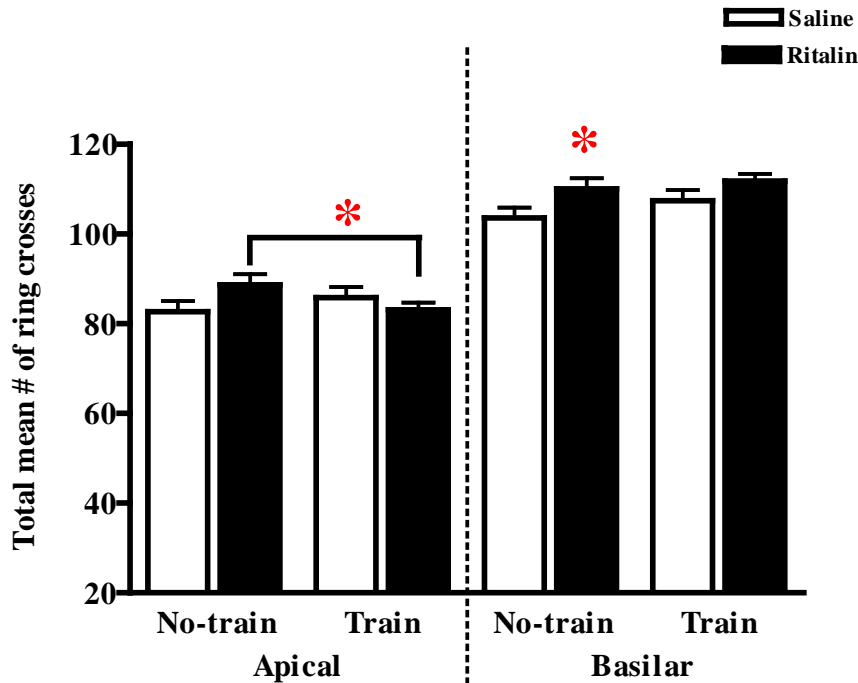


**Figure 11a.** A summary of mean branch orders in layer III pyramidal neurons. The graph shows a Ritalin-induced increase in apical branch order that was blocked by subsequent training. In contrast, both Ritalin and training alone increased branch order in basilar dendrites but the combination had no further effect.

In contrast, an ANOVA of apical dendritic length (Sholl) showed no significant main effect of treatment  $[F(1,38) = 0.29, p = 0.59]$ , or training,  $[F(1,38) = 0.55, p = 0.46]$ , but a significant interaction  $[F(1,38) = 3.96, p = 0.05]$ . Whereas, an ANOVA of basilar

dendritic length showed a significant main effect of treatment [ $F(1,38) = 6.22, p = 0.017$ ], but not training, [ $F(1,38) = 1.62, p = 0.21$ ], nor interaction [ $F(1,38) = 0.22, p = 0.64$ ].

### Cg3 III Sholl Analysis



**Figure 2.3.11b.** A summary of dendritic length in layer III Cg3 neurons. The graph demonstrates a significant reduction in apical dendritic length as a result of combined Ritalin use and training, whereas overall Ritalin increased basilar length, although only significantly in the untrained group.

**NAC core and shell.** Long-term use of Ritalin in juvenile rats did not result in any significant changes in dendritic morphology of medium spiny neurons in either the shell or core of the nucleus accumbens. ANOVA's with treatment and training as variables showed no significant effects of treatment [ $F(1, 36) = 0.62, p = 0.44$ ], training [ $F(1, 36) = 0.01, p = 0.91$ ], or interaction [ $F(1, 36) = 0.17, p = 0.90$ ] in the shell. Similarly, the core also showed no significant effect of treatment [ $F(1, 36) = 2.19, p = 0.15$ ], training [ $F(1, 36) = 0.26, p = 0.61$ ], or interaction [ $F(1, 36) = 0.48, p = 0.49$ ].

**Par I.** We found no effect of either treatment or training in the dendritic field of layer III Par1 neurons. An ANOVA of apical dendritic branching showed no significant main effect of treatment [ $F(1,38) = 0.007, p = 0.93$ ], training [ $F(1,38) = 0.91, p = 0.35$ ], nor interaction [ $F(1,38) = 0.25, p = 0.62$ ]. An ANOVA of apical dendritic length also showed no significant main effect of treatment [ $F(1,38) = 0.41, p = 0.53$ ], training [ $F(1,38) = 0.49, p = 0.49$ ], nor interaction [ $F(1,38) = 0.10, p = 0.75$ ]. Similar results were found for basilar branch order and length ( $p$ 's all  $> 0.40$ ).

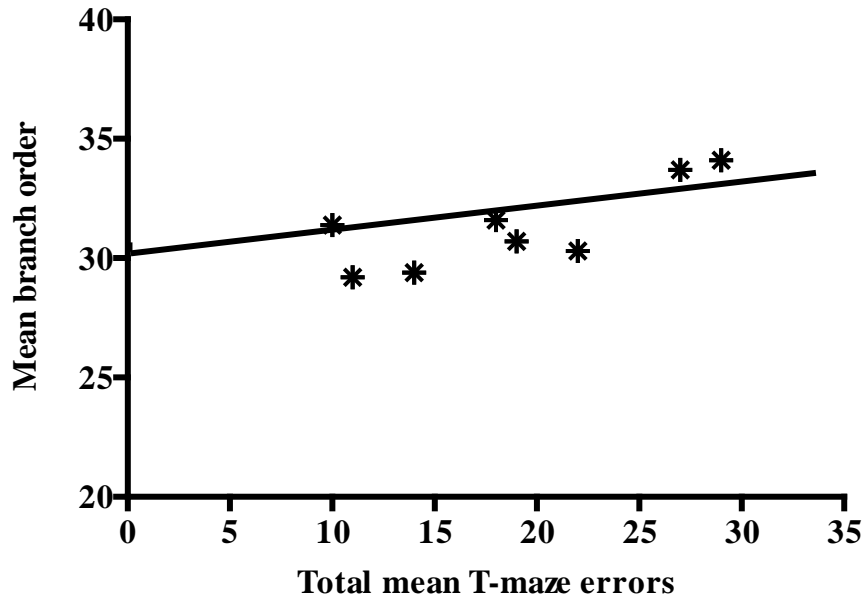
**CAI.** Analysis of basilar branch order and length showed no influence of either condition. An ANOVA of basilar dendritic branch order showed no significant main effect of treatment [ $F(1,38) = 1.08, p = 0.31$ ], training [ $F(1,38) = 0.14, p = 0.71$ ], nor interaction [ $F(1,38) = 1.34, p = 0.25$ ]. An ANOVA of basilar dendritic length also showed no significant main effect of treatment [ $F(1,38) = 0.09, p = 0.76$ ], training [ $F(1,38) = 0.32, p = 0.57$ ], nor interaction [ $F(1,38) = 0.64, p = 0.43$ ].

### ***Correlational data.***

Hyperactivity of the NAc has been linked to reduced attentiveness and poor performance on cognitive tasks. In light of the poor performance of Ritalin-treated animals in the T-maze that was hypothesized to be at least in part due to inattentiveness, we were rather surprised by the absence of a drug-induced effect in the NAc. Thus, a correlational analysis was conducted to determine if a relationship between NAc stellate cell morphology and T-maze errors existed, regardless of treatment. Indeed, a bivariate correlational analysis did reveal a significant positive correlation between NAc core

branch order and the number of errors during the T-maze task in Ritalin ( $r = .80$ ,  $p = 0.01$ ) but not Saline ( $r = -.45$ ,  $p = 0.70$ ) treated rats (Figure 2.3.12).

### T-maze errors and NAc core correlations



**Figure 2.3.12.** The graph illustrates a positive correlation exists between T-maze errors and NAc core dendritic branch order in Ritalin-treated.

**Summary of Experiments in Study Three**

	Basilar						Apical				Basilar Spine	
	Cg3		OFC	Par1	CA1	NAc	Cg3		OFC	Par1	Cg3	OFC
<b>Ritalin vs Saline</b>	LV	LIII	LIII	LIII		LIII	LV	LIII	LIII	LIII	LV	LIII
No Training	∅	<b>h</b>	∅	∅	∅	∅	∅	<b>h</b>	∅	∅	∅	i
Training	∅	<b>h</b>	∅	∅	∅	∅	<b>h</b>	∅	∅	∅	h	∅
<b>Ritalin</b>												
Training	<b>h</b>	∅	∅	∅	∅	∅	∅	<b>i</b>	∅	∅	h	h
(vs No train)												
<b>(Saline)</b>												
Training	<b>h</b>	∅	∅	∅	∅	∅	<b>i</b>	∅	∅	∅	∅	h
(vs No train)												

**Table 2.3.1.** Arrows indicate direction of dendritic changes relative to Saline-treated controls in each experiment. **Blue arrows = branch order; Red arrows = Sholl (length); Spine = spine density;** ∅ = no change; Cg3 = region three of the cingulate gyrus; OFC (AID) = orbital prefrontal cortex; Par 1 = primary somatosensory cortex; CA1 = subfield of the hippocampus; NAc = nucleus accumbens core or shell; LV = layer five neurons and LIII = layer three neurons.

## **Discussion**

Recent research into potential long-term consequences of Ritalin-use during postnatal developmental periods has mainly focused on the possible increased propensity for later drug addiction, with little attention to the potential of stimulant use to adversely affect developing behaviours. Yet, when additional behavioural measures are conducted studies report detrimental behavioural effects such as impaired memory processing [11], increased anxiety-like behaviours [16], increased reactivity to novelty [19], aberrant play behaviour and cognitive deficits [25, 35, 43, 59] as a result of early psychostimulant exposure. Therefore, the current study included behavioural measures such as social interactions and cognitive skills, as well as behavioural sensitization to gain a better understanding of the scope of the behavioural impact that early stimulant use may have. Additionally the influence of prolonged Ritalin use on developing behavioural systems was assessed via an extensive analysis of dendritic morphology in several brain regions that have previously been shown to be influenced by psychostimulant use.

The results of the current experiment highlight a number of novel phenomena that support previous findings demonstrating the potential for permanent behavioural and anatomical alterations following early Ritalin exposure. First, the motor activating effects of Ritalin use continued to escalate over the treatment period, showing no inclination towards tolerance [41] after such extended use. The effect was transient, however, as animals pretreated with Ritalin did not display an enhanced responsiveness to amphetamine in adulthood. Secondly, a rather surprising finding was that training, but not Ritalin pretreatment, increased the motor activating effects of an acute amphetamine challenge in adulthood. Furthermore, the enhanced motor activating effects of training in

saline-treated rats was absent in those rats pretreated with Ritalin, demonstrating the capacity for early drug experience to block subsequent experiential effects. Third, early Ritalin exposure adversely affected social behaviour in young rats and produced a persistent deficit in cognitive functioning. Finally, adult rats pretreated with Ritalin during the preadolescent and adolescent period showed unique patterns of PFC circuitry. In addition, Ritalin use had the capacity to block future training-induced plasticity; producing alternative patterns of connectivity in the PFC. The findings will be discussed separately.

***Increased motor activating effects of Ritalin during/following prolonged use***

Early Ritalin exposure produced a subtle increase in motor activity by the twelfth day of injections as has already been found in earlier studies in this lab (unpublished data). There was, however, a marked increase in motor response by the final day (day 37) of the treatment period that persisted through a five day washout period. As we know of no other studies that have implemented such a prolonged period of treatment, we would expect that previous reports of either no (e.g., [19, 53]) or moderate increases in motor activity (e.g.,[19]) following early Ritalin exposure may only be applicable with short-term use. Although the increased motor response in the current experiment was not indicative of behavioural sensitization as demonstrated by the transient effect, the activity data indicated that prolonged Ritalin use through the preadolescent and adolescent period produced a somewhat heightened motor response relative to shorter- term use that occurs during a single developmental phase (i.e.,[52]).

The potential for early Ritalin use to produce chronic sensitivity to stimulants, thereby increasing the risk for addiction in later life, was not supported by the current

results. We found no evidence of a persistent stimulant sensitivity following early drug-exposure as demonstrated by the results of the amphetamine challenge in adulthood.

It should be noted that the behavioural effects reported here were produced with what is considered a very low dose of Ritalin, even by clinical standards. The absence of behavioural sensitization and later sensitivity to stimulant use may very well be related to the dose used. Direct comparisons with other studies that have focused on the addictive capabilities of early Ritalin exposure are therefore not practical. Nonetheless, based on the current findings we may expect that increasing the dose over a prolonged treatment period during development could result in one of two scenarios. It may be that a higher dose would produce tolerance, resulting in a decline in the motor activating effects. Alternatively, the behavioural effects produced by a higher dose may more closely mimic addictive behaviour, including an increased sensitivity to drugs of abuse in later life.

One of the limitations in extrapolating the results of the current study to the role of early stimulant use in the development of later addictive-like behaviour is that lack of stimulant sensitivity was inferred from an acute amphetamine challenge. Care should be taken here, as we cannot rule out the possibility that early stimulant exposure, even at low doses, may facilitate an escalation from drug abuse to drug addiction. In any case, the current findings point to the importance of treatment duration as an important factor when considering the long-term consequences of early stimulant use. Additionally, duration of treatment is likely further influenced by the developmental process itself, an implication that is especially relevant when taking into consideration the age of treatment onset in clinical settings.

***Ritalin pretreatment blocks the training-induced behavioural response to amphetamine in adulthood.***

The implications of the results from the amphetamine challenge were twofold. First, it would appear that experience, but not early drug exposure, augments the motor activating effects of amphetamine in adult rats. Secondly, early Ritalin exposure blocked the training-induced behavioural effect of the challenge.

The enhanced motor response of trained saline-treated rats during the amphetamine-challenge was very interesting. A possible explanation may be related to the ability of experience to reduce basal motor activity levels. Seeman and Madras [12, 13] have proposed a hypothesis of Ritalin action in reducing hyperactivity that may be applicable here. The researchers have proposed that the level of resting (basal) dopamine (DA) is a determining factor in nerve impulse DA release that promotes the activating effects of stimulants such as Ritalin and amphetamine. The greater the ratio between the two, the greater the DA release from presynaptic terminals, producing elevated extracellular DA at postsynaptic sites. Lower basal levels of DA would thereby enhance the release of DA and increase the effect of psychostimulants. Altered basal levels of DA and DA release would undoubtedly have an impact on neuronal activity in the nucleus accumbens (NAc), a subcortical region that is heavily innervated by dopaminergic neurons of the ventral tegmentum (VTA) and, which plays a major role in motor activity. Compatible with this hypothesis, Bowling and colleagues [58] have shown that rats raised in enriched-environments during post-weaning development have lower basal levels of activity, yet show an elevated motor responsiveness when administered an acute amphetamine challenge in adulthood. Furthermore, the researchers showed that the

increased amphetamine response was correlated with an increase in DA metabolism in the NAc and an increase in DA synthesis in the striatum.

It may be that T-maze training also reduces basal activity level, although this has not been studied directly to our knowledge and remains conjecture at this point.

Nonetheless, if this is the case then one might speculate that Ritalin, which increases extracellular DA levels through the blockade of DAT (i.e., [19, 37]), may generate the opposite effect. Prolonged use of Ritalin then may result in persistent increases in basal DA levels that would in effect reduce the motor activating effects of amphetamine in later life as reported in the current study.

Although the mechanism(s) by which altered DA levels influence PFC function and behaviour remain largely unknown, the ability of Ritalin-pretreatment to block subsequent experience-induced behavioural changes has profound implications, especially when considering the length of time between the two events.

***Altered social and cognitive functioning as a consequence of long-term Ritalin use.***

Altered social interaction as a consequence of adolescent Ritalin use has been reported elsewhere [45, 54]. To our knowledge, however, this is the first time that the effects of prolonged, chronic Ritalin use on play behavior have been examined using triads. The rationale for using triads in the current study was to determine if there were peculiarities in social behaviour that may go undetected by the observer but that could be inferred by the response/acceptance of saline and Ritalin-treated play partners. Indeed, it appears that there are. The play behaviour results of the current study highlight two very interesting phenomena. First, although experimenters detected no obvious abnormalities in the behaviour of Ritalin-treated rats, the reluctance of saline-treated rats to initiate play

with Ritalin rats indicates that the treatment altered behaviour in such a way that was undesirable to 'normal' rats. In contrast, Ritalin-treated rats initiated play equally among their conspecific partners. Similar results have been found in play interactions in juvenile rats following neonatal OFC lesions, in which case controls initiated fewer 'play' attacks on OFC lesion-partners relative to intact play-partners [44]. Secondly, when Ritalin-treated rats were placed in triads that included another Ritalin-treated rat they used avoidance behavior more often when play was initiated by the saline-treated partner. One explanation for the latter finding may be that Ritalin rats were able to recognize the play intentions of same-treated animals but may have had difficulty in interpreting the intentions of 'normals'. This interpretation is also supported by the findings of Pellis and colleagues that show that rats with neonatal lesions of the OFC fail to incorporate subordinate/dominant modulation of play behaviour that is typical in juvenile play behaviour of intact rats [44].

It is interesting that two of the regions that are known to facilitate play behaviour in rats are also known to be influenced by psychostimulant use. For example, play is a behaviour believed to have reward value [15, 29], and the reinforcing properties of both natural and drug-rewards are subserved by the NAc (i.e.,[44]). As well, the orbitofrontal cortex (OFC) plays a role in the development of play behaviour [27, 33] and is also affected by psychoactive drugs [45].

It is noteworthy, that in the current study play behaviour was assessed when the rats were still undergoing treatment. The rationale was that in doing so we could take advantage of the peak play phase that occurs between P30 and P40 in rats [21]. Owing to extended time between last injection and play behaviour (about 18 hours) and the fast

metabolism rate of low doses of stimulants such as Ritalin (see [22]) however, it is unlikely that there was an acute stimulant-induced effect on observed play behaviour.

Along similar lines, it should also be noted that although previous studies have reported a stimulant-induced reduction in play behaviour as a consequence of the analgesic effects of stimulant injections into the nape of the neck [26, 31, 38, 49], this does not appear to be the case in the current experiment. Ritalin-treated rats did not significantly differ in the number of avoidance behaviours with same-treated rats, but only with saline-treated partners suggesting that it was not related to a reduction in sensory sensation.

Cognitive skills in adulthood also appeared to be impaired by early Ritalin exposure. Ritalin-pretreated animals made almost twice as many errors as saline-treated in the T-maze task. It may be surprising that given the low dose of Ritalin used in the current study, we would find deficits in cognitive function in adulthood. The findings suggest that the effects of early drug experience are persistent and may indeed influence subsequent behaviour and learning.

#### ***Altered connectivity in the adult brain as a consequence early Ritalin use***

The potential for psychostimulants to produce enduring, alterations in cortical and subcortical connectivity has been reported previously by this lab and in collaboration with others following adult stimulant use [2, 5, 30]. Importantly, these studies have linked the aberrant connectivity to behavioural indices of psychostimulant addiction and alterations in subsequent experience-induced plasticity. One of the questions asked in the current study was whether similar effects would be produced by low dose psychostimulant use in young animals. It was predicted that, owing to the late

development of the PFC and related circuitry, and the fact that the immature brain is both highly plastic and vulnerable to experiential influences into adolescence (see [21]), Ritalin use during this period would influence developing circuitry. Further, as training may also influence circuitry, it was predicted that Ritalin use would disrupt these changes, providing a possible explanation for some of the behavioural effects reported previously (unpublished data). Indeed, the area that was most affected by stimulant use in the current study was the prefrontal cortex, with no effect of either Ritalin or training in the NAc, hippocampus, or parietal cortex.

The anatomical findings in the current study illustrate that both prolonged Ritalin use and training result in pronounced alterations in PFC circuitry. Especially interesting was that training and Ritalin produced very distinct patterns of connectivity, as did the combination of the two experiences. Whereas Ritalin use produced a decrease in the dendritic field of basilar AID pyramidal cells, and increases in both the basilar and apical dendritic fields of layer III Cg3 neurons, there was no apparent effect of early Ritalin use on layer V Cg3 neurons. In contrast, the effects of training were restricted to basilar fields of layer III neurons, producing increases in both the AID and Cg3. Further, unlike Ritalin, training altered connectivity of layer V Cg3 neurons, producing a decrease in the apical dendritic field that was blocked by Ritalin exposure.

Interestingly, although persistent changes in the dendritic morphology of prefrontal and nucleus accumbens neurons in both young [50] and adult rats have been reported following psychostimulant use, we found no changes in circuitry of NAc neurons. The latter finding however is in keeping with the absence of behavioural sensitization as well in the current study.

These findings indicate that the pattern of experience-induced plasticity is regulated by the type of experience. Furthermore, as Ritalin blocked training-induced modifications in Cg3 layer III and V neurons, it also suggests that experience-induced alterations in circuitry have the capacity to modulate subsequent changes, perhaps adversely. Combined with the behavioural influence of early Ritalin use, we may now begin to elucidate the functional significance of at least some of these anatomical alterations.

### *Conclusions*

Based on the behavioural and anatomical findings of the current study it would appear that cognitive tasks such as the T-maze produce enhanced dendritic morphology in basilar fields of both layer III AID and Cg3 neurons. The training-induced effects, however, can be reversed or blocked by early Ritalin exposure that may explain the poor performance of these animals on this task. Furthermore, as layer II/III neurons in the Cg3 are known to project to the NAc, it may also help to explain the high, positive correlation between neuronal branching of NAc core neurons and T-maze performance in Ritalin, but not saline-treated rats that was reported here.

Finally, the enhanced response of trained saline-treated rats to an adult amphetamine challenge may be linked to the training-induced decrease in Cg3 layer V neurons. As the major output neurons of the PFC, alterations in the input received by apical dendrites would most certainly influence the output produced.

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## **IV. Caffeine exposure alters the effect of early methylphenidate exposure in rats**

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## **Abstract**

In the current study we investigated the behavioural and anatomical effects of early methylphenidate (Ritalin) exposure in rats pre-treated with caffeine. Beginning the first day of weaning (postnatal day (P) 22), caffeine was delivered every other day to half of the rat pups via the drinking water (0.5mg/ml), whereas as the other half of the subjects were maintained on regular tap water. At the end of the first week (P29) of caffeine exposure the animals received subcutaneous injections of either Ritalin (0.5mg/kg) or saline (0.9%) twice daily. All treatment ended when pups were 41 days old. Motor activity in an open field apparatus was monitored intermittently throughout the drug treatment period. Animals were tested on the Elevated-plus maze and a non-match to sample T-maze paradigm to assess the long-term effects of early stimulant exposure. All rats were given an amphetamine challenge (1 mg/kg) on the final day of the experiment. The behavioural results showed that neither Ritalin nor caffeine influenced motor activity during the treatment phase. Further, the motor response to an acute amphetamine challenge in adulthood showed no apparent influence of either early stimulant experience. Interestingly, although Ritalin alone increased the number of errors in the T-maze task in adulthood, this effect was reversed in rats that received the combined treatment of Ritalin and caffeine in the pre-and periadolescent period. All animals behaved comparably in the Elevated plus maze. Ritalin alone produced extensive dendritic changes in the prefrontal cortex. Interestingly, the combined treatment of Ritalin and caffeine not only altered the pattern of connectivity, but as in the case of AID spine density, actually reversed it relative to Ritalin treatment alone (producing increased spine density opposed to the decrease found with Ritalin alone).

## **Introduction**

Psychostimulants, such as cocaine and amphetamine have been shown to permanently alter brain structure [29,37,38]. Although most often studied in reference to addiction, therapeutic doses of psychostimulants also appear to have the capacity to bring about changes in brain connectivity, at least in young animals. For example, chronic administration of low-doses (0.5 mg/kg) of either *d*-amphetamine or Ritalin during preadolescent development alter dendritic morphology in prefrontal cortical (PFC) pyramidal neurons [16,18]. Interestingly, low doses of these same psychostimulants do not appear to alter synaptic connectivity in the nucleus accumbens (NAc), a region that has reliably been implicated in the addictive potential of psychostimulants. Thus it would seem that late maturing cortical regions, such as the PFC, are most susceptible to experiential factors, such as drugs and gonadal hormones. Although the functional significance of these cortical changes in connectivity has not yet been determined, there is evidence to suggest a correlation between persistent deficits in cognitive performance in adulthood as a result of early drug experience and altered connectivity in the medial prefrontal cortex [16,18]. Additionally, it has been shown that psychostimulant use has the potential to block subsequent experience-induced changes in connectivity following either adult [27] or periadolescent exposure [17] in rats. Alterations in connectivity are not restricted to any particular class of psychostimulant and have also been reported following chronic caffeine exposure in adult [10] and neonatal [26] rats.

Caffeine is considered an atypical psychostimulant in that it does not appear to influence NAc functioning and thus is considered somewhat benign in its addictive potential [19]. Nonetheless, there is the possibility that caffeine may augment the effects

of other psychostimulants, perhaps contributing to their addictive potential [12]. For example, caffeine in low doses has been found to produce cross-sensitization to drugs of abuse such as amphetamine [32,40]. Additionally, pre-exposure to potentially addictive substances such as nicotine [13] or Ritalin [16] have been found to augment the behavioural response to subsequent acute intraperitoneal injections of caffeine. Other similarities to Ritalin include the finding that the motor activating effects of caffeine are attributable to modulation of dopamine-mediated circuitry in the PFC [1] and that chronic caffeine exposure has been found to alter dendritic morphology in the cingulate region of the PFC [26], a region that also influenced by early Ritalin use [18]. Few studies have investigated the interaction of psychostimulants such as Ritalin and caffeine beyond their potential to produce cross-sensitization (e.g.[11,40]) or cross-tolerance (e.g.[24,25]).

In the current study we investigated the potential interaction of caffeine and Ritalin exposure on the developing PFC in male rats. Subchronic exposure to caffeinated water was initiated at the time of weaning and one week later rats began treatment with low dose Ritalin injections twice a day. In addition to evaluation of the motor activating effects during the treatment phase, the animals underwent behavioural testing in adulthood to assess the effects of the early psychostimulant use on cognitive development. Alterations in brain connectivity were inferred using analyses of dendritic morphology.

## **Methods and Procedures**

**Subjects.** Twenty four, male pups from six litters (4 from each litter) were used in the current experiment. At weaning, the pups were randomly assigned to one of two groups, 1) non-modified H<sub>2</sub>O (Con) group, and 2) caffeine and H<sub>2</sub>O (Caff) group. The groups were housed in two large plexi-glass hanging tubs in groups of twelve and kept on a 12 hour light/dark schedule. Food and water were provided *ad libitum*. As the pups matured they were divided again to form four groups of six where they remained until the end of the experiment. On completion of the final task, rats were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline solution. The brains were harvested and post-fixed for two weeks in a Golgi-Cox solution before being transferred to 30% sucrose solution for at least 4 days prior to being sectioned. The brains were cut into 200µm sections with a Vibratome and mounted onto gelatin-coated slides. After a minimum 24 hour ‘setting’ period the slides were stained using the Golgi-staining protocol as outlined by Gibb and Kolb [22].

## **Drug Administration**

**Caffeine.** On the day of weaning (postnatal day 22) the pups were assigned to one of two groups in a pseudo-random fashion (2 pups from each litter). In one group (Caff) the regular drinking water bottles were replaced with bottles of caffeinated water (0.5mg/ml) every other day (subchronic) for a period of three weeks. The bottles were weighed each day, providing daily fluid intake measures that were then used to determine the average caffeine consumption per rat. On average the pups consumed about 127mg/kg (range of 107 – 140 mg/kg) of caffeine per 24 hour period of availability (Table 2.4.1). The second

group of rats (Con) was maintained on unmodified tap water. The initial exposure to caffeine began on the day of weaning to lessen the likelihood of the pups developing a taste-aversion to the caffeinated water. Water intake remained comparable between groups and we did not see any substantial variations between caffeine on/off days (Table 1).

Group	Water(ml)/per rat		Food Intake (% of body wt)
	On	Off	
Control	24.91 ml	23.21 ml	12.5 % avg.
Caffeine	23.97 ml	18.82 ml	11.7 % avg.

**Table 2.4.1.** The table shows the average water in milliliters consumed per animal during both the ON/OFF caffeine schedule. Food intake represents the average amount of food consumed as a percentage (%) of body weight. Food intake was monitored on the last four days of the caffeine schedule only and once again during a 24 hour period 2 weeks later.

***Methylphenidate (Ritalin;MPD).*** On P28/29, the caffeine and control groups were further divided into (Rit)alin or (Sal)ine treatment groups in a pseudo-random manner, keeping the groups more or less weight-matched. Four groups were formed; 1) Con+Sal, 2) Con+Rit, 3) Caff+Sal and, 4) Caff+Rit. All pups received subcutaneous (s.c.) injections of either Ritalin (0.5mg/kg) at a concentration of 0.5mg/ml or saline (0.9%) twice a day (a.m. & p.m.) for 12 consecutive days (P41).

## **Behavioural Analyses**

***Activity Apparatus.*** Motor response to caffeine, Ritalin, and amphetamine were assessed in an open-field apparatus. Activity was assessed at the beginning of the non-caffeine days (following 24 hour caffeine exposure), and five minutes following the a.m. injection once Ritalin treatment began. On testing days the animals were transported from their home cages to the activity testing room and activity was individually monitored in the AccuScan Instruments Inc., Versamax animal activity monitoring system (open field). The activity apparatus consisted of clear plexi-glass cages measuring 42cm x 42cm x 30cm with removable plexi-glass lids. The cages were equipped with horizontal and vertical sensors (infra red beams) that monitored activity in selected intervals for a total of 20 minutes. Recorded measures of horizontal activity (number of beam breaks on the lower bar in each sample period), total distance (path traveled in centimeters), and the number of movements (number of separate horizontal movements identified as such by a one second break in ambulatory activity) were retrieved for analysis. Once the testing period was complete, the VersaDat function scanned the files for errors to verify the integrity of data collected. The files were then converted for import to an excel spreadsheet.

***Elevated plus-maze.*** The elevated plus-maze task was used to assess exploration behaviour. The maze consisted of two arms that had high walls (closed arms) and two that had no walls (open arms). The open arms were perpendicular to the closed arms forming a 't' shape. The apparatus was elevated about 50 cm off of the floor with a camera elevated above the apparatus that was used to record the individual animal's behaviour for 10 minutes. Time spent in the open arms of the apparatus, and the number

of entries into the closed/open arms were calculated and used as an index of anxiety-like behaviour [30].

***T-maze task.*** At P68/P69 the subjects began habituation to the T-maze (a walled maze in the shape of a “T”). On the first day of habituation the animals were introduced to the apparatus in groups for a one hour period, each day thereafter animals were individually habituated. Once habituated, the rats were trained to run to the end of the arms of the apparatus to obtain a food reward (piece of fruit loop). Subjects were rewarded regardless of the arm they chose during the training phase. The testing phase began once all of the animals readily entered the arms to retrieve a reward. In the testing phase, rats were required to learn a non-match to sample paradigm of the T-maze task (for review of task details[44]). In short, each trial consisted of two components. In the first, one arm was blocked forcing the subject into the open arm. In the second component the block was removed and the rat was reintroduced to the maze (10 sec. delay). To retrieve a reward, the rat had to choose the arm opposite of where the reward had just been obtained (component one). Subsequent trials began about 30 seconds after the end of the second component. Each subject was given 10 consecutive trials per day. A correct response on at least 8 out of 10 trials for four consecutive days was required to reach criterion.

### **Anatomical Analyses**

***Brain weight.*** At the end of the experiment the brains were harvested. The olfactory bulbs and spinal cord were uniformly trimmed and the brain weight recorded prior to being placed in the post-fixative solution.

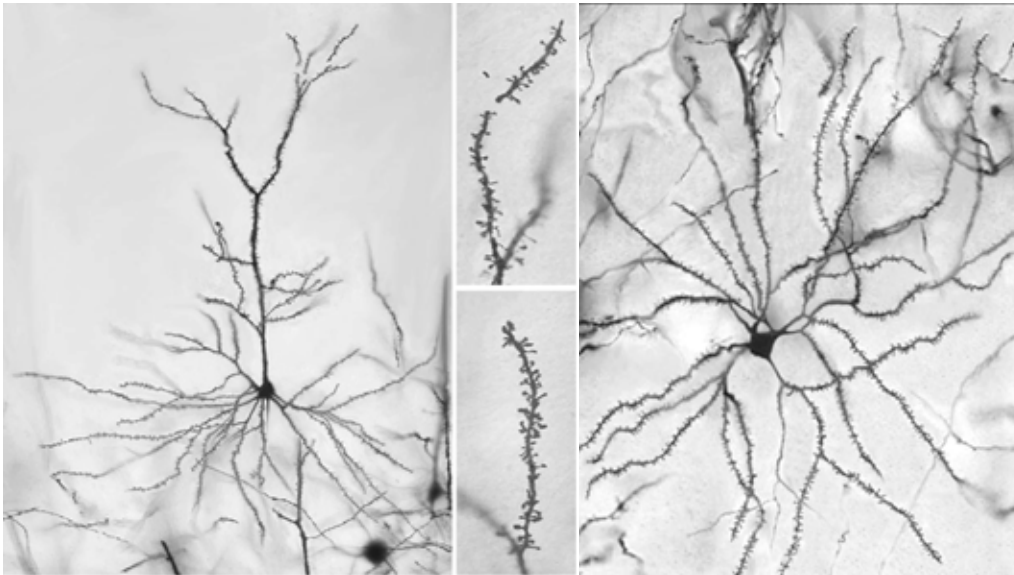
**Body weight.** Caffeine and Ritalin have been reported to moderate body weight in lab animals. Therefore, a thorough analysis of body weight over the course of the experiment was conducted to determine the affect of either experimental condition.

**Dendritic morphology.** Neurons from select brain regions known to be affected by psychostimulant use were reconstructed using a camera lucida (Figure 2.4.1) and dendritic morphology quantified for analyses. The cells included in the current study were pyramidal cells (e.g., Figure 2.4.1, left) of the primary somatosensory cortex (Par 1, layer III), region three of the anterior cingulate cortex (Zilles Cg3, layer III and layer V) as well as pyramidal neurons of the dorsal agranular insular cortex (AID, layer III) described by Zilles [28] and stellate cells of the nucleus accumbens [33].

To be included in the analysis, cells had to be; 1) thoroughly impregnated by the Golgi-Cox solution so that the dendritic trees were clearly visible at 100X and not obscured by blood vessels, neighboring processes or debris, and 2) relatively intact with minimal breakage. The cells were drawn using camera lucida (200X) and quantified by means of; 1) dendritic branch order; a quantification of the number of bifurcations off each dendritic process that extends from the soma, is useful means of determining alterations in dendritic length, and 2) Sholl analysis; a quantification of the number of processes that cross the rings (spaced 20  $\mu$ m apart) of a concentric circle placed over the cell representation, is another method useful method of quantifying dendritic length. The means from five neurons in each hemisphere were calculated and used for later comparisons.

Spine density was also determined for fourth-order terminals of basilar dendritic tress in Cg3 LV and AID LIII. As with dendritic branch order and Sholl analysis, spine

density for each hemisphere was determined by calculating the mean of five terminals (10 mm in length). Spines were drawn at 1000X (see Figure 2.4.1, center) and included all spine protrusions that had a visible head. It was recognized that this method of evaluation is an underestimation of the total spine density as those spines on both the bottom and top of the dendrite would not be visible to the experimenter (see[14] for additional details). Cells within each region were drawn by experimenters blind to the experimental groups.



**Figure 2.4.1.** Photographs of Golgi-Cox stained cells. **Left:** A photograph of a layer III pyramidal cell (200X) in the PAR1 cortex. **Center:** A photograph of a segment of basilar dendrite (1000X magnification) depicting dendritic spines. **Right:** A photograph of a stellate cell (200X) from the NAc.

## Results

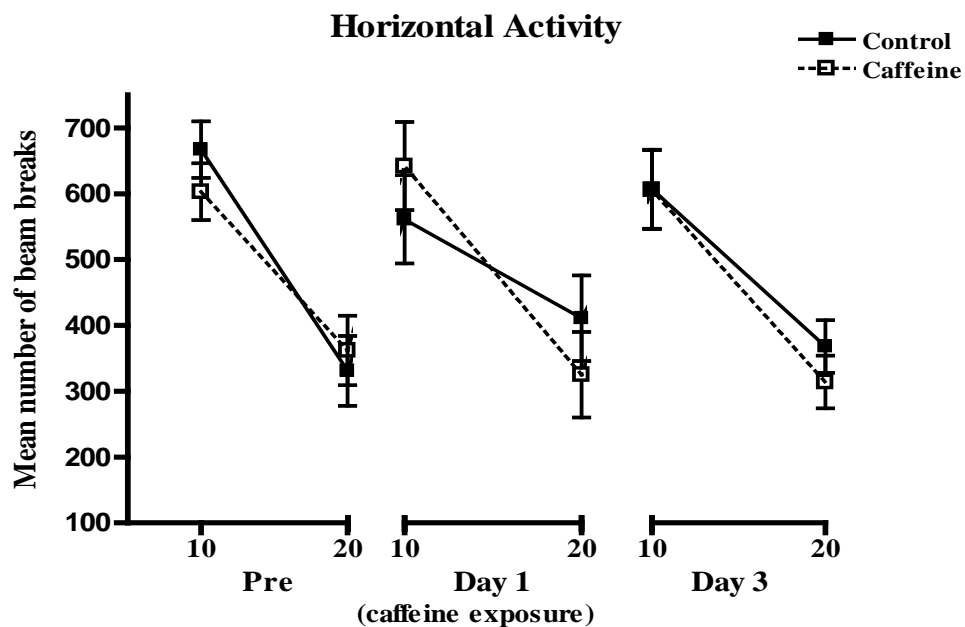
### Behavioral Results

#### *Motor Activity.*

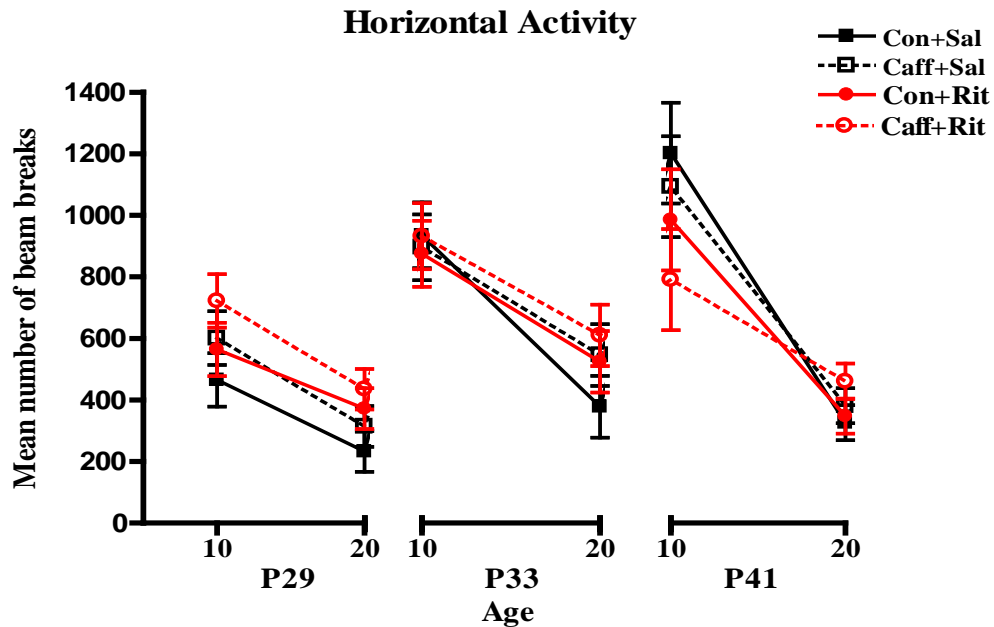
**Caffeine & Ritalin.** The motor activity of animals monitored over the course of the caffeine and Ritalin treatment periods revealed no apparent affect of either experience. Activity levels following the first and third caffeine exposure (Figure 2.4.2), as well as

activity on the initial, fifth and thirteenth day of concurrent Ritalin exposure (Figure 2.4.3) showed neither the development of tolerance nor sensitization. A repeated measures ANOVA of horizontal activity during the initial caffeine exposure showed no significant effect caffeine [ $F(1,22) = 0.12, p = 0.73$ ]. A repeated measures ANOVA of activity with continued caffeine exposure and/or concurrent Ritalin use also showed no main effect of group [ $F(1,20) = 0.42, p = 0.52$ ], treatment [ $F(1,20) = 0.08, p = 0.78$ ], or the interaction [ $F(1,20) \leq 0.01, p = 0.99$ ].

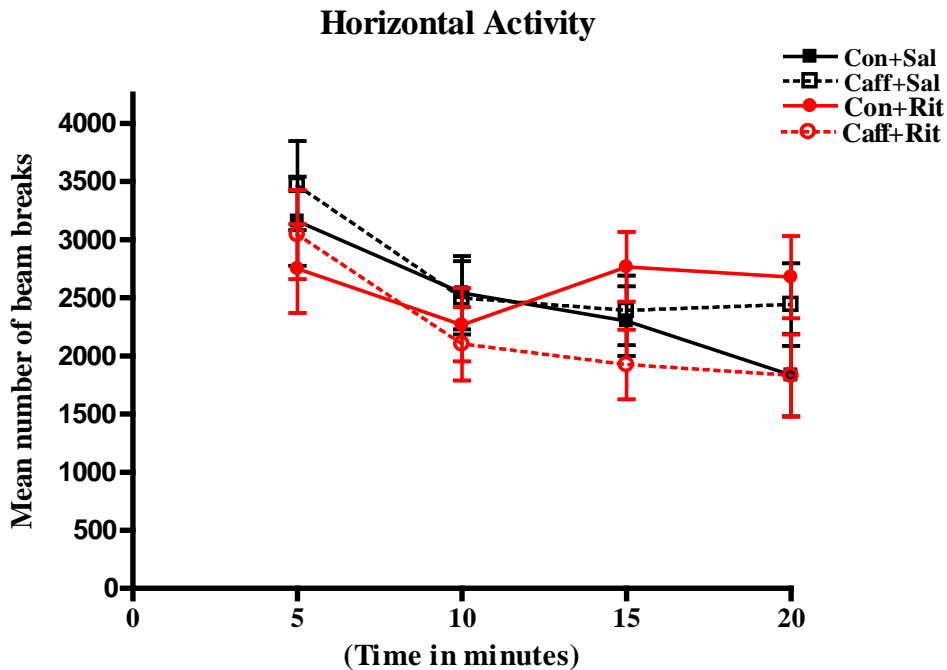
**Amphetamine challenge.** The results of the acute amphetamine challenge at the conclusion of the experiment showed that there were no apparent residual effects of early stimulant experience on later drug response in adulthood (Figure 2.4.4). A repeated measures ANOVA of activity during the amphetamine challenge revealed no main effect of group [ $F(1,20) = 0.75, p = 0.79$ ], treatment [ $F(1,20) = 0.34, p = 0.57$ ], or the interaction [ $F(1,20) \leq 1.32, p = 0.26$ ].



**Figure 2.4.2.** The line graph depicts motor activity in 10 minute intervals on the day prior to the first caffeine exposure (Pre), and on the day following the first (Day 1) and third (Day 3) exposure. Caffeine did not influence motor activity during the first week of treatment.



**Figure 2.4.3.** The line graph depicts motor activity in 10 minute intervals on the first (P29), fifth (P33) and last (P41) day of Ritalin exposure. The results show that neither caffeine nor Ritalin, alone or combined, influenced motor activity over the treatment period.

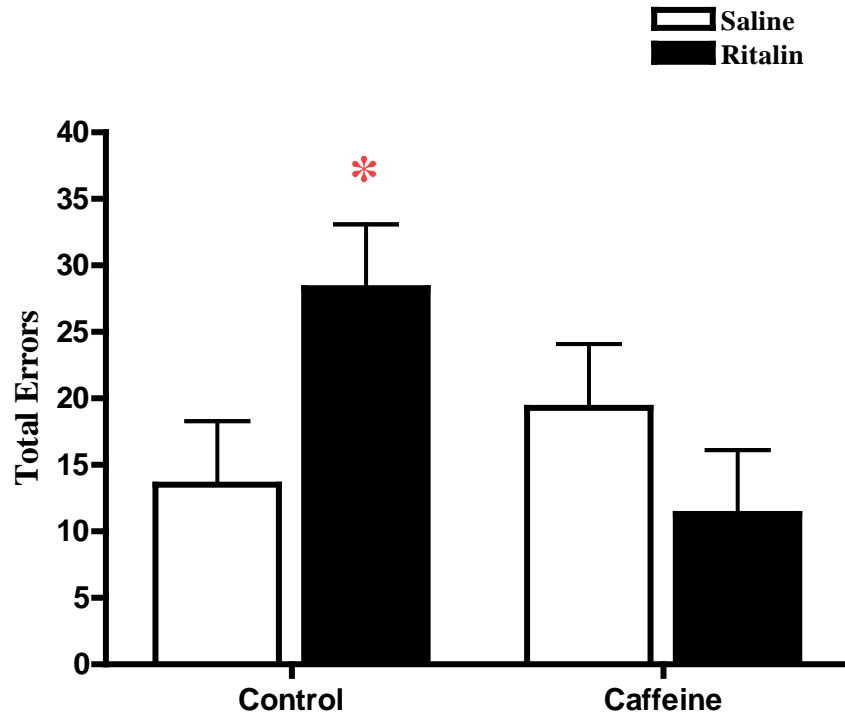


**Figure 2.4.4.** A line graph illustrating that early exposure to either caffeine/Ritalin had no apparent influence on later motor response to an acute amphetamine challenge in adulthood.

***Elevated plus maze.*** We found no differences in the time spent in open and closed arms, nor the number of entries into each among the groups. An ANOVA of time spent in the open arms with group and treatment as variables showed no main effect of group [ $F(1,20) = 0.02$ ,  $p = 0.89$ ], treatment [ $F(1,20) = 0.01$ ,  $p = 0.92$ ], nor interaction [ $F(1,20) = 1.34$ ,  $p = 0.26$ ]. Similar results were found with time spent in the closed arms ( $p$ 's  $> 0.10$ ).

***T-maze task.*** Animals treated with Ritalin during the preadolescent period made substantially more errors than either the Con+Sal or Caff+Rit rats (Figure 2.4.5). Yet, caffeine itself appeared to have no influence on T-maze performance. An ANOVA with group and treatment as variables showed a significant interaction [ $F(1,20) = 5.66$ ,  $p = 0.03$ ], but no main effect of either group [ $F(1,20) = 1.35$ ,  $p = 0.26$ ], or treatment [ $F(1,20) = 0.51$ ,  $p = 0.49$ ]. The pairwise comparison showed that Con+Rit rats made significantly more errors than either the Con + Sal or Caff+Rit groups (0.04; 0.02, respectively). No other differences were found among the groups ( $p$ 's  $> 0.30$ ).

## Errors in T-maze task



**Figure 2.4.5.** The bar graph shows that early Ritalin use affected cognitive skills in adulthood. The Ritalin-induced deficit was inhibited by the pre and concurrent use of caffeine.

### *Anatomical Results.*

**Brain weight.** The brain weights of the Caff + Rit group were increased relative to the Caff + Sal group by the end of the experiment (Table 2.4.2). Yet, neither caffeine nor Ritalin alone resulted in any changes in brain weight. An ANOVA of brain weight with group and treatment as variables showed a significant interaction of the two [ $F(1,18) = 4.89, p = 0.04$ ], but no main effect of treatment [ $F(1,18) = 0.69, p = 0.42$ ], nor group [ $F(1,18) = 0.24, p = 0.63$ ]. The pairwise comparison revealed a significant effect of Caff + Rit relative to the Caff + Sal group, but no significant differences between Con + Rit

and Con + Sal ( $p = 0.34$ ). There were no significant differences between the Con + Sal and the Caff + Sal rats ( $p = 0.24$ ) or the Con + Rit and the Caff + Rit rats ( $p = 0.07$ ).

**Brain weight (g)**

Treatment	Group	
	Control	Caffeine
Saline	2.11 +/- 0.03	2.06 +/- 0.03
Ritalin	2.07 +/- 0.03	* 2.15 +/- 0.03

**Table 2.4.2.** The table shows the mean brain weight of each group. Caff+Rit animals had heavier average brain weights relative to Caff+Sal rats as illustrated by the asterisk (\*), but there were no other group differences. +/- depicts the standard error.

***Body weight.***

***Caffeine (P22-P29).*** Subchronic caffeine use produced a slight reduction in the weight during the initial caffeine only phase (first week of the experiment). Although the groups did not differ on the first day of treatment ( $p = 0.30$ ), by the end of the week there was a trend ( $p = 0.07$ ) towards a lower body weight in the caffeine group (Figure 2.4.6).

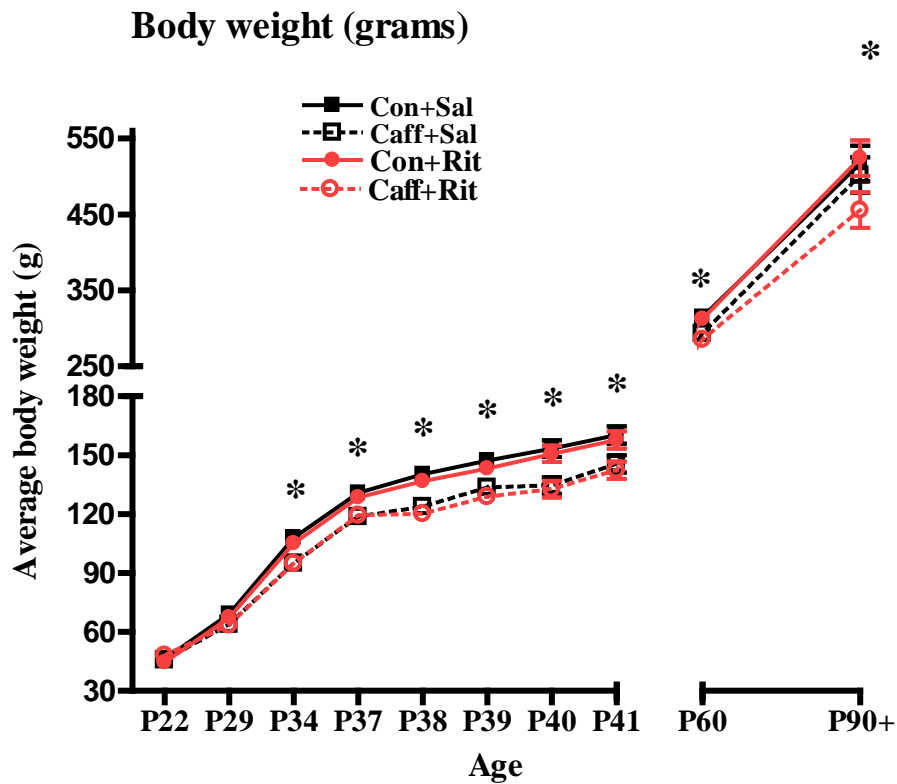
***Caffeine and Ritalin (P34-P41).*** Over the course of exposure, caffeine use continued to moderate weight gain (Figure 2.4.6). There were no apparent influences with the addition of Ritalin, however. By P34 the Caff + Sal rats showed a significantly slower weight gain relative to the Con + Sal rats ( $p$ 's  $< 0.005$ ). Similarly, Caff + Rit moderated weight gain relative to Con + Rit rats ( $p$ 's  $< 0.02$ ), with the exception of the P37 ( $p = 0.11$ ). No differences were found between saline and Ritalin rats ( $p$ 's  $> 0.40$ ).

***Caffeine and Ritalin (P60).*** More than two weeks following the last stimulant exposure the rats that had been exposed to caffeine continued to lag behind on weight gain relative to those animals that had not received caffeine (Figure 2.4.6). Ritalin did not appear to have any additional effects on body weight as Con + Rit had higher body weights relative to Caff+Rit ( $p = 0.02$ ) that was similar to the higher weight of Con+Sal relative to the Caff+ Sal rats ( $p = 0.05$ ). There were no differences between saline and Ritalin treated rats ( $p$ 's  $> 0.20$ ).

***Caffeine and Ritalin (P90+).*** By the end of the experiment only those rats that had been treated with both caffeine and Ritalin lagged behind in body weight (Figure 2.4.6) relative to the Con + Rit group ( $p = 0.05$ ). The Caff + Sal group no longer differed from the Con + Sal group ( $p = 0.65$ ).

A repeated measures ANOVA of body weight with group and treatment as variables showed a significant main effect of group [ $F(1,20) = 10.42, p = 0.004$ ], but not treatment [ $F(1,20) = 0.72, p = 0.41$ ], nor interaction [ $F(1,20) = 0.23, p = 0.64$ ].

**Food consumption.** Owing to the caffeine-induced moderation of body weight, the average percentage of food intake per animal was monitored over a period of three days near the end of the stimulant treatment period and again at P58 (Table 2.4.1). The rats consumed almost identical quantities of food. Thus, changes in body weight were unlikely caused by differences in food intake.



**Figure 2.4.6.** The line graph shows that a caffeine-induced difference in weight gain was evident by P34. Although caffeine treated rats continued to lag behind, by the end of the experiment only the Caff+Rit showed a moderation in body weight.

### *Dendritic morphology*

In summary, Ritalin exposure during the periadolescent period *increased* the apical dendritic field, but *decreased* basilar spine density in layer III neurons of the orbital prefrontal cortex (AID). Layer V neurons in the Cg3 showed a Ritalin-induced *increase* in the basilar branch order and a *decrease* in spine density. In contrast, layer III neurons of the Cg3 showed only a Ritalin-induced *decrease* in apical dendritic length.

Caffeine, on the other hand produced no apparent changes in layer III pyramidal cells in the OFC and Cg3. The only obvious caffeine-induced change in dendritic

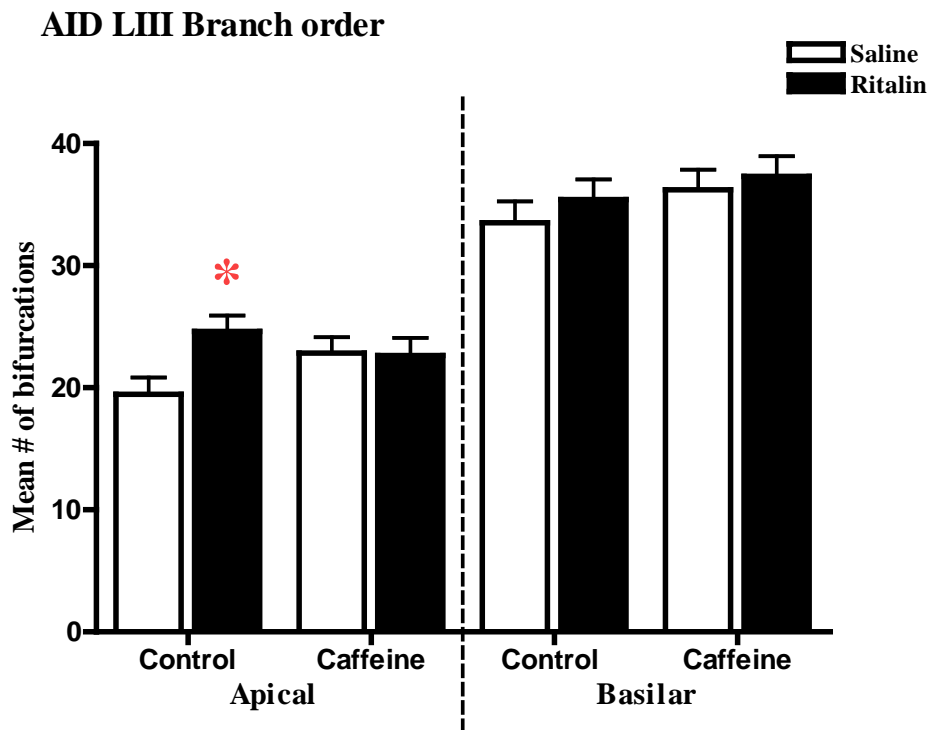
morphology was a *decrease* in apical branch order and length (Sholl), and a contrasting *increase* in the basilar dendritic field of layer V Cg3 neurons.

Interestingly, caffeine inhibited/blocked the Ritalin-induced increase in OFC apical branch order and in combination with Ritalin produced the opposite effect of Ritalin itself on basilar spine density. Further, the addition of caffeine blocked both the Ritalin-induced increase in layer V Cg3 basilar dendritic field and the decrease of layer III apical dendritic length. Ritalin in combination with caffeine blocked only the caffeine-induced increase in the basilar dendritic field of layer V pyramidal neurons in the Cg3. Neither Ritalin, nor caffeine had any influence on dendritic morphology of NAc or Par 1 neurons (see summary Table 2.4.3)

### **AID Layer III**

***Branch order & Sholl analysis.*** Ritalin alone produced an increase in the number of bifurcations in apical but not basilar dendrites. The Ritalin effect on branch order was inhibited by pre-and concurrent exposure to caffeine however (Figure 2.4.7). Yet, caffeine itself did not produce any obvious changes in branch order. In fact, the only apparent affect of caffeine itself was an overall marginal increase in apical dendritic length, irrespective of treatment. An ANOVA of apical branch order with treatment and group as variables showed a trend towards an interaction of the two [ $F(1,33) = 3.86, p = 0.058$ ], but no main effect of either treatment [ $F(1,33) = 3.31, p = 0.078$ ] or group [ $F(1,33) = 0.26, p = 0.61$ ]. The pairwise comparison revealed that the interaction was due to Ritalin producing a significant increase in the Control, but not the Caffeine group ( $p = 0.01$ ;  $p = 0.92$ , respectively). An ANOVA of apical dendritic length (Sholl) with treatment and group as variables showed a marginal main effect of group [ $F(1,35) = 4.12,$

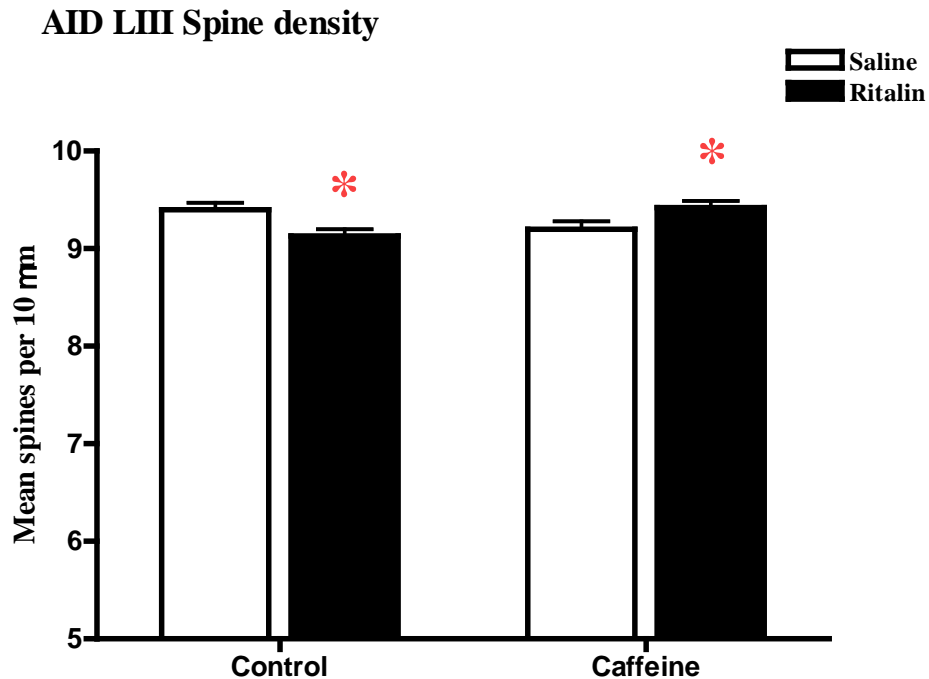
$p = 0.05$ ], but no significant main effect of treatment [ $F(1,35) = 0.09$ ,  $p = 0.77$ ], nor interaction [ $F(1,35) = 0.30$ ,  $p = 0.59$ ]. There were no significant effects of Ritalin or caffeine on basilar branch order (all  $p$ 's  $> 0.10$ ), or basilar dendritic length (all  $p$ 's  $> 0.05$ ).



**Figure 2.4.7.** The graph illustrates Ritalin alone increased apical branch order, an effect that was inhibited by caffeine treatment. Caffeine itself produced no changes in branch order.

**Spine density.** Ritalin alone produced a decrease in AID basilar spine density relative to saline-controls. When pretreated with caffeine, however, the effect was the opposite, with Ritalin now producing an increase in AID spine density relative to Ritalin controls as well as Saline controls (Figure 2.4.8). An ANOVA of mean spine density with treatment and group as variables showed a significant interaction between the two [ $F(1,36) = 10.27$ ,

$p = 0.003$ ], but no main effect of treatment [ $F(1,36) = 0.09$ ,  $p = 0.76$ ], nor group [ $F(1,36) = 0.38$ ,  $p = 0.54$ ].



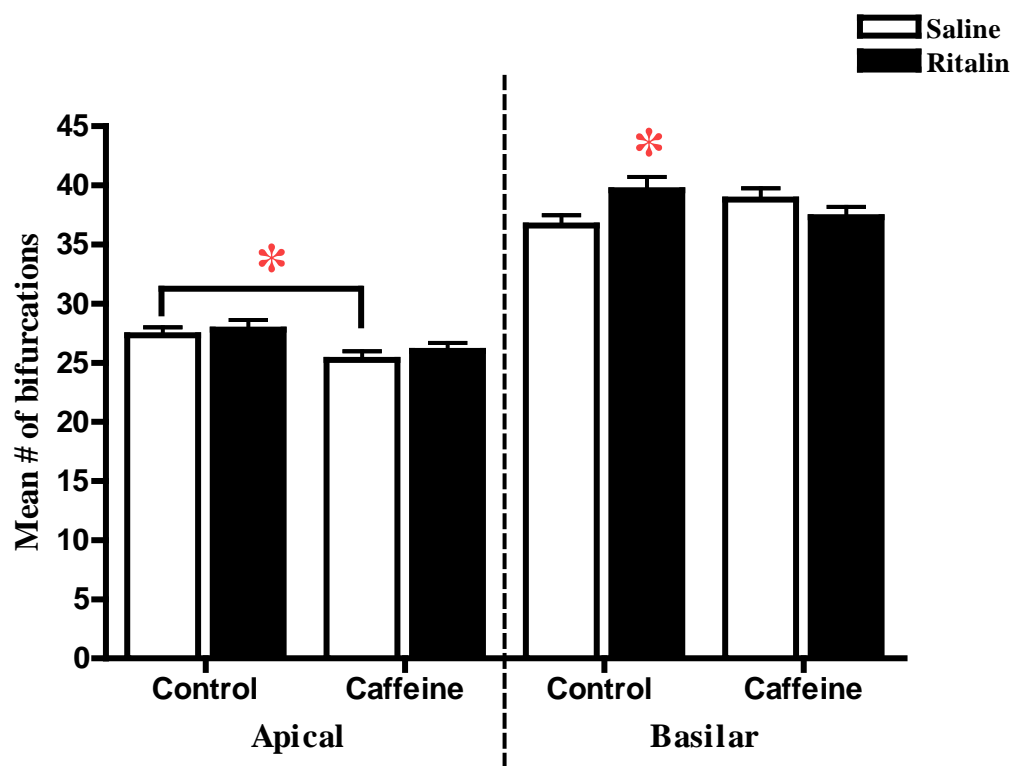
**Figure 2.4.8.** The graph illustrates Ritalin alone produced a significant decrease in spine density, whereas Ritalin + caffeine had the reversed effect; increasing spine density.

### Cg3 LV

**Branch order.** Early caffeine exposure selectively reduced apical branch order of Cg3 layer V neurons in saline treated animals in adulthood (Figure 2.4.9). The decline was attenuated by co-administration of Ritalin. Ritalin, on the other hand produced an increase in basilar branch order that was repressed in the caffeine-treated rats. An ANOVA of apical branch order with treatment and group as variables showed a significant main effect of group [ $F(1,38) = 7.31$ ,  $p = 0.01$ ], but not treatment [ $F(1,38) = 0.65$ ,  $p = 0.43$ ], or interaction [ $F(1,38) = 0.03$ ,  $p = 0.87$ ]. The pairwise comparison

revealed that the group effect was mainly due to a caffeine-induced decrease in branch order of saline ( $p = 0.04$ ), but not Ritalin ( $p = 0.09$ ) treated animals. An ANOVA of basilar branch order showed a significant interaction of treatment and group [ $F(1,38) = 5.76, p = 0.02$ ], but no main effect of either ( $p$ 's  $> 0.40$ ). The pairwise comparison revealed that the interaction was caused by a Ritalin-induced increase in

### Cg3 LV Branch order



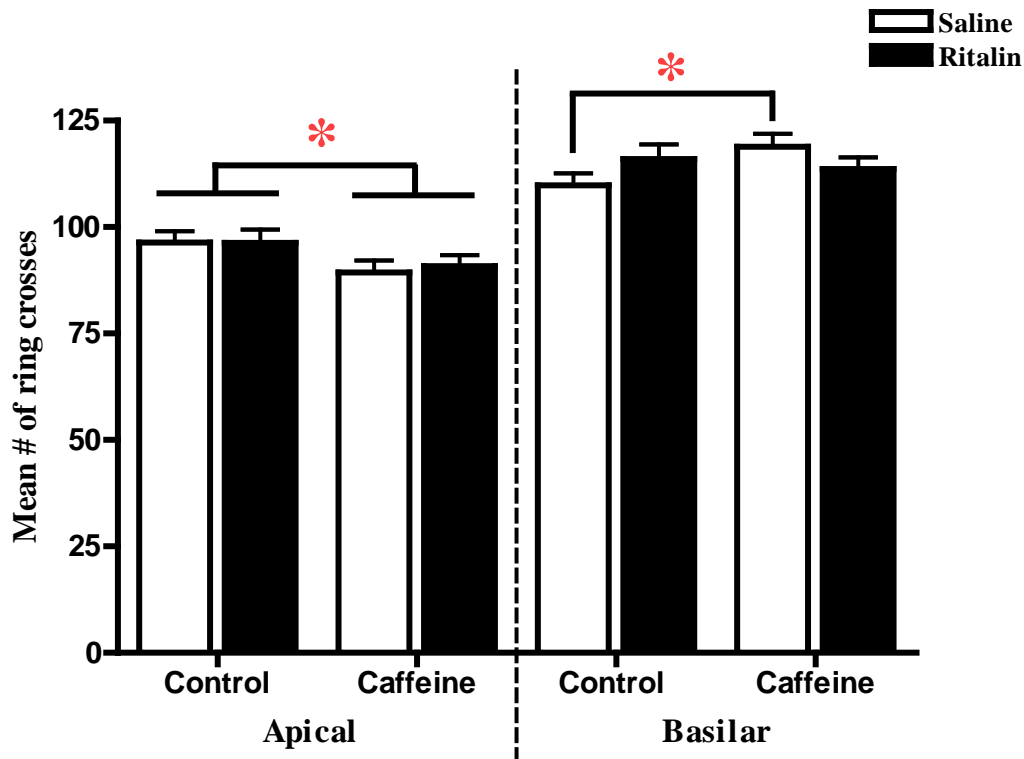
**Figure 2.4.9.** The bar graph illustrates that caffeine produced a decrease in apical, but not basilar branch order. The effects were found in saline-treated rats only, suggesting Ritalin blocked the caffeine-induced decrease of apical dendritic fields. Further, Ritalin produced an increase in basilar branch order that was inhibited by the concurrent exposure to caffeine.

**Sholl analysis.** The sholl analysis revealed that early caffeine exposure produced an overall decrease in apical dendritic length in adulthood, irrespective of whether or not the animals had also received Ritalin (Figure 2.4.10). In contrast, basilar dendritic length was

increased by the early caffeine exposure but only in saline-treated animals, suggesting that Ritalin blocked the caffeine-induced changes in connectivity.

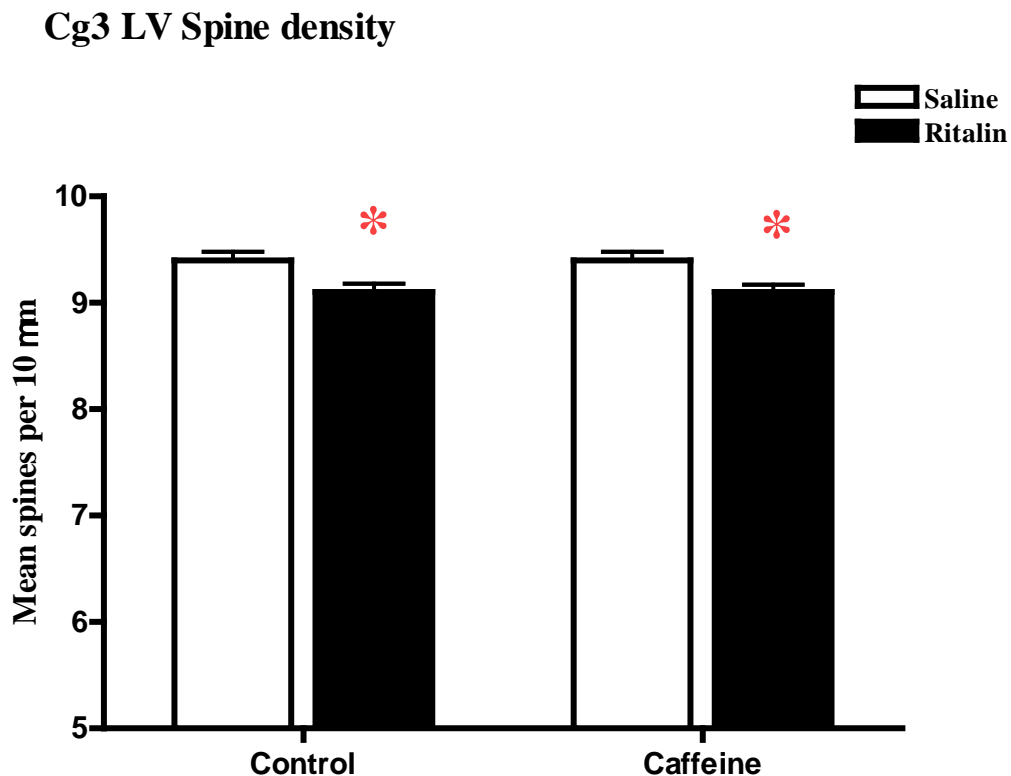
An ANOVA of apical dendritic length with group and treatment as variables showed a significant main effect of group [F(1,38) = 5.20, p = 0.03] but not treatment [F(1,38) = 0.06, p = 0.81], or interaction [F(1,38) = 0.08, p = 0.78]. In contrast, an ANOVA of basilar dendritic length showed a trend for interaction [F(1,38) = 3.66, p = 0.06], but no main effect of group [F(1,38) = 1.23, p = 0.27], or treatment [F(1,38) = 0.02, p = 0.89]. The interaction was caused by a caffeine-induced increase in saline (p = 0.03), but not Ritalin (p = 0.59) treated rats.

### Cg3 LV Sholl analysis



**Figure 2.4.10.** The bar graph depicts an overall decrease in apical dendritic length in animals that had received early caffeine exposure. In contrast, there is an increase in basilar dendritic length, but only in saline-treated rats. Ritalin alone did not appear to influence either apical or basilar dendritic length of Cg3 LV neurons.

**Spine density.** Early Ritalin exposure produced a decrease in spine density of layer V Cg3 neurons irrespective of whether or not the animals had also received caffeine (Figure 2.4.11). An ANOVA of spine density showed a significant main effect of treatment [ $F(1,38) = 14.79, p \leq 0.001$ ], but not group [ $F(1,38) = 0.001, p = 0.97$ ], or interaction [ $F(1,38) = 0.14, p = 0.71$ ]



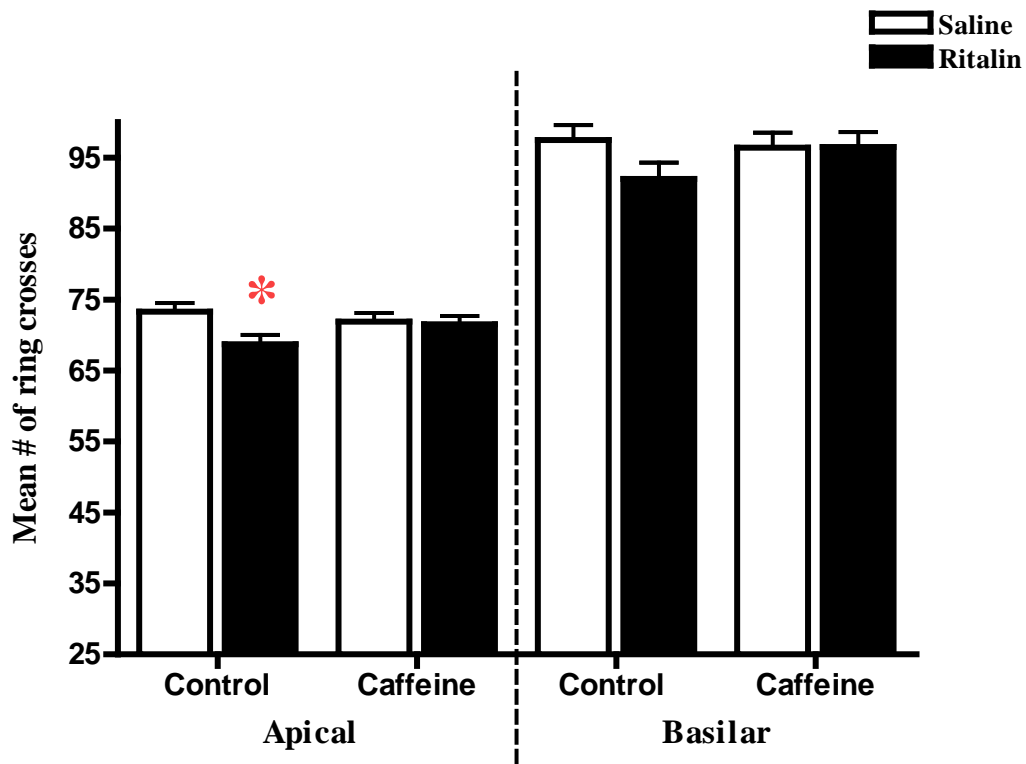
**Figure 2.4.11.** A bar graph showing that Ritalin produced a decrease in basilar spine density of Cg3 layer V neurons in both caffeine and non caffeine groups. Caffeine itself had no obvious influence on basilar spine density.

### Cg3 LIII.

**Branch order & sholl analysis.** Early Ritalin treatment produced a decrease in the apical dendritic field of layer III Cg3 neurons in adulthood (Figure 2.4.12) but did not influence the basilar field of these same neurons. The effect however was blocked by the pre and

concurrent addition of caffeine. ANOVA's of apical and basilar dendritic length (Sholl) showed a significant main effect of treatment [ $F(1,42) = 3.98, p = 0.05$ ], but not group [ $F(1,42) = 0.29, p = 0.59$ ] nor the interaction [ $F(1,42) = 2.86, p = 0.10$ ]. Apical and basilar branch order was unaffected by either condition (all  $p$ 's  $>0.06$ ).

### Cg3 LIII Sholl analysis



**Figure 2.4.12.** The bar graph illustrates that Ritalin alone produced a decrease in apical dendritic length, but this effect was inhibited by combined treatment with caffeine. Neither Ritalin or caffeine produced any other effects in LIII dendritic length.

**Par I.** Early exposure to caffeine and/or Ritalin had no apparent influence the dendritic morphology of layer III Par 1 pyramidal cells in adulthood. ANOVA's of apical and basilar branch order and the sholl analysis of dendritic length revealed no significant differences among the groups (all  $p$ 's  $> 0.30$ ).

**NAc core.** Dendritic morphology of stellate cells in the NAc core showed no apparent influence of early caffeine or Ritalin use in adulthood. ANOVA's of branch order and dendritic length showed no effect of either experience (all p's > 0.40).

**Summary of Experiments in Study Four**

	Basilar					Apical				Spine (basilar)	
	Cg3		OFC	Par1	NAc	Cg3		OFC	Par1	Cg3	OFC
	LV	LIII	LIII	LIII	LIII	LV	LIII	LIII	LIII	LV	LIII
<b>Ritalin</b>	h	∅	∅	∅	∅	∅	i	h	∅	i	i
<b>Ritalin + Caffeine</b>	∅	∅	∅	∅	∅	i	∅	∅	∅	i	h
<b>Caffeine</b>	h	∅	∅	∅	∅	ii	∅	h	∅	∅	∅

**Table 2.4.3.** Arrows indicate direction of dendritic changes relative to untreated counterparts. **Blue arrows = branch order; Red arrows = Sholl (length); Spine = spine density; ∅ = no change;** Cg3 = region three of the cingulate gyrus; OFC (AID) = orbital prefrontal cortex; Par 1 = primary somatosensory cortex; CA1 = subfield of the hippocampus; NAc = nucleus accumbens core or shell; LV = layer five neurons and LIII = layer three neurons.

**Discussion**

Concurrent administration of Ritalin and caffeine during pre- and periadolescent development reversed Ritalin-induced deficits in cognitive performance, increased brain weight, while decreasing body weight, and produced a unique pattern of connectivity in the prefrontal cortex relative to either treatment alone. Although both Ritalin and caffeine have been found to influence motor activity in a dose-dependent manner, we found no evidence of altered motor response, nor any evidence to support enhanced drug sensitivity in adulthood.

***Ritalin-induced deficits in cognitive performance are reversed by additional caffeine experience.***

Contrary to studies that have found cognitive benefits of acute Ritalin exposure, and to a much lesser degree caffeine [6-8], the current study found that early Ritalin exposure produced deficits in cognitive performance in the T-maze task in adulthood. The results presented here are in keeping with previous studies in our lab that have found increased T-maze errors in adult rats chronically exposed to Ritalin during the preadolescent stage of development (P22-P34), as well as after extended exposure that transcended through the pre-pubertal and post-pubertal phases of development ([16,18], respectively). Together these findings indicate that 1) the effects of chronic Ritalin use are fundamentally different than acute exposure and 2) early drug experience has the capacity to produce permanent alterations in brain functioning. The implications of the latter are widespread, illustrating the extraordinary capacity of experiential factors to permanently alter the trajectory of normal brain development. Further, owing to the delayed introduction of Ritalin relative to our earlier studies, the results of the current study show that the period of vulnerability for drug-induced alterations in cognitive development are not restricted to the preadolescent period but include adolescence as well.

The stimulatory effects of caffeine have been widely studied (e.g. [5,15,36,43]), yet few studies have investigated the long-term effects of postnatal caffeine on adult cognitive behavior and none, to our knowledge, have done so following exposure during the pre and periadolescent developmental period. There is evidence that chronic juvenile exposure alters play behaviour, however [23]. Additionally, Zimmerberg and colleagues

[45] have found that relatively short-term caffeine exposure in the first week of life produced impairments in an operant spatial learning task in adulthood. Contrary, to these earlier studies, we found no deficits in cognitive skills in adult rats pre-exposed to caffeine. The differences in dose, age, and treatment duration, along with variations in tasks make it difficult to make direct comparisons, however. Age may be especially relevant here as variations in vulnerability to stimulant drugs have been noted during distinct developmental periods [2,3,41]. Future research will be needed to clarify the individual effects of these factors.

The finding that concurrent caffeine and Ritalin exposure completely abolished the Ritalin-induced cognitive deficit was entirely unexpected. Owing to the fact that both of these compounds have similar therapeutic effects in the reduction of ADHD symptomology [21], and that both have the capacity to increase extracellular dopamine levels [1,20,39,42], albeit via different mechanisms, one might predict that the combined drugs would augment the effects produced by either drug alone. Thus, the reversal of Ritalin-induced deficits in adulthood by early co-administration of caffeine seems paradox. The mechanism(s) by which early exposure to the combination of Ritalin and caffeine might have altered prefrontal functioning in the T-maze are unknown at this time.

Research into alterations in the effects of receptor activation may provide a direction for future research. For example, Bonci and Williams [9] have shown that activation of D1 receptors in rats pretreated with cocaine or morphine produce opposite effects of those found in controls, inhibiting rather than enhancing gamma-aminobutyric acid type B (GABA(B)) inhibitory postsynaptic potentials (IPSPs). Interestingly, this

drug-induced effect was blocked by an adenosine A1 receptor antagonist. Although the study was conducted in adult animals, the implications are especially interesting when considering the potential effects of co-administration of Ritalin (D1 agonist) and caffeine (non-specific adenosine receptor antagonist) during development. Zimmerberg and colleagues [45] also provide some important clues that would suggest chronic exposure to caffeine may alter the effects of caffeine at the behavioural, as well as molecular, level. They report behavioural effects of chronic caffeine use that are opposite those of acute use and in fact more closely resemble the behavioural effects of adenosine. Thus, as the researchers suggest, chronic caffeine exposure may cause an upregulation of adenosine receptors.

***Altered dendritic morphology in Ritalin-treated rats is blocked by simultaneous caffeine use.***

The combination of early Ritalin and caffeine produced a unique pattern of adult prefrontal cortex connectivity relative to either drug alone, as inferred by alterations in dendritic morphology of pyramidal neurons. Ritalin had the most widespread effects, altering connectivity in layer III of the OFC and Cg3, as well as layer V of the Cg3. In contrast, the effects of early caffeine exposure were limited to major output neurons (layer V) of the Cg3. The most notable finding however, was the ability of combined Ritalin and caffeine use to block/inhibit the induction of Ritalin-induced alterations in almost all cortical regions examined.

Similar to psychostimulants of abuse, early Ritalin exposure has consistently produced alterations in the PFC as shown in the present as well as past studies in our lab.

In contrast to these same studies we have not established that any alterations occurred in the NAc core. This is an important discovery as it suggests that the influence of Ritalin on the brain may be limited to the prefrontal cortex.

Although the functional significance of these dendritic changes have not been established, taken in combination with the T-maze data, one might speculate that at least some of the extensive alterations in Ritalin-treated rats were responsible for the decline in cognitive skills in adulthood. Taken one step further, one could offer the absence of alterations in the dendritic fields, as well as cognitive deficits in rats pretreated with both Ritalin and caffeine as support.

On a similar note, the absence of alterations in NAc dendritic morphology supports the behavioural data as well, as we found no changes in the motor activity in any of the groups, nor did we see evidence of tolerance or sensitization to subsequent psychostimulant exposure.

***Caffeine decreased weight gain that endured into adulthood.***

Ritalin during early postnatal development (P5 -24) has been shown to produce decreases in weight gain but there is a rebound effect that begins shortly after drug termination [35]. The influence on body weight appears to be restricted to the neonatal period as body weight was not influenced by Ritalin exposure during the periadolescent (P35 -54) period. Similarly, Ritalin exposure in the current experiment also failed to produce changes in weight gain. In contrast, early caffeine exposure from P22 –P41 produced a marked reduction in weight gain over the treatment period. Weight gain in caffeine-treated rats continued to lag behind into adulthood relative to non-caffeine groups, irrespective of whether or not the animals had also received Ritalin. Caffeine-

induced weight loss has been reported elsewhere [31]. It has been typical however, to see a relatively rapid rebound once caffeine exposure ends [45]. The rapid rebound did not occur in the current study. Further, although the body weight of saline-treated rats co-administered caffeine eventually reached non-caffeine treated rats in adulthood, this was not the case for those treated with both Ritalin and caffeine. The absence of a rebound effect in the combined treatment group was unexpected as we found no substantial differences in food consumption in adulthood, suggesting that motivation to feed was not the cause of the decline. The low body weights of adults pretreated with Ritalin and caffeine indicates a widespread influence on overall development.

***Increased adult brain weight following combined treatment of Ritalin and caffeine.***

The decreased body weight in rats that had received the combined Ritalin and caffeine treatment makes the finding of increased brain weight in the same group even more curious. As with body weight, previous studies have found no persistent effects of early Ritalin or caffeine exposure on adult brain weight (e.g. [34,45]). It is intriguing that the Ritalin and caffeine together would have such opposite effects on brain and body weight. The increase in brain weight cannot be attributed to increases in dendritic fields alone but may reflect 1) increased neuronal or glial numbers, 2) increased neuropil, or 3) increased vascularization. In the current study the brain were processed for Golgi-Cox analysis, which restricts the measures that can be taken, and leaves any interpretation of the source of the increase conjecture at best. Future studies would benefit from a more in

depth analysis of brain anatomy that might elucidate the cause of the increased brain weight.

***Absence of motor-activating effects of Ritalin and caffeine, alone and combined***

We found no affect of either Ritalin or caffeine, alone or combined, on motor behaviour in the current study. The absence of motor activating effects during the Ritalin treatment phase was somewhat surprising as previous studies in this lab have found that Ritalin alone produces an overall increase in activity levels during the drug treatment period (Comeau, unpublished data). As dose, duration, testing protocol and mode of administration were identical to these past studies, the most likely explanation for the absence of a Ritalin-induced motor effect in the current study would be the age at which treatment was initiated. In previous experiments we have introduced Ritalin treatment at weaning (preadolescence), whereas in the present study Ritalin treatment did not begin until late in the preadolescent period, continuing into the periadolescent phase (P29 – P41). Spear and Brake [41] have reported a reduced sensitivity to catecholaminergic agonists (which would include Ritalin) during the periadolescent period that is not present in earlier or later stages of development. Thus it would seem that timing of the first exposure is an important contributing factor to the motor activating effects of Ritalin.

Interestingly, motor activity of caffeine-treated rats in the open field task did not mimic either tolerance or sensitization. As sensitization is typically associated with low doses of caffeine that is eliminated at high doses [4], the absence of increased motor behaviour over the treatment phase in the current study may not be so surprising. One might have expected then to find tolerance. In the current study, however, the average dose of caffeine was 127 mg/kg, but was dispensed orally over a 24 hour period via the

drinking water. The dose response curves to caffeine have been reported based on an intraperitoneal (i.p.) mode of administration [40], making comparison very difficult. Nonetheless, it would be reasonable to suggest that i.p. injections, whether acute or repeated, would deliver caffeine much quicker and may account for the discrepancy in motor behaviour reported here.

Owing to the fact that the motor activating effects of the combined Ritalin and caffeine treatments were comparable to either treatment alone indicates that the combined effects are not synergistic, and together are unlikely to increase the risk for later stimulant abuse. The latter view was supported by failure of any groups to display an enhanced motor response to an amphetamine challenge in adulthood.

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# **I. Adult methylphenidate use: Short and long-term effects on behaviour and dendritic morphology**

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## **Abstract**

In the current study we examined the short and long-term effects of low-dose (0.5mg/kg) methylphenidate (Ritalin) exposure in adult male rats and the potential impact of chronic stimulant exposure on social and cognitive skills. The rats were given 2X daily injections of either saline or Ritalin for 12 consecutive days and motor activity monitored periodically throughout the treatment phase. All rats received an amphetamine (1.0mg/kg) or caffeine (0.015mg/kg) challenge after a drug-free period. Animals given the amphetamine challenge were sacrificed immediately following the challenge, whereas the remainder of the animals continued on to the training phase of the experiment in which the pretreated rats were assigned to either a training or non-training group until the end of the experiment. All rats were assessed on social interaction, whereas only the training group was included in the T-maze training. At the end of training all rats received an amphetamine challenge and were sacrificed immediately afterward. Chronic Ritalin exposure in adulthood enhanced activity during the treatment period but did not produce increased sensitivity to other stimulants once treatment had ended. As well there were no apparent changes in social or cognitive performance in Ritalin-treated rats. In contrast, all three conditions, Ritalin, training and the combination of the two, produced changes in PFC circuitry. Whereas Ritalin alone produced changes only in layer V of region three cingulate cortex (Cg3) pyramidal cells, training alone produced changes in layer III of the Cg3 and orbital frontal cortical neurons. In combination, training inhibited the Ritalin-induced changes in layer V of the Cg3 and reversed the training-induced effects in layer Cg3 layer III neurons. These findings indicate that repeated low-dose psychostimulant use alters PFC connectivity. Further study will be needed to determine whether or not these

anatomical changes alter cognitive function in tasks other than the T-maze. No changes were found in the sensory cortex.

## **Introduction**

Repeated use of psychostimulants such as amphetamine and cocaine produces neurobehavioural changes, altering synaptic circuitry in the prefrontal cortex (PFC) and nucleus accumbens (NAc), and inducing behavioural sensitization [5,16,20,24,29]. Further, chronic drug experience alters synaptic plasticity in such a way as to inhibit subsequent experience-induced changes [19].

Nonetheless, there is accumulating evidence that early exposure to therapeutic doses of psychostimulants such as amphetamine and methylphenidate (Ritalin; MPD), also has the capacity to produce enduring alterations in cortical connectivity and behaviour in adulthood. For example, Diaz-Heijtj [14] has shown that chronic preadolescent *d*-amphetamine exposure alters prefrontal cortical (PFC) circuitry at a dose that is insufficient to induce behavioural sensitization, a marker of psychostimulant addiction [26,27]. Similar results have been found with low doses of Ritalin given during the preadolescent [9] and periadolescent period [11]. Additionally, investigation of the behavioural effects in the latter studies found that early Ritalin exposure altered play behaviour and produced deficits in cognitive skills in adulthood, and cross-sensitized to caffeine. Caffeine, an atypical psychostimulant (see [13]) used to treat apnea in pre-term infants [30], has also been shown to produce alterations in PFC synaptic connectivity [18] and behaviour in neonatal rats [25]. These studies indicate that drug-experience, even when the dose or drug is otherwise considered benign, may have a substantial impact on the developing brain which may influence later learning and behaviour.

The reported increased vulnerability of the developing brain [1,2,17,22] make it tempting to conclude that the structural and behavioural changes produced with even low

doses of psychostimulants is demonstrative of increased susceptibility and thus related to age at the time of the initial exposure. There is certainly evidence that would support this line of reasoning including the fact the brain areas that show the most profound structural plasticity as a consequence of early drug experience are located in late developing regions of the PFC (see [3]). Nonetheless, there are no known studies that have investigated the structural and behavioural following therapeutic doses of psychostimulants in adulthood, leaving the possibility that low doses of psychostimulants may alter connectivity and behaviour, regardless of age, though perhaps differently.

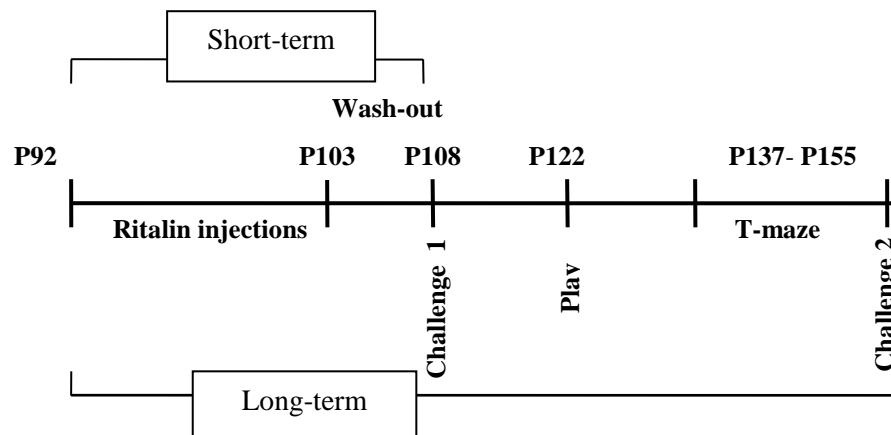
Therefore, the aim of the current study was to examine the short and long-term effects of a repeated low-dose of Ritalin in adult rats. To do this we examined the motor response to Ritalin during the treatment phase as well as using challenges of caffeine and amphetamine to assess sensitization to other stimulants. We also examined social and cognitive performance of the subjects following a prolonged wash-out period to assess the long-term risk of Ritalin use on social and cognitive behaviour.

## **Methods and Procedures**

### ***Subjects***

In the current study 32 adult, male Long-Evans hooded rats were used. The rats were obtained from 8 different litters on about postnatal day (P) 90. All animals were housed with litter mates in groups of 4-6 (depending on the litter size) in large, hanging tubs and kept on a 12 hour light/dark cycle with ad lib food and water. At the beginning of the experiment animals from each cage were randomly assigned to receive either; 1) saline or, 2) Ritalin injections. In order to assess the persistence of any drug-

effects the treatment groups were further divided into; 1) short-term (Saline = 4; Ritalin = 4) and, 2) long-term (Saline = 12; Ritalin= 12) groups at the completion of the initial drug-treatment phase. The long-term group was divided once more into 1) a group that received training (N = 12) and, 2) a group that did not participate in the training paradigms (N = 12) but were handled and transported as in the training group (Figure 3.1.1). Two rats in the latter group died unexpectedly before the end of the experiment and therefore all data obtained from the animals was removed from analyses.



**Figure 3.1.1.** An illustration of the study design illustrating the experimental time lines for both the short and long-term groups.

After a five day drug-washout period rats were randomly assigned to the short-term groups received an amphetamine challenge and were sacrificed immediately. The long-term groups, on the other hand underwent a caffeine challenge at the same point in time that was followed by an amphetamine challenge at the end of behavioural testing. At the end of the experiment all rats were given an overdose of sodium pentobarbital and intracardially perfused with a 0.9% saline solution. The brains were harvested and post-fixed in a Golgi-cox solution for two weeks. The brains were transferred to a 30% sucrose

solution at the end of the two week period where they remained until being sectioned. Brains were sectioned at 200  $\mu\text{m}$  on a Vibratome and mounted for staining. Mounted sections were stained using the Golgi staining procedures set out by Gibb and Kolb [15].

### ***Drug administration***

***Methylphenidate (Ritalin;MPD).*** On the first day of testing rats were weighed and taken to what would become the drug-administration/testing area. Subcutaneous injections of either vehicle (0.9% NaCl in dH<sub>2</sub>O) or Ritalin (0.5 mg/kg) were administered shortly thereafter. Injections were given twice daily (6 hours apart) for 14 consecutive days, with the exception of the final day in which rats were returned to their home cages at the completion of behavioral testing and did not receive the p.m. injection. At this point rats went through a 5-day wash-out period in which they were left undisturbed in their home cages.

***Amphetamine Challenge One.*** All rats underwent a 'challenge' six days following the last Ritalin injection to assess cross-sensitization to a novel stimulant. For the challenge test, the animals were taken to the drug-testing room and administered an interperitoneal (i.p.) injection of either 1mg/kg amphetamine (short-term group) or 0.015 g/kg caffeine (long-term group) and placed in the activity monitoring apparatus for 20 minutes.

***Amphetamine Challenge Two.*** At the end of the experiment, all remaining rats (long-term training/no training groups) were given a 1 mg/kg i.p. amphetamine injection and activity monitored for 20 minutes. The animals were sacrificed immediately following the challenge.

### ***Behavioural Testing***

***Activity Apparatus.*** Animals were individually tested in the AccuScan Instruments Inc., Versamax animal activity monitoring system (open field) to measure motor response. The activity apparatus consisted of a clear plexi-glass cage measuring 42cm x 42cm x 30cm with a removable plexi-glass lid. The cage(s) was equipped with sensors (infra red beams) that monitored activity in intervals for a total of 20 minutes. Each activity box had a horizontal sensor on the lower section of each cage wall (four) and two vertical bars on opposing walls that were located about mid-way between the 'floor' and 'ceiling' of the cage. The activity measures used in the current experiment included; horizontal activity (number of beam breaks on the lower bar in each sample period) that measures overall motor activity, number of movements (number of separate horizontal movements identified as such by a one second break in ambulatory activity) and rearing (number of beam breaks in the upper bar) that measures exploratory behavior. Once the testing period was complete, the VersaDat function scanned the files for errors to verify the integrity of data collected. The files were then converted for import to an excel spread sheet.

***Social Behaviour.*** The question asked here was whether there was a difference in the social interactions of cage-mates based on treatment experience in adults. In other words, were the relationships between saline/saline pairs different than between ritalin/saline or ritalin/ritalin pairs of animals? Therefore, groups were formed using existing cage mates. Each group consisted of 2 saline-treated and one Ritalin-treated rat or two Ritalin-treated and one saline-treated rat. In order to test rats in both triad configurations, we repeated the play behavior test one week later, forming new triads. All rats in the long-term Ritalin group were included in the assessment of play behavior. Rats began habituation in a play

arena one week following the caffeine challenge. Animals were introduced to the area in groups of 3-4 and left to acclimate to the surroundings for 20-30 minutes each day for three consecutive days. Following the third habituation period, the rats were individually housed (isolated) for a 24 hour period. To begin testing, groups of three rats were taken to the test room. Each rat was coded with a livestock marker for ease of identification during later data analysis. The animals were then placed inside the enclosure in pre-determined triads. Social interactions of the triads were filmed for a 10 minute period with the lights out and the experimenter out of the room. After each test subject completed the task, they were removed from the enclosure and returned to their home cage.

***T-maze Task.*** Rats were habituated to the T-maze apparatus prior to the actual training phase. Once habituated, rats were trained to run to the end of the arms of the apparatus to obtain a food reward (piece of fruit loop). During training subjects were rewarded regardless of the arm they chose. When all rats readily sought out the reward the testing phase began. In the testing phase, rats were required to learn a non-match to sample model of the t-maze task (for review of task details[8]). In short, each trial consisted of two components. In the first, one arm was blocked forcing the subject to retrieve the reward from the open arm. In the second component the block was removed and the rat was reintroduced to the maze (10 sec. delay). The rat had to choose the arm opposite of where the reward had just been obtained (component one) in order to obtain the second reward. Subsequent trials began about 30 seconds after the end of the second component. Each subject was given 10 consecutive trials per day. A correct response on at least 8 out of the trials 10 for four consecutive days was required to reach criterion.

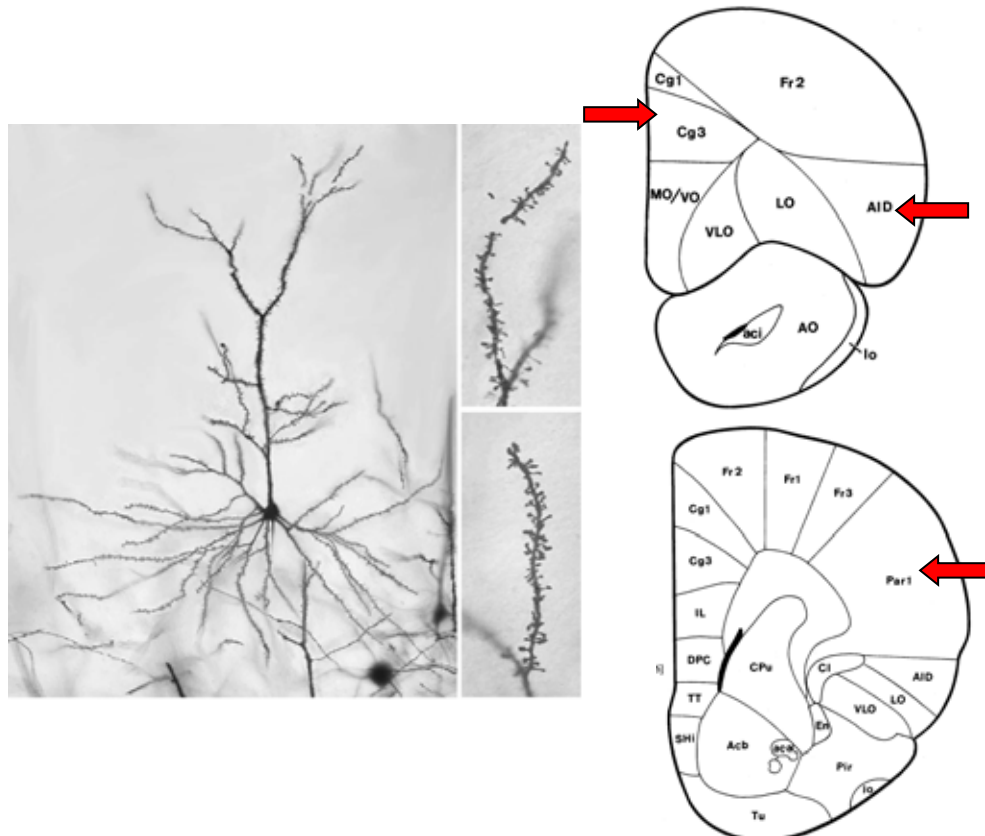
## **Anatomical Analyses**

***Dendritic morphology.*** Neurons from select brain regions known to be affected by psychostimulant use were reconstructed using a camera lucida (Figure 3.1.2.) and dendritic morphology quantified for analyses. The cells included in the current study were pyramidal cells (e.g., Figure 2, left) of the primary somatosensory cortex (Par 1, layer III), region three of the anterior cingulate cortex (Zilles [32] Cg3, layer III and layer V) as well as pyramidal neurons of the dorsal agranular insular cortex of the orbital frontal cortex (Zilles, AID, layer III).

To be included in the analysis, cells had to be; 1) thoroughly impregnated by the Golgi-Cox solution so that the dendritic trees were clearly visible at 100X and not obscured by blood vessels, neighboring processes or debris, and 2) relatively intact with minimal breakage. The cells were drawn using camera lucida (200X) and quantified by means of; 1) dendritic branch order; a quantification of the number of bifurcations off each dendritic process that extends from the soma, is useful means of determining alterations in dendritic length, and 2) Sholl analysis; a quantification of the number of processes that cross the rings (spaced 20  $\mu\text{m}$  apart) of a concentric circle placed over the cell representation to estimate dendritic length. The means from five neurons in each hemisphere were calculated and used for later comparisons.

Spine density was also determined for fourth-order terminals of basilar dendritic trees in Cg3 LV and AID LIII. As with dendritic branch order and Sholl analyses, spine density for each hemisphere was determined by calculating the mean of five terminals (10  $\mu\text{m}$  in length). Spines were drawn at 1000X (see Figure 3.1.2, center) and included all spine protrusions that had a visible head. It was recognized that this method of evaluation

is an underestimation of the total spine density as those spines on both the bottom and top of the dendrite would not be visible to the experimenter (see[7] for additional details). Cells within each region were drawn by experimenters blind to the experimental groups (WC, KK, & CC).



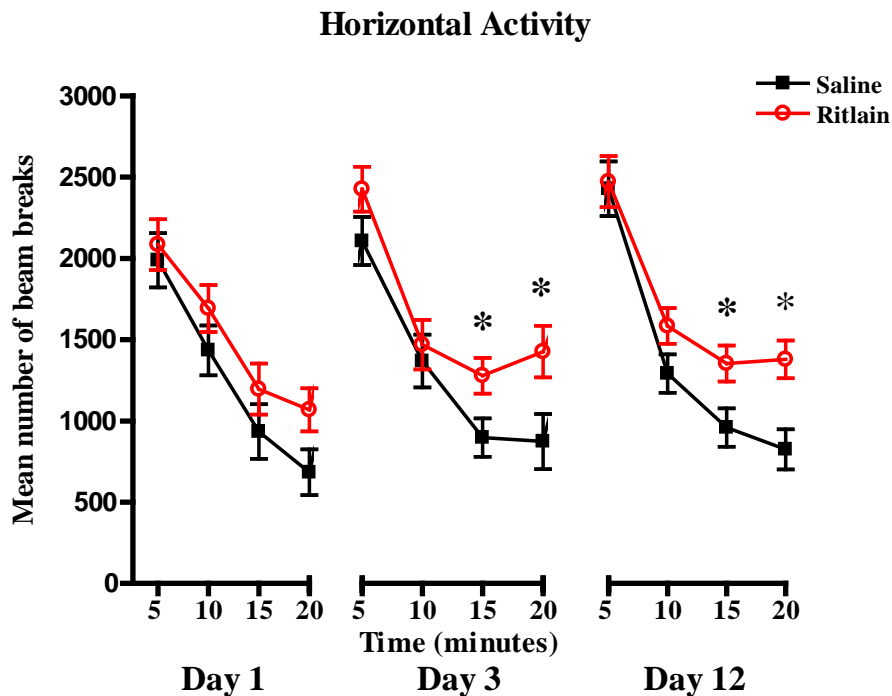
**Figure 3.1.2. Left:** A photograph of a layer III pyramidal cell (200X) in the PAR1 cortex. **Center:** A photograph illustrating a segment of basilar dendrite (1000X magnification) from which dendritic spine data were obtained. **Right:** Top and bottom. Illustration of regions included (revised from Zilles [32]).

## Results

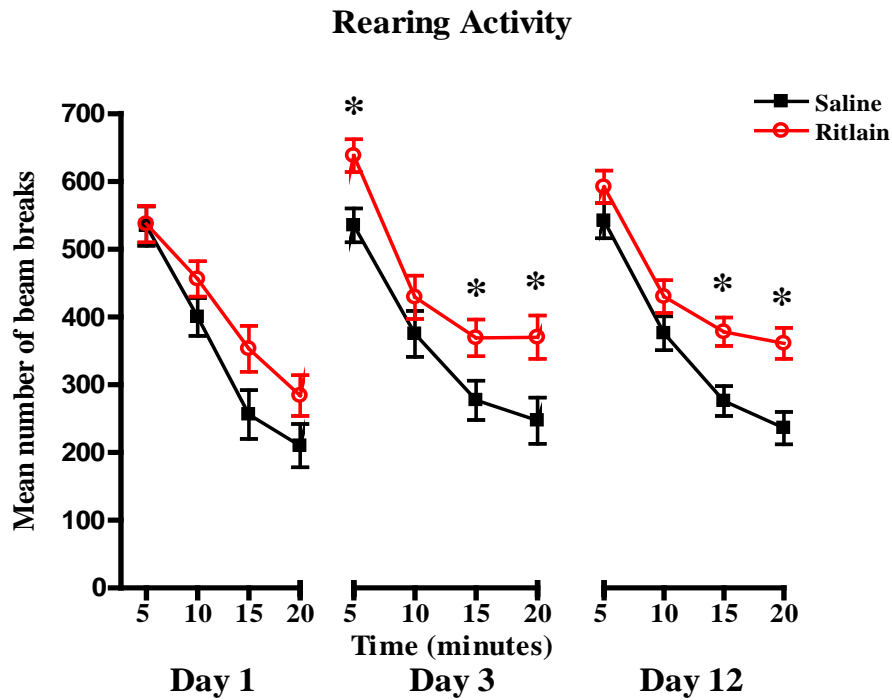
**General Observations.** At the end of the treatment phase there were no apparent differences in the disposition or grooming behavior of rats that had received chronic Ritalin injections. All animals appeared healthy and continued to gain weight throughout the experiment.

**Behavioral Results.**

**Motor activity.** Chronic Ritalin treatment enhanced both horizontal (Figure 3.1.3a) and rearing (Figure 3.1.3b) activity in adult rats. Although, we found no initial effect of Ritalin (Day 1) on either horizontal ( $p = 0.21$ ) or rearing ( $p = 0.12$ ) activity, an increase in both was evident by Day 3 of treatment ( $p = 0.035$ ;  $p = 0.006$ , respectively) and was maintained on the final day (Day 12) of treatment ( $p = 0.029$ ;  $p = 0.002$ , respectively). A repeated-measures ANOVA's of horizontal and rearing activity on days 1, 3 and 12 showed a treatment effect on each measure ( $[F(1, 28) = 4.93, p = 0.035]$  ;  $[F(1,28) = 9.95, p = 0.004]$ , respectively).



**Figure 3.1.3a.** A line graphs of horizontal activity that illustrates a Ritalin-induced enhancement of motor activity over the initial treatment period.



**Figure 3.1.3b.** An illustration of the mean activity levels within each 5 minute interval. Beginning Day 3 Ritalin treatment augmented rearing activity.

**Challenges.** Following a 5-day drug wash-out period all animals received a psychostimulant challenge. The short-term group received amphetamine (1.0mg/kg), whereas the long-term group received caffeine (0.015mg/kg). The statistical analysis was performed separately for each group. At the end of the experiment all remaining animals received an amphetamine challenge (1.0mg/kg). The statistical analyses included training as a variable to examine separately the effect that this factor. The results from the horizontal and rearing activity analyses were almost identical, thus only the horizontal activity is presented for the challenges.

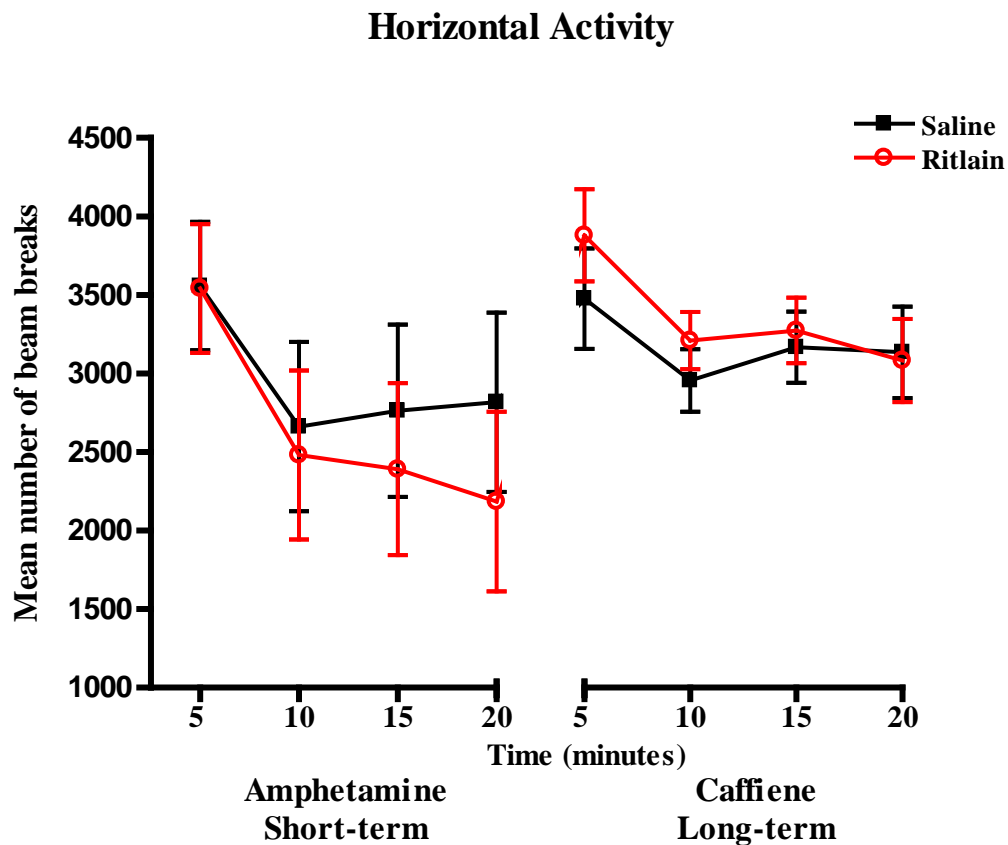
#### **Short-term**

**Amphetamine challenge.** The response of rats pretreated with Ritalin was comparable to the animals that had received saline injections over the treatment period (Figure 3.1.4). An

ANOVA of horizontal activity with treatment as the variable showed no significant effect of treatment on horizontal activity [ $F(1,6) = 0.18, p = 0.69$ ].

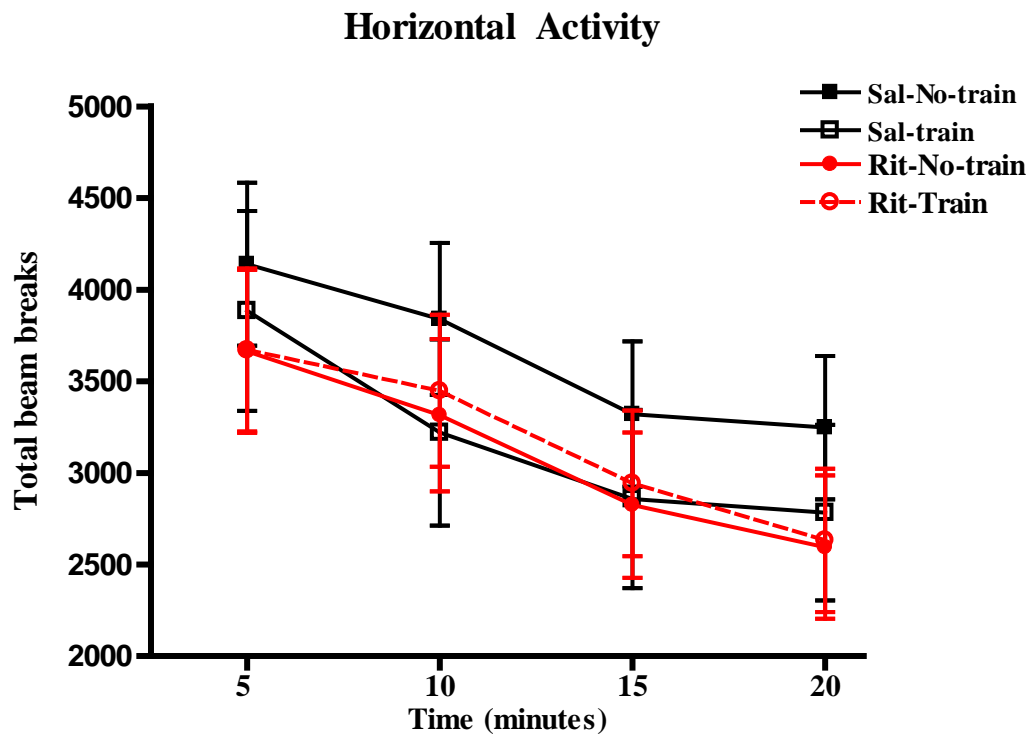
### *Long-term*

**Caffeine Challenge.** Animals that were to continue in the experiment received a caffeine rather than amphetamine challenge (Figure 3.1.4). An ANOVA of horizontal activity with treatment as a variable showed no significant effect of treatment on horizontal activity [ $F(1,20) = 0.32, p = 0.58$ ].



**Figure 3.1.4.** An illustration of the activity response to the amphetamine (1.0 mg/kg) and caffeine (0.015mg/kg) challenges. The graph shows that the initial Ritalin effect did not appear to produce any enduring sensitivity to subsequent stimulant exposure.

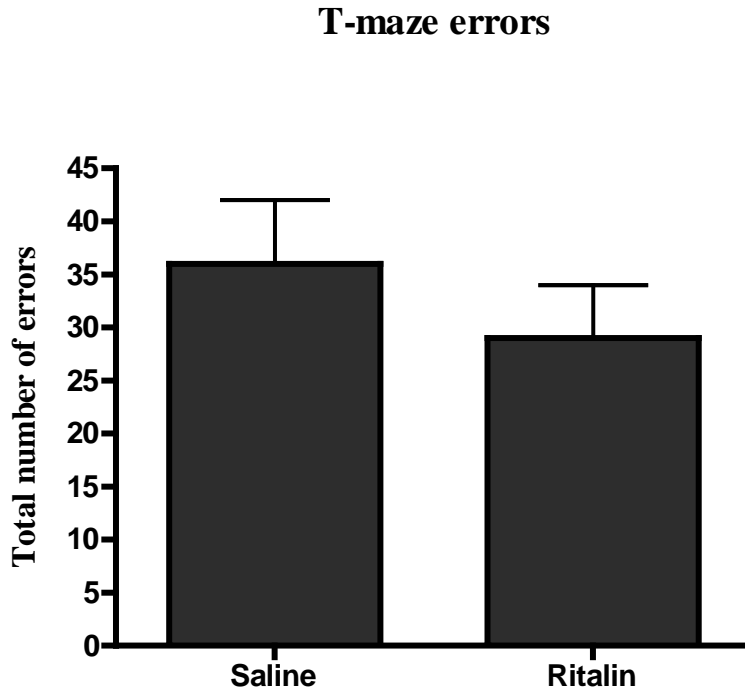
**Amphetamine challenge.** At the conclusion of the training tasks, all animals in the long-term groups received a final amphetamine challenge (Figure 3.1.5). All groups, irrespective of treatment or training responded similarly in the challenge. An ANOVA of horizontal activity with treatment and training as variables showed no main effect of treatment [ $F(1,18) = 0.45, p = 0.51$ ], training [ $F(1,18) = 0.21, p = 0.65$ ], nor interaction [ $F(1,18) = 0.41, p = 0.53$ ].



**Figure 3.1.5.** The line graph illustrates the motor response to an amphetamine in 5 minute intervals. There were no apparent differences among the groups.

**T-maze task.** Ritalin exposure in adulthood produced no enduring effects on cognitive performance in the T-maze. Saline and Ritalin-treated animals made a comparable

number of errors before reaching criterion (Figure 3.1.6). An ANOVA of total errors showed no effect of treatment [ $F(1,8) = 0.73, p = 0.42$ ].



**Figure 3.1.6.** The bar graph depicts the total number of errors to reach criterion in the T-maze task. There were no significant differences between the treatment groups.

**Social Interactions.** Chronic Ritalin exposure produced no differences in the way that Ritalin-treated rats (R) interacted with Ritalin (R/R) or Saline (R/S)-treated cage-mates. Saline-treated rats (S) also showed no differences in their interactions towards Ritalin (S/R) and saline (S/S) -treated animals. For both triad groups (RRS and SSR), a multi-factorial ANOVA of social interactions showed no significant differences between R/R and R/S interactions ( $p's > 0.20$ ), nor S/S and S/R interactions ( $p's > 0.20$ ). Data not shown.

**Anatomical Results- Dendritic Morphology.**

In summary, all three conditions, Ritalin, training and the combination of the two, produced changes in PFC circuitry but to different degrees. Whereas Ritalin alone

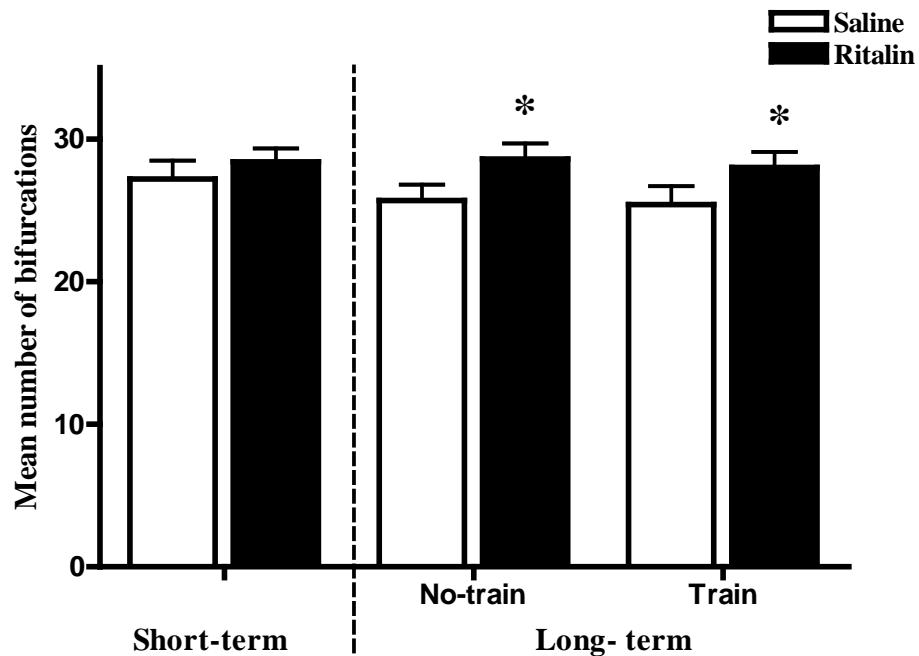
produced changes only in layer V of region three cingulate cortex (Cg3) pyramidal cells, training alone produced changes in layer III of the Cg3 and orbital frontal cortical neurons. In combination, training inhibited the Ritalin-induced changes in layer V of the Cg3 and reversed the training-induced effects in layer Cg3 layer III neurons.

***Cg3 Layer V branch order***

***Short-term.*** Chronic Ritalin exposure in adulthood had no effect on apical (Figure 3.1.7) or basilar branch order. ANOVA's of apical and basilar branch order showed no significant effect of treatment on apical [ $F(1,15) = 0.52, p = 0.48$ ], or basilar [ $F(1,15) = 1.21, p = 0.29$ ] branch order.

***Long-term.*** Chronic treatment in adulthood produced increases in apical, but not basilar branch order, irrespective of training. This finding indicates that the synaptic changes occurred at least one week after the last injection. An ANOVA of apical dendritic branch order with treatment and training as variables showed a significant main effect of treatment [ $F(1,40) = 5.83, p = 0.02$ ], but not training [ $F(1,40) = 0.20, p = 0.66$ ], nor the interaction [ $F(1,40) = 0.02, p = 0.88$ ]. An ANOVA of basilar branch order on the other hand showed no main effect of treatment [ $F(1,40) = 0.02, p = 0.89$ ], training [ $F(1,40) = 0.12, p = 0.73$ ], or interaction [ $F(1,40) = 0.25, p = 0.62$ ].

### Cg3 LV Apical branch order



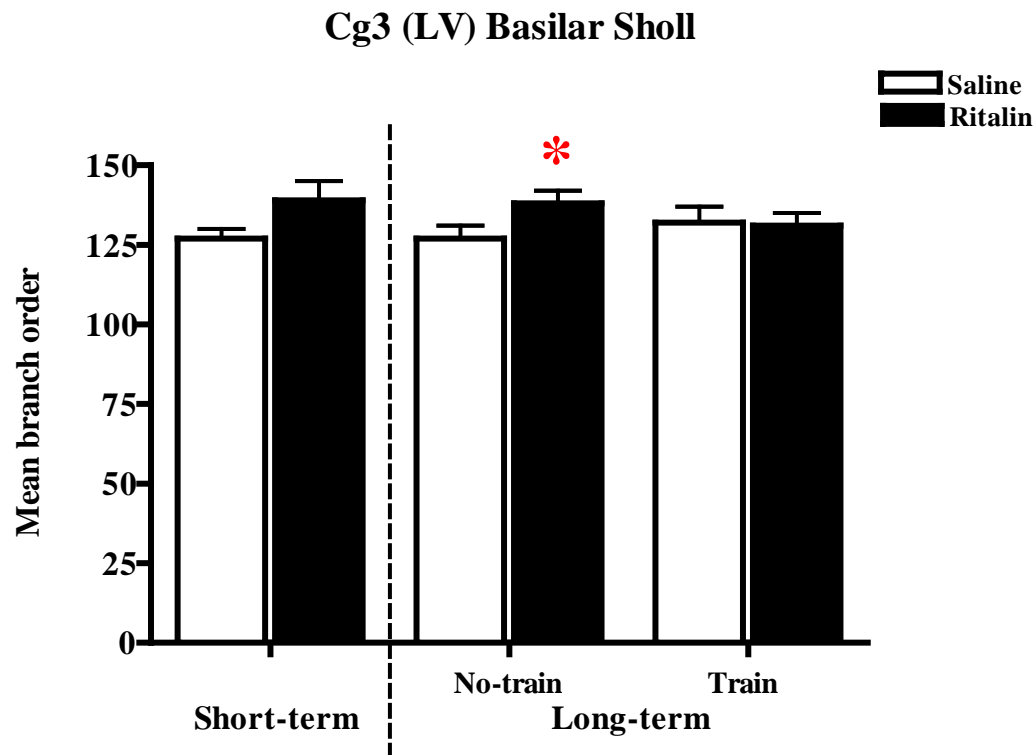
**Figure 3.1.7.** The bar graph illustrates no short-term effect of treatment an overall increase in apical branch order in Ritalin-treated rats in the long-term group. The effect was not influenced by training itself.

#### *Cg3 LV Sholl analyses.*

**Short-term.** Chronic Ritalin treatment had no influence on apical or basilar dendritic length (Figure 3.1.8). An ANOVA of dendritic length (Sholl) showed no significant effect of treatment on either apical [ $F(1,15) = 0.18, p = 0.68$ ], or basilar [ $F(1,15) = 2.91, p = 0.11$ ] fields.

**Long-term.** In contrast to the short-term effects of chronic Ritalin exposure, the long-term effects showed a drug-induced increase in basilar, but not apical, dendritic length (Figure 3.1.8). An ANOVA of basilar dendritic length (Sholl) with treatment and training as variables showed no main effect of treatment [ $F(1,40) = 1.57, p = 0.22$ ], training [ $F(1,40) = 0.06, p = 0.81$ ], or interaction [ $F(1,40) = 2.30, p = 0.14$ ]. The pairwise comparison

however, revealed that Ritalin significantly increased dendritic length in No-training ( $p = 0.04$ ) but not the training group ( $p = 0.86$ ). An ANOVA of apical dendritic length showed no significant main effect of treatment [ $F(1,40) = 1.60, p = 0.22$ ], training [ $F(1,40) = 0.07, p = 0.79$ ], or interaction [ $F(1,40) = 1.18, p = 0.28$ ]. The pairwise comparison revealed no group differences ( $p$ 's  $> 0.05$ ).



**Figure 3.1.8.** The bar graph demonstrates a Ritalin-induced increase in basilar dendritic length (Sholl) that was blocked by training.

***Cg3 LV spine density.***

***Short-term.*** Chronic Ritalin treatment had no influence on basilar spine density of Cg3 layer V cells. An ANOVA of spine density showed no significant effect of treatment [ $F(1,15) = 0.001, p = 0.92$ ].

***Long-term.*** Neither chronic Ritalin exposure, nor training had any apparent effect on basilar spine density in layer V Cg3 pyramidal neurons. An ANOVA with treatment and

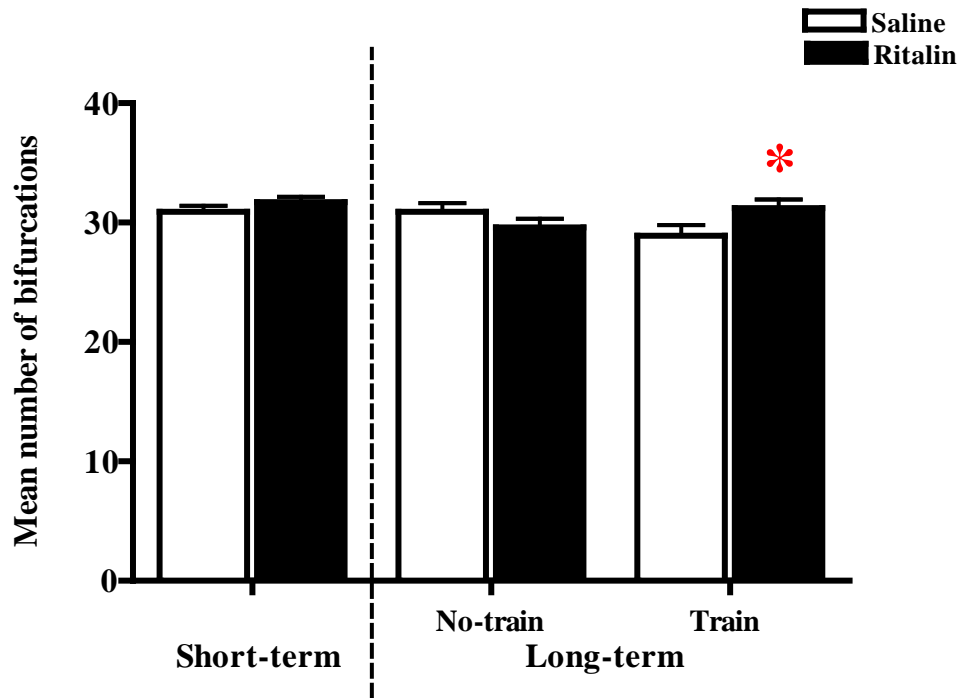
training as variables showed no significant effect of treatment [ $F(1,40) \leq 0.001$ ,  $p = 0.99$ ], training [ $F(1,40) = \leq 0.001$ ,  $p = 0.99$ ], or interaction [ $F(1,40) = 0.79$ ,  $p = 0.38$ ].

### ***Cg3 L III branch order***

***Short-term.*** Chronic pre-treatment of Ritalin in adulthood produced no changes in apical or basilar branch in layer III neurons of the Cg3 (Figure 3.1.9). An ANOVA of apical branch order showed no significant effect of treatment [ $F(1,15) = 1.27$ ,  $p = 0.28$ ]. An ANOVA of basilar branch order also showed no significant effect of treatment [ $F(1,15) = 0.43$ ,  $p = 0.84$ ].

***Long-term.*** In contrast to the short-term group, the long-term effects of chronic Ritalin exposure produced a differential effect on apical branch order, which showed an increase in rats in the Ritalin + training group, relative to Saline-treated rats (Figure 3.1.9). There was no effect however on basilar branch order. An ANOVA of apical branch order showed a significant interaction of treatment and training [ $F(1,40) = 5.30$ ,  $p = 0.03$ ]. The interaction was due to a Ritalin-induced increase in apical branch order of rats in the training (0.058), but not in the No-training group ( $p = 0.21$ ). An ANOVA of basilar branch order showed no significant main effect of treatment [ $F(1,40) = 3.50$ ,  $p = 0.07$ ], training [ $F(1,40) = 0.06$ ,  $p = 0.81$ ], or interaction [ $F(1,40) = 0.02$ ,  $p = 0.89$ ].

### Cg3 LIII Apical branch order



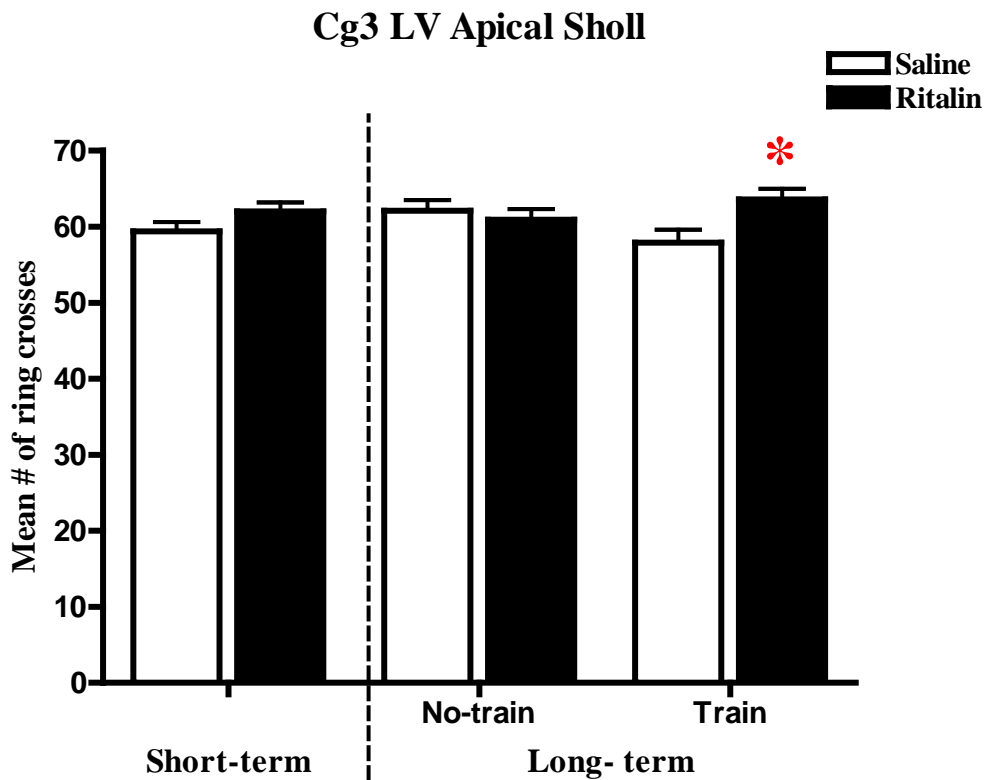
**Figure 3.1.9.** An illustration of apical branch order depicting the Ritalin-induced increase in trained rats relative to saline + Training. No other group differences were found.

#### *Cg3 LIII Sholl analyses.*

**Short-term.** Chronic Ritalin use in adulthood produced no significant changes in either apical (Figure 3.1.10) or basilar dendritic length (Sholl). ANOVA's of dendritic length showed no significant treatment effect in apical [ $F(1,15) = 2.52, p = 0.14$ ], or basilar [ $F(1,15) = 1.97, p = 0.18$ ].

**Long-term.** Training, selectively produced increases in apical dendritic length of Ritalin treated rats, whereas training selectively produced a trend towards a decrease in basilar dendritic length rats that received saline injections (Figure 3.1.10). An ANOVA of apical dendritic length (Sholl) with treatment and training as variables showed a significant interaction [ $F(1,40) = 5.72, p = 0.02$ ], with no significant effect of either treatment

[F(1,40) = 2.49, p = 0.12], or training [F(1,40) = 0.29, p = 0.59]. An ANOVA of basilar dendritic length (Sholl) with treatment and training as variables showed a strong trend towards an interaction [F(1,40) = 3.74, p = 0.060], but no significant main effect of treatment [F(1,40) ≤ 0.001, p = 0.99], or training [F(1,40) = 0.92, p = 0.34]. The pairwise comparison showed that the interaction was due to a significant increase in Ritalin + training rats relative to the untreated training group (p= 0.011), coupled with a trend towards a training induced decline in saline –treated rats (p = 0.06). The same was true for basilar dendritic length with a trend towards a training-induced decline in saline-treated rats (0.06).

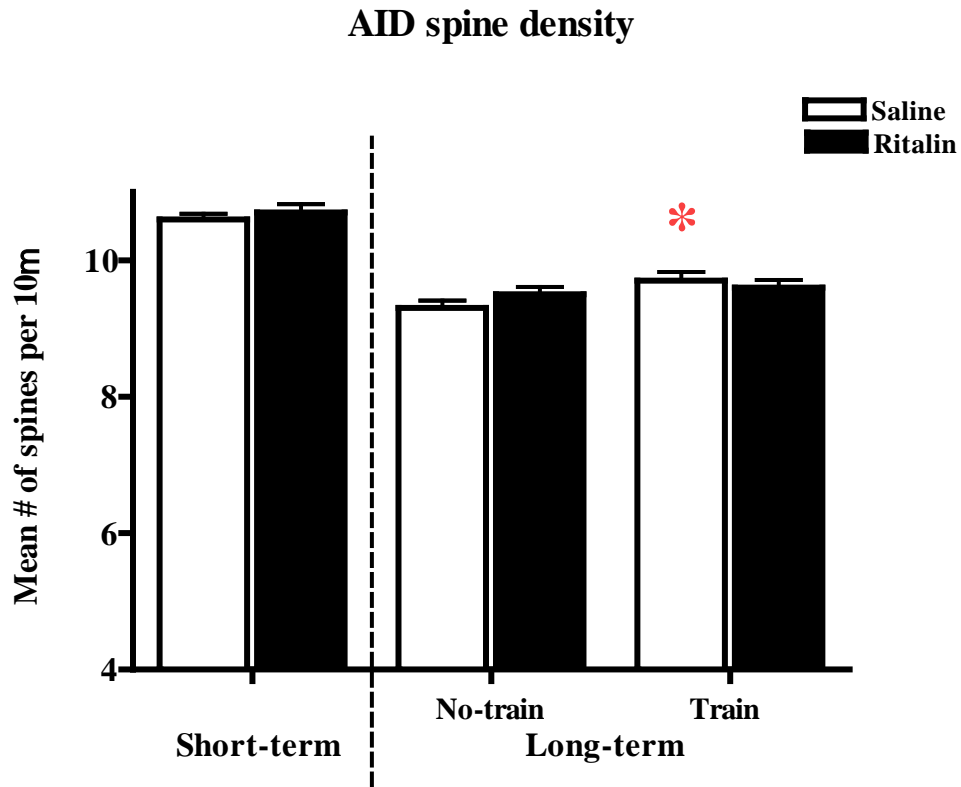


**Figure 3.1.10.** An illustration of the Ritalin-induced increase in apical dendritic length in rats that also underwent training.

**AID spine density.**

**Short-term.** As with all regions examined, Ritalin treatment had no apparent influence on AID spine density (Figure 3.1.11). An ANOVA of spine density showed no significant effect of treatment [ $F(1,15) = 0.27, p = 0.61$ ].

**Long-term.** Training, but not Ritalin increased basilar spine density in layer III neurons of the AID (Figure 3.1.11). An ANOVA of spine density with treatment and training as variables showed significant main effect of training [ $F(1,40) = 6.68, p = 0.014$ ], but not treatment [ $F(1,40) = 0.52, p = 0.47$ ], or the interaction [ $F(1,40) = 1.98, p = 0.17$ ].

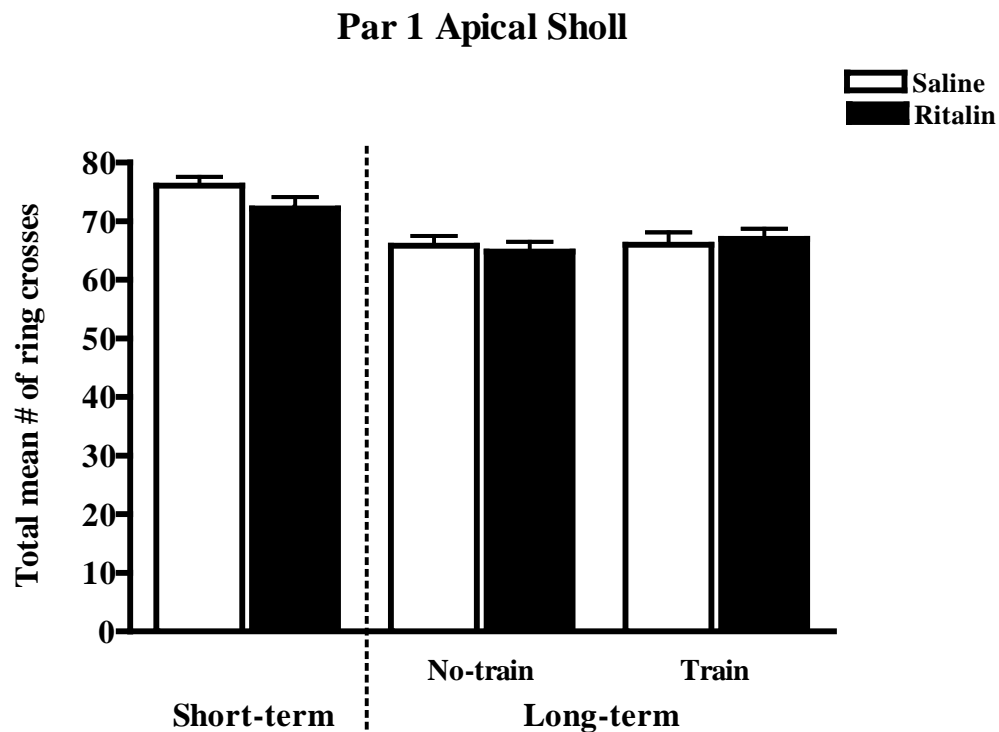


**Figure 3.1.11.** An illustration of a training-induced increase in AID spine density. There was no apparent influence of treatment.

**Par1 Layer III Sholl analyses**

**Short-term.** Chronic Ritalin use in adulthood produced no changes in apical or basilar dendritic length in the Par 1 (Figure 3.1.12). ANOVA's of apical and basilar dendritic length (Sholl) showed no effect of treatment on apical [ $F(1,15) = 2.5, p = 0.14$ ], or basilar [ $F(1,15) \leq 0.001, p = 0.99$ ].

**Long-term.** Treatment and training in adult rats produced no effects on Par 1 dendritic length (Figure 3.1.12). ANOVA's with treatment and training as variables showed no main effect of treatment [ $F(1,40) = 0.003, p = 0.96$ ], training [ $F(1,40) = 0.52, p = 0.48$ ], or interaction [ $F(1,40) = 0.35, p = 0.56$ ] in apical dendrites, nor was there a significant effect of treatment [ $F(1,40) = 0.06, p = 0.82$ ], training [ $F(1,40) = 0.16, p = 0.69$ ], or interaction [ $F(1,40) = 0.31, p = 0.58$ ] in basilar dendrites.



**Figure 3.1.12.** The bar graph illustrates that neither training nor treatment had any apparent influence on synaptic connectivity in layer III of the Par 1.

**Par 1 branch order.**

**Short-term.** Chronic Ritalin treatment produced no effects on apical or basilar branch order. ANOVA's of apical and basilar branch order showed no effect of treatment [F(1,15) = 1.20, p = 0.29] on apical or basilar dendrites [F(1,15) = 0.30, p = 0.59].

**Long-term.** Rats that had either been pretreated with Ritalin and/or had received training showed no effects of the experience(s) on Par 1 branch order. ANOVA's of apical and basilar branch order with treatment and training as variables showed no effect of treatment [F(1,40) = 0.23, p = 0.64], training [F(1,40) = 3.16, p = 0.08], or interaction [F(1,40) = 0.32, p = 0.58] on apical nor was there an main effect of treatment [F(1,40) = 0.66, p = 0.42], training [F(1,40) = 0.64, p = 0.43, interaction [F(1,40) = 1.32, p = 0.26].

**Summary of Experiments in Study Five**

	Basilar			Apical			Spine (basilar)	
	Cg3		Par1	Cg3		Par1	Cg3	OFC
Saline	LV	LIII	LIII	LV	LIII	LIII	LV	LIII
Train	∅	∅	∅	∅	i	∅	∅	h
<b>Ritalin</b>								
Short-term	∅	∅	∅	∅	∅	∅	∅	∅
Long-term								
No train	h	∅	∅	h	∅	∅	∅	∅
Training	∅	∅	∅	h	hh	∅	∅	∅

**Table 3.1.1.** Arrows indicate direction of dendritic changes relative to untreated counterparts. **Blue arrows = branch order; Red arrows = Sholl (length); Spine = spine density; ∅ = no change; Cg3 = region three of the cingulate gyrus; OFC (AID) = orbital prefrontal cortex; Par 1 = primary somatosensory cortex; LV = layer five neurons and LIII = layer three neurons.**

## **Discussion**

Psychostimulant abuse in adulthood generates structural plasticity in the PFC and striatum, produces behavioural sensitization and inhibits subsequent experience-induced plasticity. Whether or not therapeutic doses of psychostimulants, such as amphetamine and methylphenidate (Ritalin), used in the treatment of juvenile and adult ADHD initiate experience-dependent structural changes has not been determined.

In the current study we investigated the behavioural and anatomical effects of chronic MPD use in adulthood. The results of the behavioural tests indicate that therapeutic doses of Ritalin produce an overall increase in motor response during the treatment period. The effect is short-term however, as we found no influence of Ritalin pretreatment on challenges conducted 5 days or 60 days post-treatment. No drug-induced changes were found in either social interactions or cognitive performance in the T-maze.

Experience-induced plasticity occurred with both chronic drug exposure and training, although each experience produced a unique pattern of structural change. In addition, the combination of the two experiences produced their own pattern of altered connectivity in the PFC. No changes were found in the Par1 as consequence of either experience.

***Low-dose Ritalin exposure augments motor activity but fails to induce sensitization, cross-sensitization.***

The increased motor response to Ritalin over the drug-treatment period in the current study is indicative of the stimulatory actions of psychostimulants. The enhanced motor response was stable over much of the treatment period however, and did not promote cross-sensitization to either caffeine or amphetamine. Thus, low doses of Ritalin

were ineffective in producing behavioural sensitization, a phenomenon that is characteristic of repeated use of psychostimulants at high doses. In addition, based on the results of the drug challenges one could infer that the potential for later drug abuse was not potentiated by the previous drug experience.

***Repeated low-dose Ritalin use does not influence cognitive or social skills***

In contrast to studies that have found benefits in cognitive performance following acute (e.g. [31]), or relatively short-term (5 days ) daily psychostimulant exposure (e.g., [4,6]), the results of the T-maze task would suggest that the benefits of low-dose psychostimulant use on performance are not permanent. One of the main differences between these past studies and the current one is the timing between drug exposures and testing. For example, Arnsten and Dudley [4] assessed T-maze performance 30 minutes post-injection and Berridge and colleagues [6] conducted similar tests 20 minutes after oral administration. In the current study T-maze testing began about 5 weeks after the last Ritalin injection. Given that the task requirements were comparable, the contrasting outcomes may reflect differences in immediate versus delayed effects on cognition. If this is the case, then we can conclude that long-term Ritalin use at low doses does not permanently alter cognitive function, a finding that is in opposition to what occurs in the developing brain where we have found that early Ritalin use at these doses in periadolescent rats cause deficits in the T-maze in adulthood [9-11].

Leblanc-Duchin and Taukulis [23] have found that repeated use of high doses of Ritalin (10mg/kg) in adult rats significantly diminishes social interactions after a wash-out period. The effects of low-dose Ritalin use on social behaviour in adults have not been well studied, however. Owing to the apparent absence of Ritalin-induced social anomalies

in the current study, however, it would appear that there is a substantial difference between the effects of low and high doses of psychostimulants on social behaviour. One caveat in the social interaction assessment was the lack of a pre-treatment baseline to determine existing within group relationships. As it stands it is not possible to determine if dominant and subordinate relationships shifted. Further study should be conducted before drawing any definitive conclusions from the current study.

***Repeated low-dose Ritalin use and training differentially alters connectivity in the PFC***

In the current study we found increases in layer V Cg3 neurons, a finding that is similar to the results of previous studies on the structural effects of psychostimulant use in adult rats in which increases in Cg3 LV neurons were also found (e.g.,[12,28,29]). It is quite remarkable that Ritalin, at a dose that is at least half of that used in most other studies with psychostimulants such as amphetamine [20,28,29] would be sufficient to produce such changes. The effects of Ritalin on PFC neurons in the current study were not widespread however, as we found no apparent changes in layer III pyramidal cells of the Cg3 or AID.

In contrast, the influence of training alone on structural plasticity in the PFC was evident in basilar dendrites of the AID and layer III, but not layer V, Cg3 pyramidal cells. Of special interest here is the fact that there were opposing effects on the AID and Cg3, in that whereas training produced an increase in AID synaptic connectivity, it produced a decrease in layer III of the Cg3. What makes this finding so intriguing is that Kolb and colleagues have repeatedly found opposing experience-induced structural changes in these PFC regions (for review [21]). Further, the opposing effects found here with training are exactly opposite to what is found in cases of psychostimulant use, where there is an

increase in the Cg3 region and a decrease in the OFC. The results here are similar however, to the structural changes that occurred during bar press training where the control animal received a sucrose reward rather than a drug-reward [12], in that there was a training/sucrose increase in AID spine density but no effect in Cg3 layer V spines. The current data then may fit very well with the hypothesis proposed by the authors, experience differentially affects regions of the PFC.

The opposing effects of psychostimulants and training may also help to explain the unique pattern of structural changes that occurred with the combination of training and Ritalin treatment in the current study. For example, whereas Ritalin alone produced an increase in Cg3 LV neurons, the addition of training appeared to reverse the drug effect, producing a decrease rather than increase in these neurons. As well, whereas training alone decreased layer III Cg3 neurons, the combination of the two experiences again produced opposite effects, increasing the dendritic fields of Cg3 neurons.

Together these results indicate that psychostimulant use at low doses produces a moderate, short-term motor response with limited potential for later drug abuse and has little effect on cognitive and social behaviour. The modest behavioural effects are reflected in the limited drug-induced structural changes in the PFC that are partially reversed by experience-dependent plasticity through training. Further, the unique pattern of structural-plasticity as a consequence of Ritalin treatment and training suggest that the effects of chronic psychostimulant use, at least at therapeutic doses, can be altered through experience-induced plasticity, as well as vice versa. How these experiences interact at the synapse is not known but it would appear that they may be working through different mechanisms that potentially counteract one another.

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## **I. Learning-induced alterations in prefrontal cortical circuitry**

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## **Abstract**

In the current set of studies we investigated the effects of various learning paradigms (Complex housing, T-maze task, Grice box task, and extinction/no extinction in a fear conditioning task) on dendritic morphology of the prefrontal cortex (PFC), as well as primary somatosensory cortex (Par 1). Comparisons were made between control (naïve) animals housed in standard lab cages and animals housed for 4, 8, or 16 days in complex environments. In the formal training tasks both naive and yoked animals were used. In the fear conditioning paradigm, comparisons were made between naïve controls and two groups of animals that underwent fear conditioning, with/without the addition of extinction learning. The animals were sacrificed within 24 hours of training completion and the brains harvested and prepared for later anatomical analysis using the Golgi-Cox procedure. The results demonstrated that experience in all paradigms significantly altered PFC circuitry, albeit differently. Whereas complex housing produced decreases in the dendritic fields of Layer (L) V Cg3 neurons that was time dependent (at 4 days but not at 16 days exposure), yoked animals in the T-maze and Grice box training, and all animals in the fear conditioning paradigm showed increases in this regional layer. A similar result was found in LIII of the AID (Grice box data missing), although in the opposite direction. Thus, one consistent finding was that experience produced opposing changes in layer V Cg3 and layer III of the AID, increasing one while decreasing the other. These results indicate that PFC circuitry is altered by experiential factors but the changes may be transient, evident only after short exposure periods in complex environments, or in animals sacrificed immediately after training completion.

## **Introduction**

Experience-induced changes in cortical dendritic morphology have been well documented. Greenough and colleagues were some of the first to show experience-induced alterations in neuronal structure (e.g.[12,33]), although the original work by Hebb [14], in which he reported enhanced cognitive performance in rats raised in enriched conditions, and the Berkley group (e.g.[2,7]) in the 1960's, that showed experience-induced changes in gross brain anatomy, were equally formative in demonstrating that the brain could be altered by experience.

Complex housing is one of the most widely studied models of experience-dependent structural plasticity, repeatedly producing robust changes in neuronal morphology in the visual and sensory cortex in the developing and the mature (e.g.,[9,20,32]) brain, and alterations in subcortical structures such as the nucleus accumbens (NAc) in adults [13,21] as well. Experience-related alterations in structural morphology are not restricted to complex environments, however. Training-induced changes have also been found in the occipital cortex of animals trained in visual spatial tasks (e.g.,[10]) and the motor cortex (e.g., [11]) following reach training, the piriform (olfactory) cortex [16] with odor discrimination training, as well as in the cerebellar cortex [3] following motor learning but not exercise. Paramount in these and subsequent studies is that they suggest the existence of a direct relationship between structure and function.

Given the role that the PFC plays in learning and executive functions, in addition to its extensive capacity for reorganization and change as demonstrated in lesion studies (see [19]) and response to psychostimulant use [30,31], it would seem reasonable to

expect that similar learning-related changes would also occur. Yet, surprisingly there are only two known studies that have examined structural changes in pyramidal neurons of the prefrontal cortical (PFC) [21,24]. In the first, Kolb and colleagues [21] found no changes in dendritic arborization of layer V PFC neurons in rats following 3 – 3.5 months of complex housing. In contrast, Kozorovitskiy and colleagues [24] found that complex housing increased the dendritic field of L III pyramidal PFC neurons in primates after only 30 days of complex housing. The two main differences between these studies are 1) the layer in which measures were taken (LV vs LIII, respectively), and 2) the duration of the experience.

Studies of complex housing have varied in duration, ranging from 4 – 90+ days (e.g., [20,34], with all reporting effects of differential housing on dendritic morphology. These measures, however, were taken from occipital and parietal cortices, and may not be representative of changes that occur in the PFC. Alternatively, it may be that learning-related changes in the PFC are specific to layer III and unrelated to duration of the experience. It also remains to be determined if learning-induced changes in the PFC occur under various learning paradigms.

To investigate, we examined the effects of learning on dendritic morphology of the PFC and sensory cortex using four types of learning paradigms that are known to employ PFC circuitry as well as having the potential to initiate structural changes in the sensory (Par 1) cortex. The four paradigms used were complex housing with varying durations, T-maze and Grice box training, and fear acquisition/extinction. In addition, layers III and V of the PFC were examined.

## **Materials and Methods**

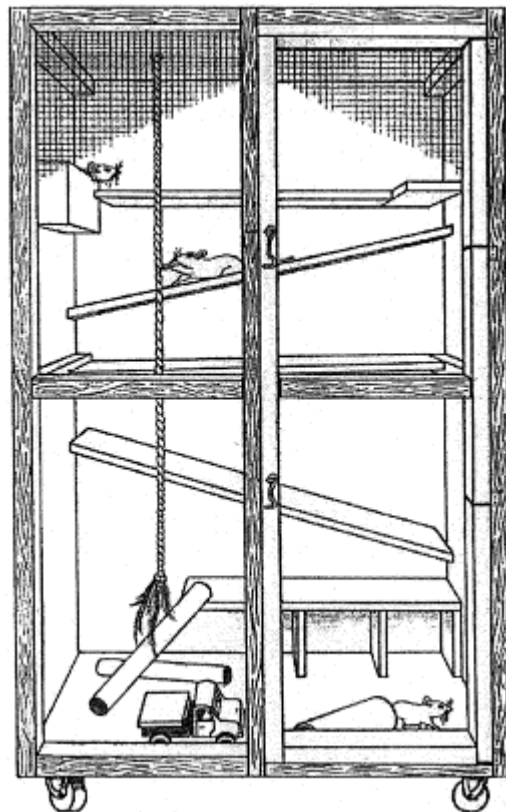
**Subjects.** Forty-nine male, Long-Evans rats aged 3 months old (about postnatal (P) 90) at the start of each experiment were used in the current set of four studies. The animals were pair-housed in standard, hanging, plexi-glass cages and maintained on a 12:12 hour light/dark cycle.

In the first experiment (Experiment a), eighteen adult rats aged P90 – P92 were randomly assigned to one of four housing conditions: 1) lab-housed (N= 5), 2) 4-day complex- housing (ENR) (N=4), 8-day ENR (N=4), and 16-day ENR (N= 5). In the second study (Experiment b) sixteen rats (P90 – P92) were assigned to one of three groups. One group formed the non-handled controls, a second group were trained in the T-maze task (N= 7), and a third group (N=4) received comparable handling and exposure to the T-maze but non-contingent rewards. In the third experiment (Experiment c), one group of rats were trained on a spatial task (N=5), a second group (cage mates of the trained group) participated without training (N=5), whereas a third group were left undisturbed (N=5). In a fourth experiment (Experiment d) the animals participated in a fear conditioning paradigm in which one group underwent extinction (N= 6), whereas the other fear-conditioned group did not (N=4). A third group of rats did not participate and were left undisturbed (N=5).

### ***Testing procedures***

***Experiment a - Complex-housing.*** As adults, rats assigned to the complex groups were housed in large structures with numerous objects for periods of 4, 8, or 16 days, whereas the lab-control group remained in standard lab-cages. The complex-housing structure

consisted of a large (61 X 122 X 183 cm) enclosure (Figure 4.1.1). The sides and front of the structure were made of heavy wire mesh and the back vertical wall was made from galvanized steel. Three platforms were attached to the back wall at varying heights with wooden ramps that provided access to each level. The floor of the structure was covered with corn-cob bedding and objects that included plastic ‘toys’, paper, cardboard boxes, and PVC pipe, were strewn over the floor and the platforms to encourage exploration. The enclosures were cleaned weekly and new objects were introduced to maximize exploration. Lab cages were the standard plexi-glass hanging tubs (39 X 57 X 21 cm) with corn-cob bedding covering the floor, but without addition objects. All animals in the study were given ad libitum access to food and water.



**Figure 4.1.1.** Schematic illustration of ‘condo’ used to house animals in the complex housing condition (picture from Kolb, Gibb & Gorny [20]).

**Experiment b – T-maze training.** In this task animals were food deprived to 95% of body weight prior to testing and maintained at this weight throughout the experiment. Adult rats that were assigned to either the trained or yoked groups were first habituated to the T-maze apparatus prior to the actual training phase. Once habituated, the animals were taught to run to the end of the arms of the apparatus to obtain a food reward (piece of fruit loop). All subjects were rewarded regardless of the arm they chose during the training phase. The testing phase began once all of the rats readily entered the arms to retrieve a reward. In the testing phase, ‘trained’ rats were required to learn a non-match to sample paradigm of the T-maze task (similar to that used by Bartolini and colleagues [1]). In short, each trial consisted of two components. In the first, one arm was blocked forcing the subject into the open arm. In the second component the block was removed and the rat was reintroduced to the maze (10 sec. delay). To retrieve a reward, the rat had to choose the arm opposite of where the reward had just been obtained (component one). Subsequent trials began about 30 seconds after the end of the second component. Each subject was given 10 consecutive trials per day. A correct response on at least 8 out of 10 trials for four consecutive days was required to reach criterion. The yoked controls were also repeatedly placed in the T-maze apparatus for the first 4 days of the training phase and rewarded regardless of which arm they entered. Thereafter, the yoked rats were simply placed into the maze and allowed to explore and retrieve treats that strewn about the maze.

**Experiment c – Grice-box training** All rats were placed on a restricted food schedule and maintained at about 95% of original body weight for the duration of the experiment. Animals were trained in a spatial reversal paradigm using a Grice box (parallel alley

maze) apparatus. The apparatus consisted of a start box (entrance) measuring 8 1/2 " in length that opened to a trapezoid decision area (12" in length) leading to two parallel alleys measuring 22" in length with food well located at the farthest end of each alley. All walls, including the outer and separation wall were 12 inches in height. Both the trained and yoked rats were acclimated to the apparatus for two days prior to beginning the pre-training phase. In short, on day 1 of the pre-training phase both alleys were baited and the animals were individually placed into the start alley and allowed to retrieve the reward (piece of fruit-loop) from both alleys before being removed. On day 2 and 3 the animals were allowed to retrieve a reward in the alley of their choice after which time they were removed and the procedure repeated (ten trials per day). If the animal chose the same alley twice, the alley was blocked on the third trial to force entry into both alleys. On day 4, the training phase began. The 'correct' alley for individual rats in the trained group was determined (the alley opposite to the last entry of the final trial of pre-training phase) and baited, whereas the incorrect choice was left unbaited. Each animal was given 10 non-correction trials a day with intertrial intervals of 10 seconds until they reached criterion (eight correct entries in a single session). Once criterion was reached the 'correct' alley was reversed and the procedure repeated as above until the rats once again reached criterion. All animals in the trained group underwent 4 reversal trials, whereas the yoked controls continued to be rewarded in the alley of their choice (see [22] for detailed description). Within 24 hours of reaching criterion on the final reversal the animals were sacrificed along with their yoked cage mate.

***Experiment d –Extinction of fear conditioning.*** The protocol used for the extinction of fear conditioning task can be found in detail in Quirk [27]. In summary, the rats were

taken to a testing room and individually placed in conditioning chambers. Each animal was given a habituation trial (tone alone) followed by seven trials of foot shock (scrambled 0.6 mA shock with 0.5s duration) /tone (4 kHz sine wave at 80 dB with 30 second duration) pairings spaced about 4 minutes apart. Three hours after conditioning all the rats were returned to the chambers and half (extinction group) were given 15 extinction trials consisting of tone only, whereas the other half received no tone (non-extinction group). On the following day all rats received 15 extinction trials with tone only. All rats were perfused immediately following the last extinction trial.

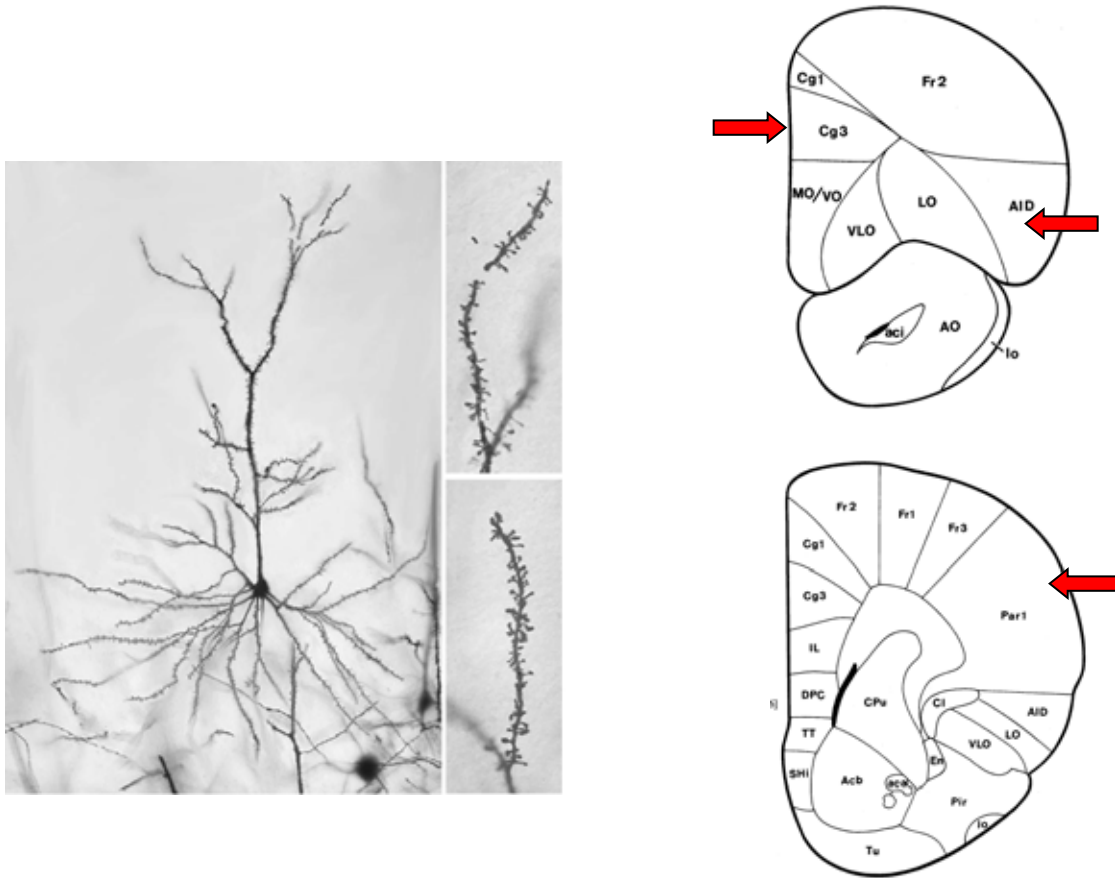
***Tissue preparation.*** All of the subjects from the above studies were intracardially perfused with 0.9% saline solution and the brains harvested and post-fixed in Golgi-Cox solution for 14 days before being transferred to a 30% sucrose solution for an additional 4 days. Once the brains were removed from the sucrose solution they were cut into 200  $\mu$ m sections using a vibratome and placed on gelatin-dipped slides. The slides were stored in the dark for a minimum 24 hours before being processed using the revised Golgi procedure outlined in Gibb and Kolb[8].

***Assessment of dendritic morphology.*** Neurons from select regions of the prefrontal and somatosensory cortex were used to assess experience-induced plasticity based on dendritic morphology. The cells included in the current study were layer III pyramidal cells (e.g., Figure 4.1.2, left) of the primary somatosensory cortex (Par 1), layer III and V of region three of the anterior cingulate cortex (Zilles Cg3), as well as layer III pyramidal

neurons of the dorsal agranular insular region of the orbital frontal cortex (AID) described by Zilles [36].

To be included in the analyses cells had to meet the following criterion: 1) all chosen cells needed to be thoroughly impregnated by the Golgi-Cox solution, with dendritic trees that were clearly visible at 100X and not obscured by blood vessels, neighboring processes or debris, and 2) relatively intact with minimal breakage. The cells were drawn using camera lucida (200X) and quantified using analyses of dendritic branch order (number of branch bifurcations), and Sholl (number of branches that crossed an overlay of concentric rings spaced 20  $\mu\text{m}$  apart). The means from five neurons in each hemisphere were calculated and used for later comparisons.

Spine density was also determined for fourth-order terminals of basilar dendritic trees in Cg3 LV and AID LIII. As with dendritic branch order and Sholl, spine density for each hemisphere was determined by calculating the mean of five terminals (10  $\mu\text{m}$  in length). Spines were drawn at 1000X (see Figure 4.1.2, center) and included all spine protrusions that had a visible head. It was recognized that this method of evaluation is an underestimation of the total spine density as those spines on both the bottom and top of the dendrite would not be visible to the experimenter (see[21] for additional details). Cells within each region were drawn by experimenters blind to the groups until analysis.



**Figure 4.1.2. Left:** A photograph of a layer III pyramidal cell (200X). **Center:** A photograph illustrating a segment of basilar dendrite (1000X magnification) from which dendritic spine data were obtained. **Right:** Top and bottom. Illustration of regions included (revised from Zilles [36]).

## ***Results.***

***General observations.*** Animals housed in the complex-enclosures (Experiment a) were active and freely moved about their environment, exploring all levels of the enclosure.

The rats in the formal training experiments (Experiments b-d) learned the tasks without any obvious difficulties, with all animals successfully reaching criterion.

***Dendritic morphology.***

***Experiment a- Complex housing.*** In summary, complex housing produced opposing effects on Cg3 and AID dendritic morphology, decreasing the dendritic field of Cg3 pyramidal neurons while increasing the dendritic field of AID neurons. Further, the influence of housing condition on Cg3 LV and AID neuronal dendrites was time dependent with changes occurring within the first 4 days that for the most part diminished with continued enrichment. Complex-induced dendritic changes in the sensory cortex (Par 1) on the other hand, were only apparent in animals housed for 16 days, with no obvious changes with either the 4 or 8 day exposure periods.

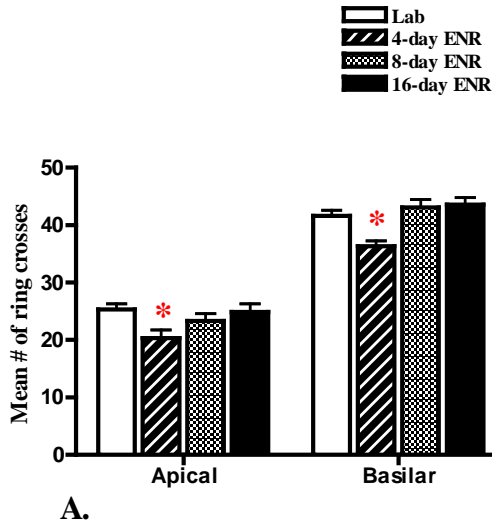
***Cg3 LV branch order and Sholl analyses.*** Complex housing produced an initial decrease in apical and basilar branch order as illustrated by the 4-day housing condition (Figure 4.1.3a & b). The effect however, was transient as layer V Cg3 neurons in rats housed for 8 or 16 days showed no apparent effect of housing. ANOVA's of branch order showed a significant effect of housing on apical [ $F(3,25) = 3.23, p = 0.04$ ] and basilar [ $F(3,29) = 8.12, p \leq 0.001$ ] branch order. The LSD post hoc analysis revealed that the effect of housing on apical branch order was due to a significant decrease in day 4 animals relative to controls ( $p = 0.009$ ) and day 16 animals ( $p = 0.02$ ). Day 8 and day 16 rats did not significantly differ from controls ( $p$ 's  $> 0.30$ ). The post hoc revealed similar results for basilar branch order with day 4 rats having significantly lower branch orders than all other groups ( $p$ 's  $\leq 0.002$ ) but no other group differences ( $p$ 's  $> 0.20$ ).

The Sholl analyses of dendritic length on the other hand showed no significant effect of housing on apical [ $F(3,26) = 1.28, p = 0.30$ ] or basilar [ $F(3,29) = 1.10, p = 0.37$ ] dendritic length.

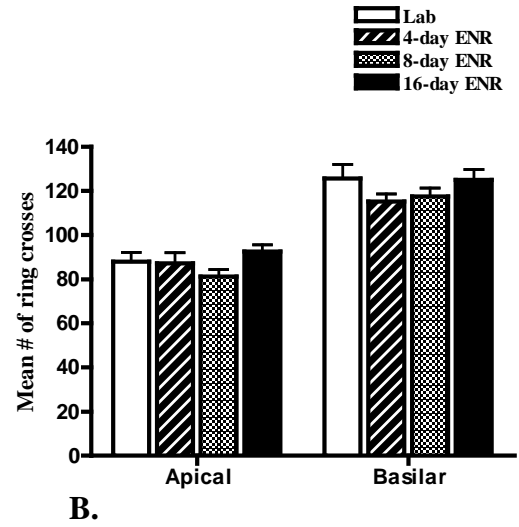
***Cg3 LIII branch order and Sholl analyses.*** In contrast to layer V Cg3 neurons, the effect of complex housing on layer III neurons did not appear to be transient (Figure 4.1.3 c & d). In the case of branch order, the effect appeared to be potentiated over time with the day 4 group showing the least amount of decline of the three complex housing groups. Dendritic length (Sholl) on the other hand appeared to be affected equally among the complex groups with all showing a significant decrease relative to lab-housed animals. ANOVA's of branch order showed a significant effect of housing on apical [ $F(3,32) = 5.75, p = 0.003$ ] and basilar [ $F(3,32) = 7.75, p = 0.001$ ] dendritic fields. The LSD post hoc analyses revealed that all groups had significantly fewer bifurcations on apical branches ( $p$ 's  $< 0.05$ ) with the exception of the day 4 group which showed only a trend ( $p = 0.06$ ) relative to controls. Basilar branch order was significantly lower in all groups ( $p$ 's  $< 0.05$ ) relative to controls.

The Sholl analyses showed very similar results with ANOVA's revealing a significant reduction in the length of apical [ $F(3,32) = 3.90, p = 0.018$ ] and basilar [ $F(3,32) = 4.38, p = 0.011$ ] dendrites with complex housing. The LSD showed the effects were evident in all complex groups (all  $p$ 's  $< 0.05$ ).

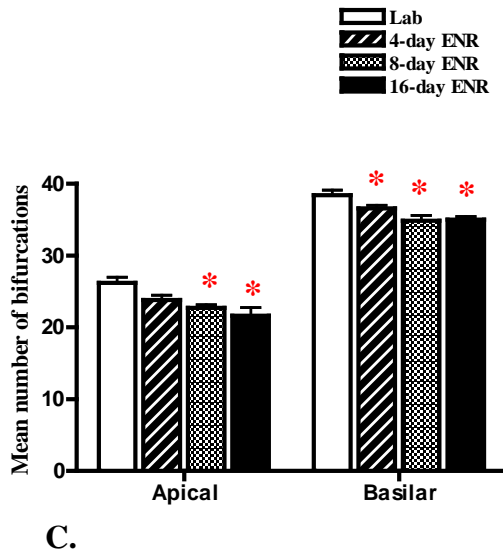
### Cg3 LV Branch Order



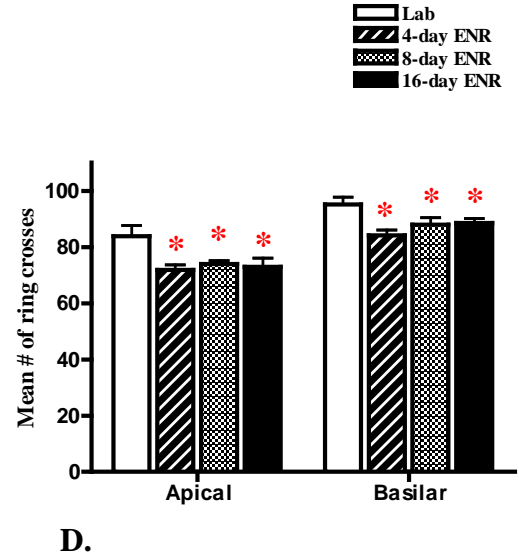
### Cg3 LV Sholl



### Cg3 LIII Branch Order



### Cg3 LIII Sholl



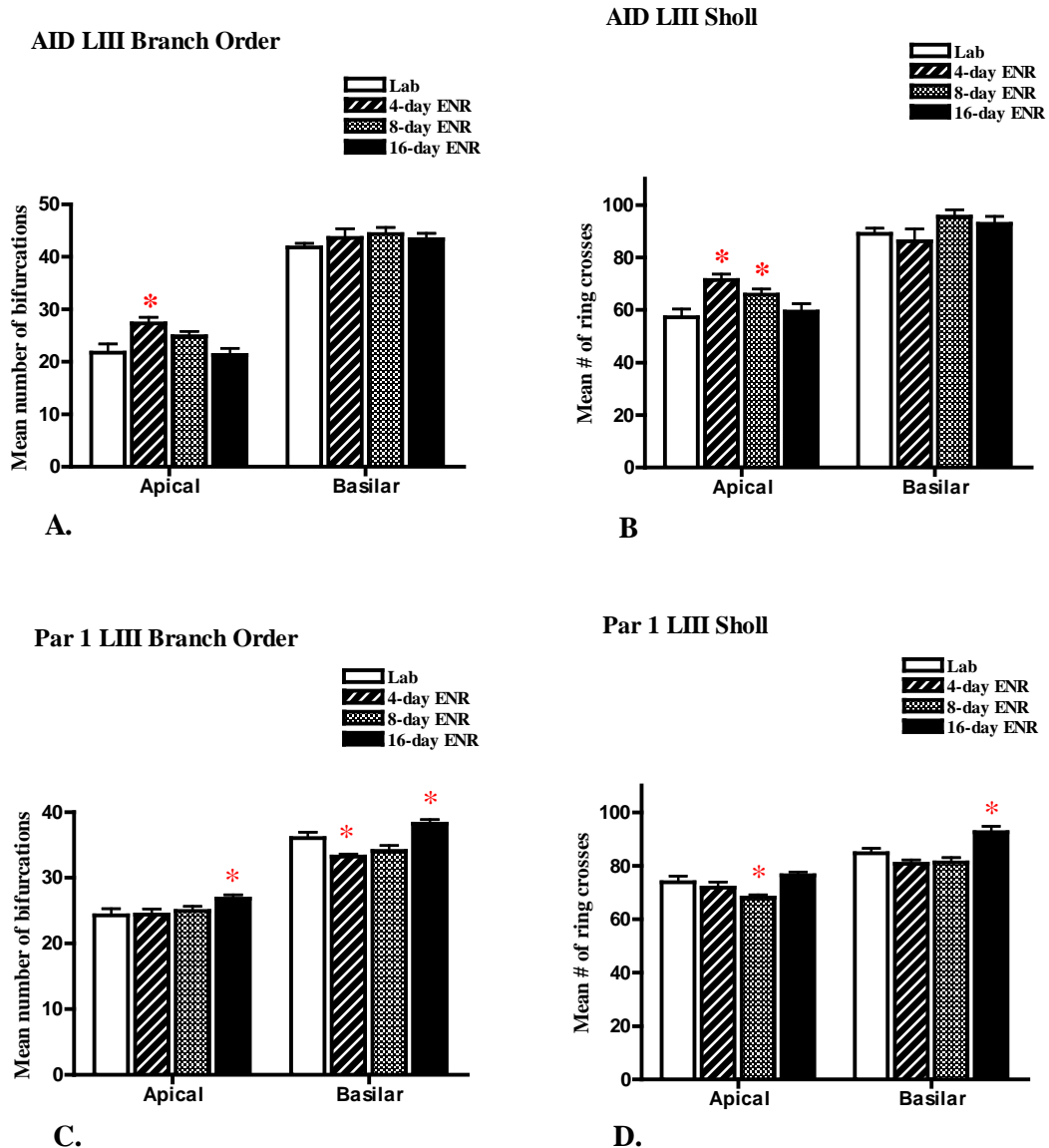
**Figure 4.1.3.** The mean number of branch bifurcations and Sholl (dendritic length) for layer V (A & B) and III (C & D) of the Cg3. Complex housing reduced the dendritic field in layers V & III, although the effects on layer V neurons were transient.

***AID L III branch order and Sholl analyses.*** Complex housing produced a transient increase in apical, but not basilar, dendritic fields in layer III AID pyramidal neurons (Figure 4.1.4a & b). ANOVA's of branch order showed a significant effect of housing on apical [ $F(3,29) = 3.74, p = 0.02$ ] but not basilar [ $F(3,29) = 0.92, p = 0.44$ ] dendrites (Figure 4a). The LSD post hoc analysis revealed that apical branching in the 4 day complex housing group was increased relative to day 16 and lab-housed animals ( $p$ 's < 0.01), with no other group differences found ( $p$ 's > 0.05).

The results of the Sholl analyses were similar with enrichment producing an increase in apical [ $F(3,29) = 4.64, p = 0.009$ ], but not basilar [ $F(3,29) = 1.84, p = 0.16$ ] dendritic length (Figure 4b). The LSD post hoc analyses showed the effect on apical length was due to a significant increase in day 4 and day 8 rats relative to lab-housed controls ( $p = 0.002$ ;  $p = 0.03$ , respectively) but no difference was found between day 16 and lab-housed controls ( $p = 0.57$ ).

***Par 1 LIII branch order and Sholl analyses.*** Complex housing produced a time-dependent increase in apical branch order, as well as basilar branch order and dendritic length (Figure 4.1.4c & d). Only those rats housed for 16 days in the complex enclosure exhibited increases in the dendritic fields of layer III neurons of the Par 1. ANOVA's of branch order showed a significant effect of housing on basilar [ $F(3,32) = 9.49, p < 0.001$ ] but not apical branch order [ $F(3,32) = 2.22, p = 0.11$ ]. Post hoc analyses however revealed that the absence of an complex effect on apical branch order was due to the fact that only the day 16 ( $p = 0.03$ ) but not the day 4 or day 8 groups showed an effect ( $p = 0.92$ ;  $p = 0.58$ , respectively). In contrast, whereas day 4 enrichment produced a decrease in basilar branch order ( $p = 0.01$ ), day 16 enrichment produced an increase ( $p = 0.03$ ).

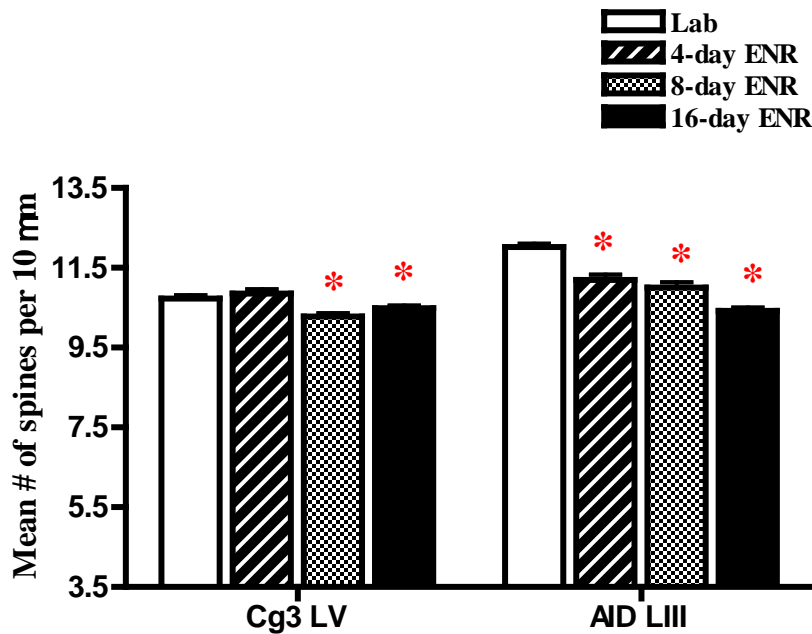
The Sholl analyses showed an effect of housing on apical dendritic length [ $F(3,32) = 3.75, p = 0.02$ ] that was due to a significant decrease in day 8 rats ( $p = 0.030$ , although no other group differences ( $p$ 's  $> 0.05$ ). In contrast, there was a significant effect of housing on basilar branch order [ $F(3,32) = 8.76, p < 0.001$ ] that was due solely to an increase in day 16 housing relative to lab-housed controls ( $p = 0.004$ ).



**Figure 4.1.4.** The mean number of branch bifurcations and Sholl (dendritic length) for layer III AID (A & B) and Par 1 (C & D) pyramidal neurons. Complex housing time-dependently increased the dendritic field in both the AID and Par 1.

**Cg3 LV and AID LIII basilar spine density.** Complex housing decreased spine density in both the Cg3 and AID (Figure 4.1.5). ANOVA's of basilar spine density showed a significant effect of housing on Cg3 [ $F(3,32) = 9.05, p < 0.001$ ], and AID [ $F(3,32) = 44.71, p < 0.001$ ] spine density.

### Basilar spines

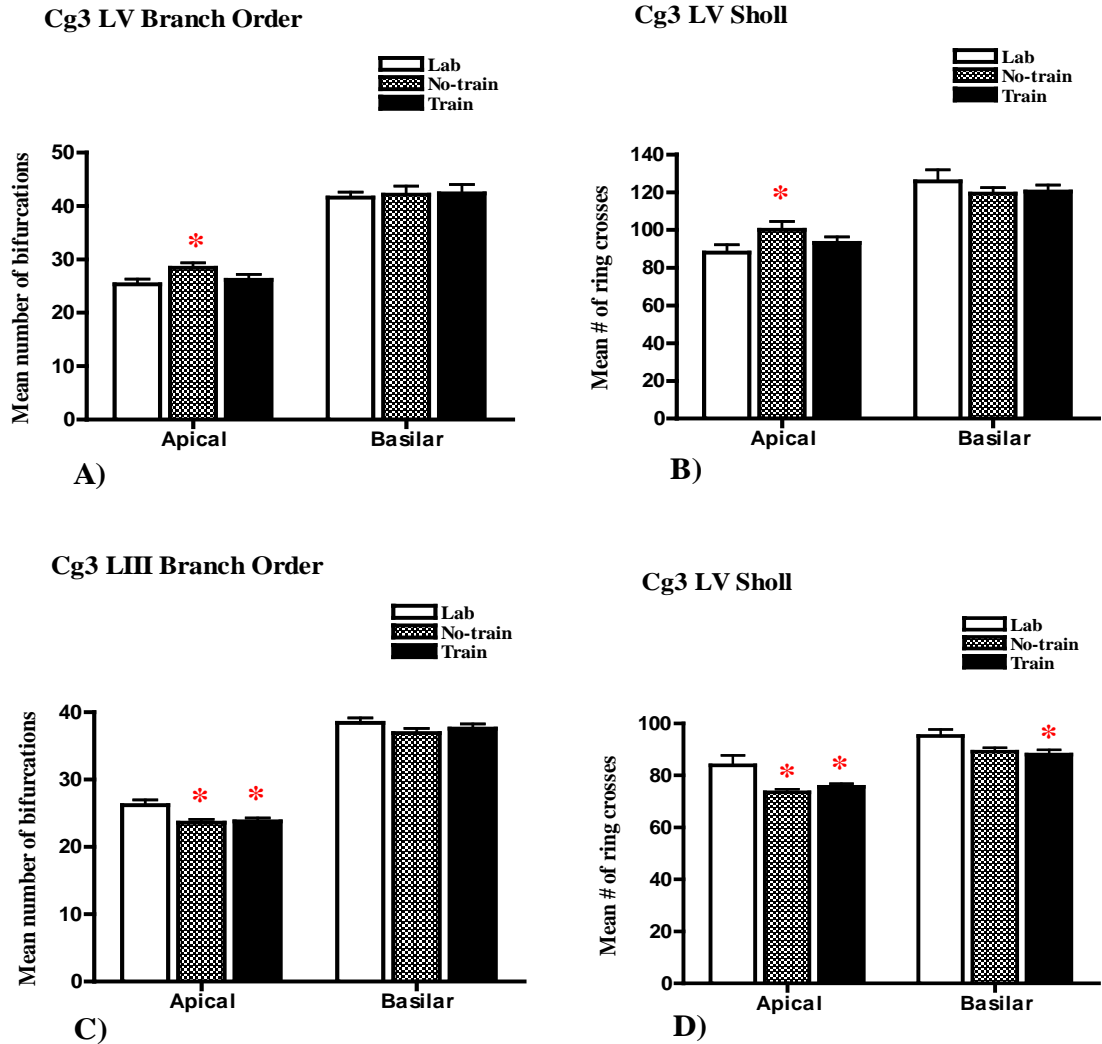


**Figure 4.1.5.** Complex housing produced a decrease in basilar spine density in both the Cg3 and AID.

**Experiment b- T-maze training.** Overall, formal training in the T-maze did not produce distinct alterations in cortical circuitry. The effects of experience on PFC neurons produced a general decline in the dendritic arborization of both trained and yoked control rats, whereas there was an increase in Par 1 neurons. The exception was Cg3 layer V neurons where there were no apparent effects of training although the yoked rats exhibited an increase in the apical dendritic field of pyramidal neurons.

***Cg3 LV branch order and Sholl analyses.*** The apical dendritic fields of Cg3 LV neurons were increased in yoked controls only, with no apparent effect of formal training on either branch order or dendritic length (Figure 4.1.6a & b). ANOVA's of branch order showed no significant effect of experience on apical [ $F(2,19) = 2.28, p = 0.13$ ], or basilar [ $F(2,26) = 0.08, p = 0.92$ ] dendrites. The post hoc however, revealed a marginal increase in apical branching of yoked controls ( $p = 0.05$ ). Similar results were found in the Sholl analysis. ANOVA's of dendritic length (Sholl) showed no significant effect of experience on apical [ $F(2,18) = 2.13, p = 0.15$ ], or basilar [ $F(2,27) = 0.55, p = 0.59$ ] dendrites. As with branch order, the post hoc revealed a marginal increase in apical dendritic length in yoked controls ( $p = 0.05$ ).

***Cg3 LIII branch order and Sholl analyses.*** In contrast to layer V of the Cg3, both training and yoked rats showed a decrease in apical dendritic fields of Cg3 LIII neurons, although T-maze training alone resulted in an additional decrease in basilar dendritic length as well (Figure 4.1.6c & d). ANOVA's of branch order showed a significant effect of experience on apical [ $F(2,29) = 5.59, p = 0.009$ ], but not basilar [ $F(2,29) = 0.87, p = 0.43$ ] dendrites. ANOVA's of dendritic length also showed a significant effect of experience on apical [ $F(2,29) = 5.14, p = 0.012$ ], as well as basilar [ $F(2,29) = 3.36, p = 0.05$ ] dendrites. The post hoc analyses revealed that although apical dendritic length was decreased in both trained and yoked controls, T-maze training alone produced a decrease in basilar dendritic length ( $p = 0.02$ ).



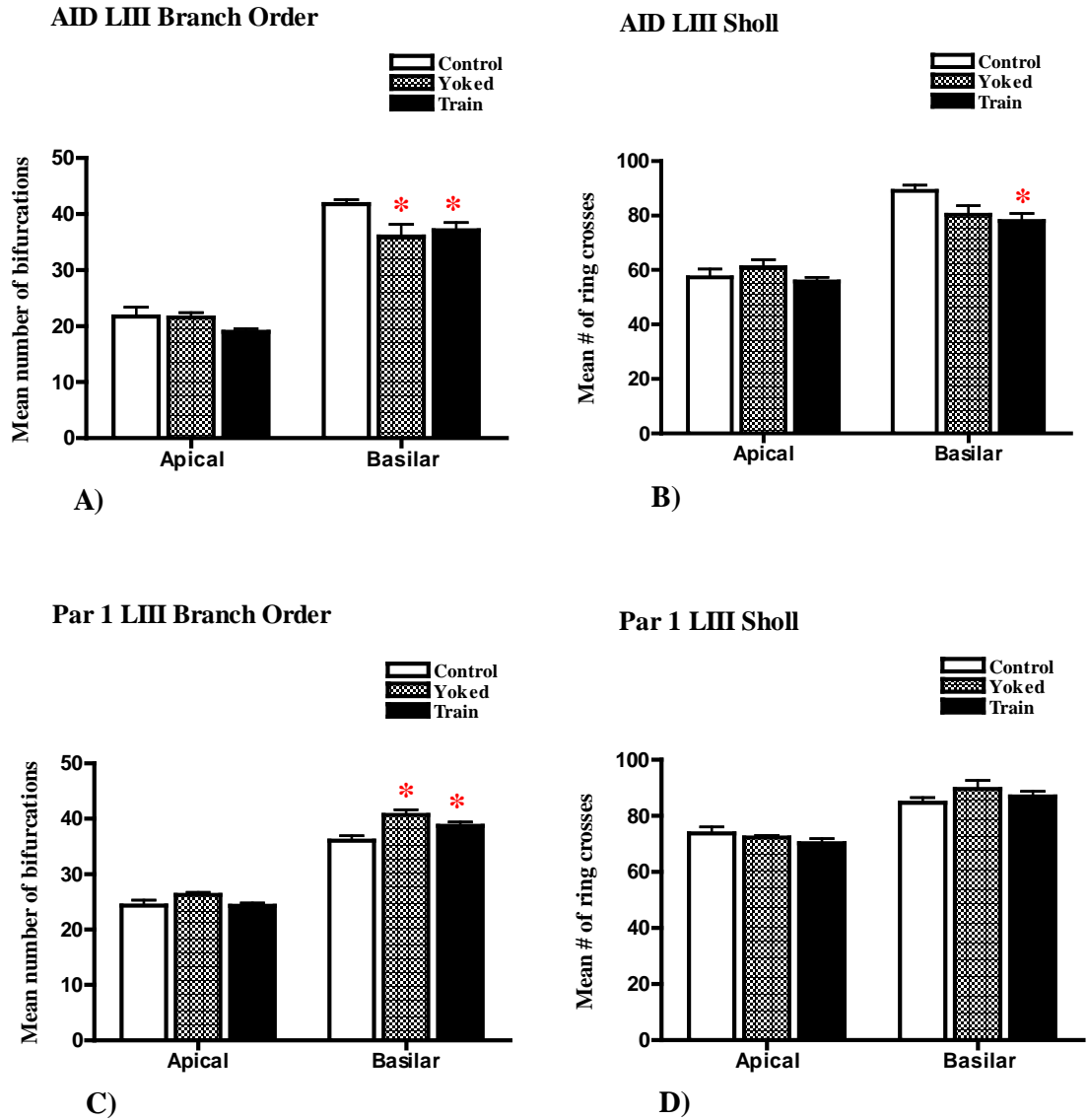
**Figure 4.1.6.** Graphs reveal that yoked animals showed significant increases in apical branch order (a) and Sholl analysis (b) in Cg3 LV pyramidal neurons. In contrast, both trained and yoked rats showed a significant decrease in apical branch order (c) and Sholl analysis (d) in Cg3 LIII neurons. Further, training alone altered basilar dendritic fields although the effect was limited to LIII Cg3 neurons (d).

***AID LIII branch order and Sholl analyses.*** Both trained and yoked controls exhibited a decrease in AID basilar branch order but only the trained rats also showed a decrease in basilar dendritic length (Figure 4.1.7a & b). Neither group showed any changes in apical dendritic fields. ANOVA's of branch order showed a significant effect of experience in basilar [ $F(2,28) = 4.13, p = 0.03$ ], but not apical [ $F(2,28) = 2.14, p = 0.14$ ] dendritic arborization. ANOVA's of dendritic length (Sholl) showed a similar effect with a significant decrease in basilar [ $F(2,28) = 4.63, p = 0.02$ ], but not apical [ $F(2,28) = 1.13, p = 0.34$ ] dendritic length. The LSD post hoc analysis revealed that only trained rats had a significant decrease in basilar length relative to both the control group ( $p = 0.006$ ). No other group differences were apparent ( $p$ 's  $> 0.05$ ).

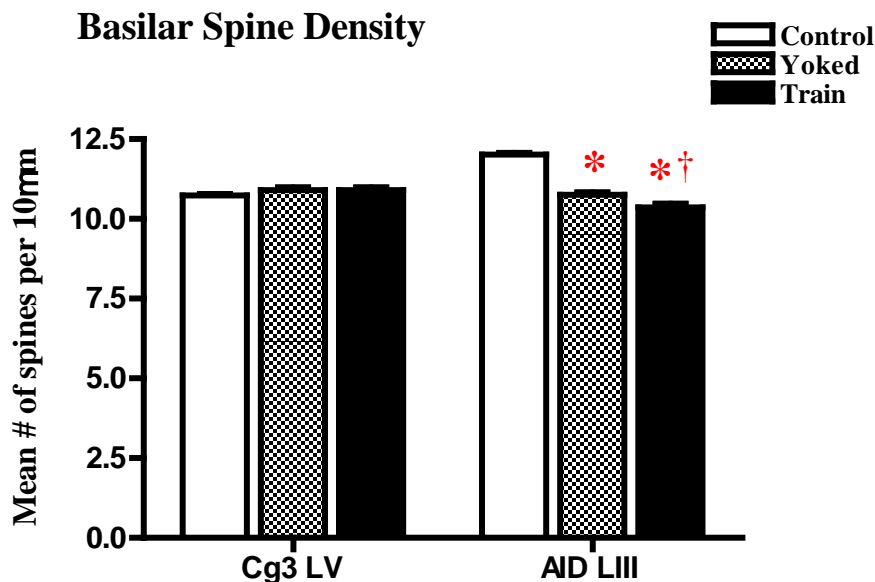
***Par 1 LIII branch order and Sholl analyses.*** Basilar branch order was increased in both trained and yoked rats (Figure 4.1.7c) but neither group showed any apparent alterations in Par 1 dendritic length (Figure 4.1.7d). ANOVA's showed a significant experience-induced increase in basilar [ $F(2,29) = 6.60, p = 0.004$ ], but not apical [ $F(2,29) = 2.29, p = 0.12$ ] branch order, whereas there were no apparent alterations in apical [ $F(2,29) = 1.00, p = 0.38$ ] nor basilar [ $F(2,29) = 1.08, p = 0.35$ ] dendritic length.

***Cg3 LV and AID LIII basilar spine density.*** Neither trained nor yoked rats showed any experience-induced changes in Cg3 LV basilar spine density (Figure 4.1.8). Both groups however, showed increased spine density in basilar dendrites of AID layer III pyramidal neurons relative to controls. An ANOVA of Cg3 spine density showed no differences among the groups [ $F(2,27) = 0.84, p = 0.44$ ]. An ANOVA of AID spine density on the

other hand showed significant group differences [ $F(2,29) = 48.72, p < 0.001$ ]. The post hoc revealed that basilar spine density was significantly reduced relative to both control and yoked groups ( $p < 0.001$ ;  $p = 0.04$ , respectively).



**Figure 4.1.7.** The graphs illustrate that both trained and yoked rats had significant decreases in basilar dendritic fields (a) of AID neurons, although training alone also produced a decrease in basilar dendritic length (b). In contrast, trained and yoked rats showed increases in basilar dendritic branching (c) in Par 1 pyramidal neurons but no significant changes in dendritic length (d).



**Figure 4.1.8.** An illustration of the decrease in AID LIII, but not Cg3 LV, basilar spine density in trained and yoked rats. The effect was most prominent in trained rats however, as the reduction was relative to both controls and yoked groups.

*Experiment c – Grice box training.* In summary, the anatomical results for trained and yoked rats in the Grice box task were very similar and showed regionally-specific alterations in PFC circuitry as a consequence of the experience, more so than the formal training itself. Whereas we found increases in Cg3 LV basilar branching in yoked rats, there were widespread decreases in basilar as well as apical dendritic branching and length in Cg3 LIII neurons in both trained and yoked rats relative to controls. Spine density was also decreased in basilar dendrites of layer III AID and layer V Cg3 neurons.

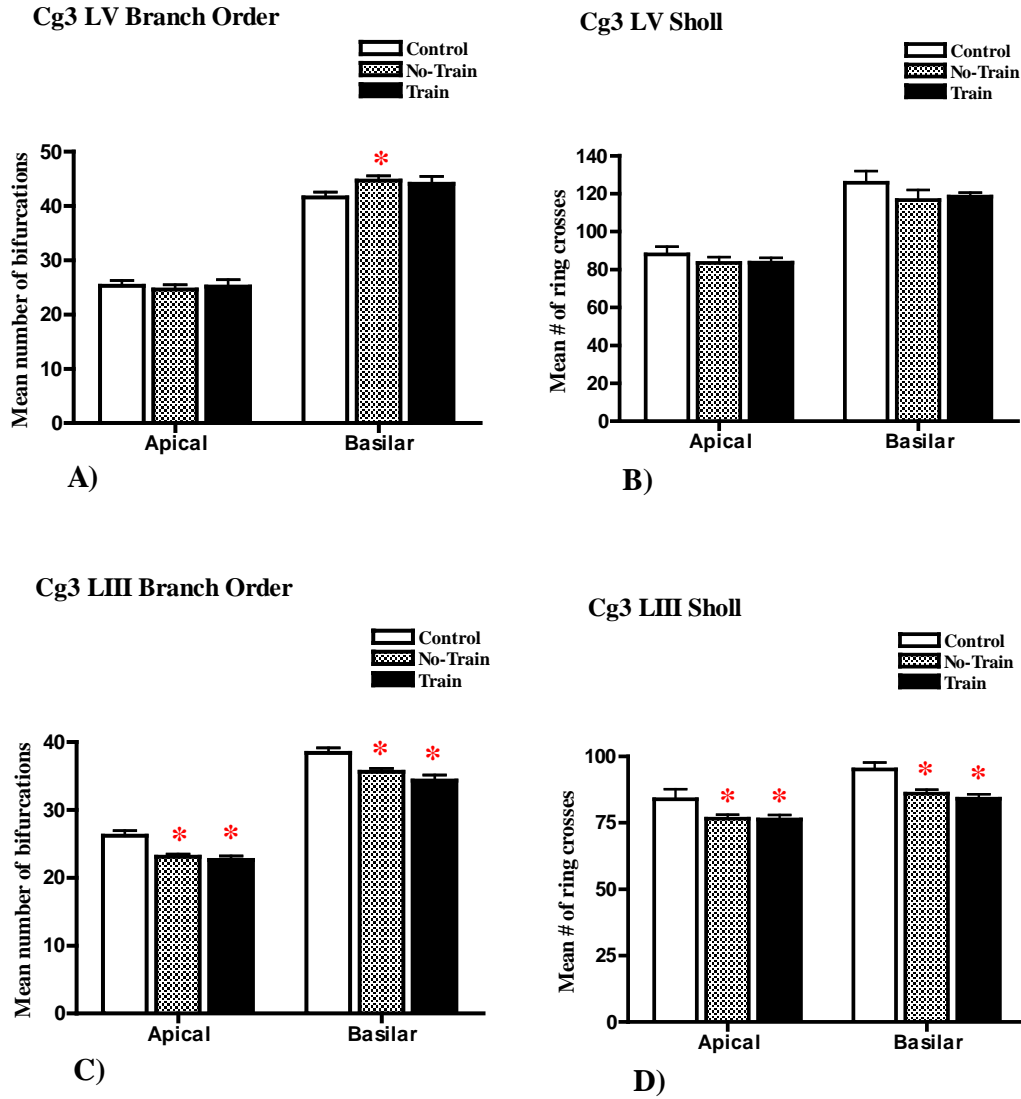
*Cg3 LV branch order and Sholl analyses.* The Grice box task produced only limited changes in Cg3 LV neurons. In fact, the only apparent effects on branch order and length were found in yoked rats, which showed an increase in basilar branching relative to

controls (Figure 4.1.9a & b). There were no group differences between trained and yoked rats, however. ANOVA's of branch order showed no significant effect of experience on apical [ $F(2,20) = 0.14, p = 0.87$ ], nor basilar [ $F(2,25) = 2.44, p = 0.11$ ] branching, although the post hoc revealed a moderate increase in basilar dendritic branching of yoked rats relative to controls ( $p = 0.05$ ), but no other group differences were found ( $p$ 's  $> 0.10$ ). ANOVA's of dendritic length (Sholl) also showed no significant effects of experience on apical [ $F(2,20) = 0.56, p = 0.58$ ], nor basilar [ $F(2,24) = 0.86, p = 0.43$ ] dendritic length.

***Cg3 LIII branch order and Sholl analyses.*** Unlike layer V neurons, Cg3 layer III neurons showed prominent, widespread, decreases in dendritic fields as a result of the Grice box experience (Figure 4.1.9c & d). ANOVA's of branch order show significant decreases in apical [ $F(2,27) = 10.65, p < 0.001$ ], and basilar [ $F(2,27) = 8.50, p = 0.001$ ] branch bifurcations as a consequence of experience. There were no differences between trained and yoked rats in either apical or basilar branch order ( $p = 0.59; p = 0.21$ , respectively). Similarly, ANOVA's of dendritic length showed a significant effect of experience on basilar [ $F(2,27) = 8.83, p = 0.001$ ] but only a trend on apical [ $F(2,27) = 2.90, p = 0.07$ ]. The LSD post hoc analyses however, revealed that although there were no differences between trained and yoked rats on either basilar or apical dendritic length ( $p = 0.48; p = 0.95$ , respectively), both groups were significantly decreased relative to controls ( $p$ 's  $\leq 0.05$ ).

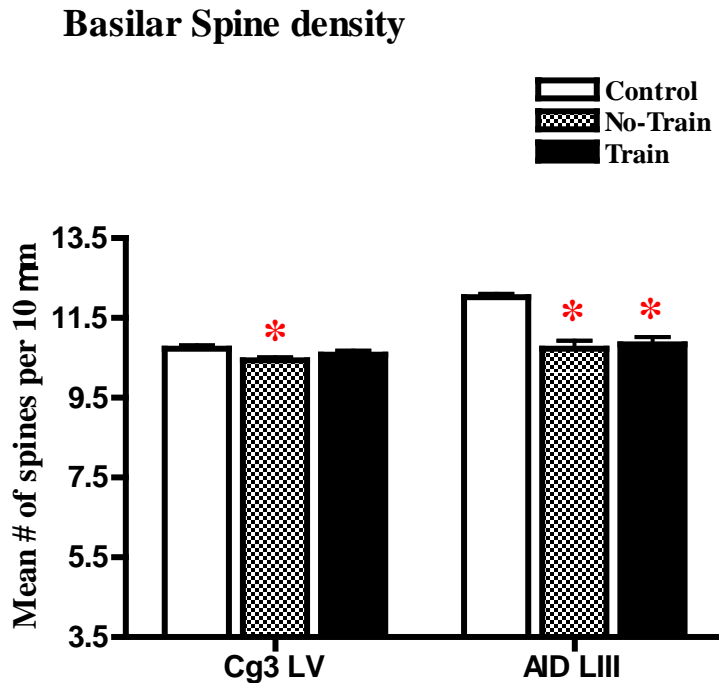
***Par 1 LIII branch order and Sholl analyses.*** Neither the trained nor yoked rats showed any apparent changes in dendritic morphology in Par 1 L III pyramidal neurons.

ANOVA's of branch order showed no effect of experience on apical [ $F(2,27) = 0.68, p = 0.51$ ] or basilar [ $F(2,27) = 0.09, p = 0.91$ ] branching. Similarly, ANOVA's of dendritic length showed no significant group differences in apical [ $F(2,27) = 0.17, p = 0.85$ ] or basilar [ $F(2,27) = 0.78, p = 0.47$ ] dendrites.



**Figure 4.1.9.** An illustration of the increase in Cg3 LV basilar branch order (a) in yoked rats, but no changes in Cg3 LV dendritic length (b). Layer III Cg3 neurons, on the other hand showed a decrease in both apical and basilar branch order (c) and length (d) in trained and yoked controls.

**Cg3 LV and AID LIII basilar spine density.** The effects of experience on basilar spine density were similar among trained and yoked rats (Figure 4.1.10). An ANOVA of Cg3 LV spine density showed no significant effect of experience [ $F(2,27) = 2.96, p = 0.069$ ], although the LSD post hoc revealed that yoked rats had reduced density relative to controls ( $p = 0.02$ ) but not in relation to the trained rats ( $p = 0.24$ ). In contrast, an ANOVA of AID LIII spine density showed a significant effect of experience [ $F(2,27) = 19.58, p < 0.001$ ] that was due to reduced density in both trained and yoked rats relative to controls ( $p$ 's  $< 0.001$ ).



**Figure 4.1.10.** An illustration of the experience-induced decline in both Cg3 LV and AID LIII basilar spine density.

**Experiment d- fear conditioning and extinction.** Extinction of fear conditioning produced changes in PFC circuitry that were for the most part limited to the basilar dendrites with the exception of a decrease in apical dendritic branching in Cg3 LIII

neurons. Fear conditioning in the absence of extinction on the other hand did not appear to produce such selective alterations in circuitry. In either case, the direction of change was opposite in layer V compared to layer III neurons, except in the instance of spine density in which there was a decrease in both Cg3 LV and AID LIII basilar spine density.

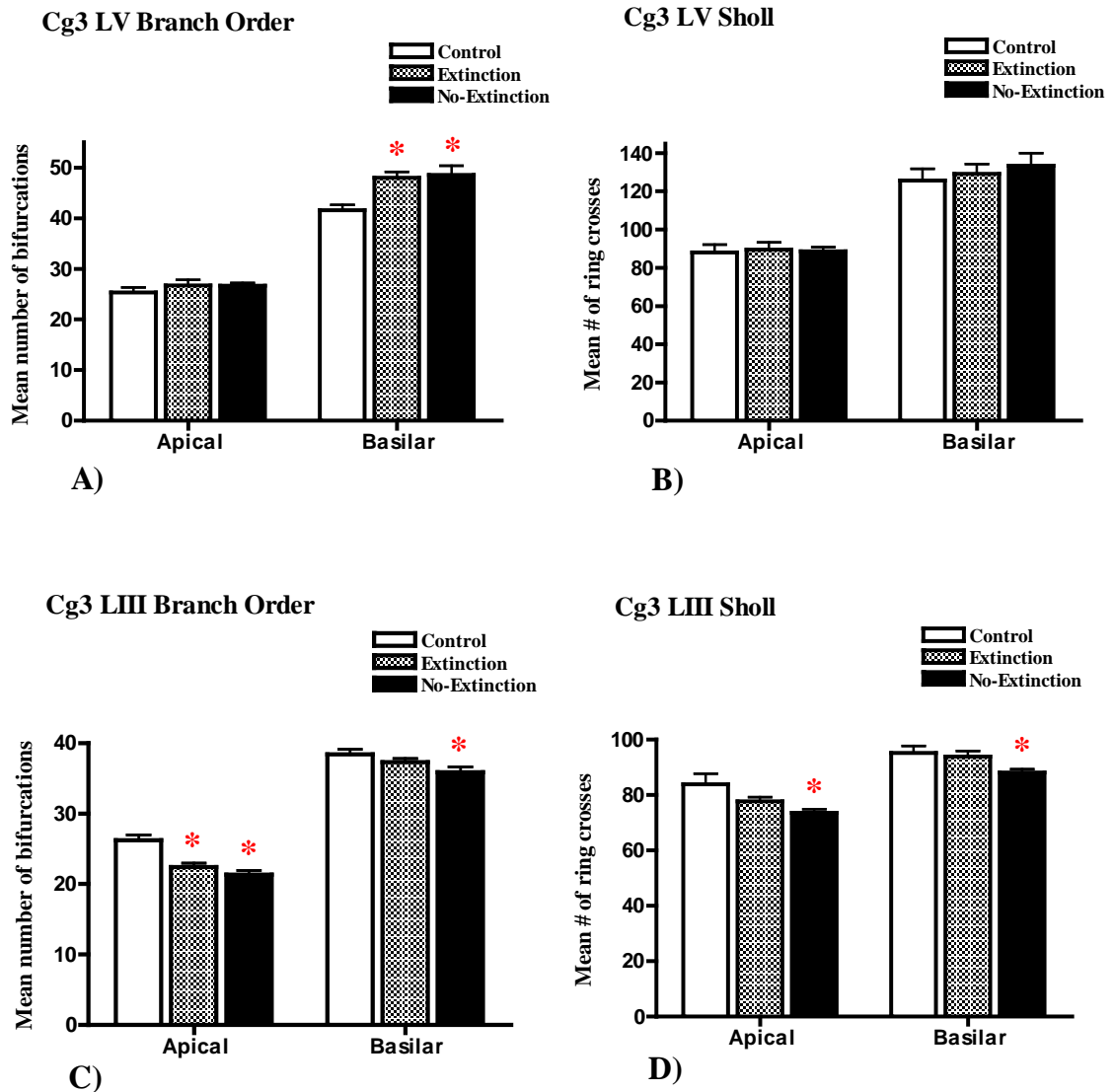
***Cg3 LV branch order and Sholl analyses.*** Fear conditioning irrespective of extinction, produced an increase in basilar Cg3 LV branch order relative to control animals (Figure 4.1.11 a & b). No differences were found between the extinction and no-extinction group. An ANOVA of branch order showed a significant effect of experience [ $F(2,26) = 8.59$ ,  $p = 0.001$ ], but not apical [ $F(2,18) = 0.64$ ,  $p = 0.54$ ] branch order. ANOVA's of dendritic length revealed no significant effect of experience on basilar [ $F(2,18) = 0.04$ ,  $p = 0.96$ ], nor apical [ $F(2,26) = 0.36$ ,  $p = 0.70$ ] dendritic length.

***Cg3 LIII branch order and Sholl analyses.*** In contrast to layer V neurons, fear conditioning in the absence of extinction produced decreases in both apical and basilar branch order (Figure 4.1.11c) as well as dendritic length (Figure 4.1.11d). Extinction however, produced a decline in apical branch order but did not influence basilar branch order or dendritic length. ANOVA's of branch order showed a significant effect of experience on apical [ $F(2,27) = 14.58$ ,  $p < 0.001$ ], and basilar [ $F(2,27) = 3.45$ ,  $p < 0.05$ ] branch order. In the latter case, the LSD post hoc showed that the no-extinction group but not the extinction group showed significant decreases in basilar branching ( $p = 0.01$ ;  $p = 0.23$ , respectively). The results of the Sholl show a significant effect of experience on apical [ $F(2,27) = 3.88$ ,  $p = 0.03$ ], but not basilar [ $F(2,27) = 2.72$ ,  $p = 0.08$ ] dendritic length. The LSD post hoc comparisons however, revealed that the no-extinction groups alone showed decreases in apical and basilar length relative to controls ( $p = 0.01$ ;  $p =$

0.04, respectively) whereas, there were no group differences between the extinction group and controls ( $p$ 's  $> 0.08$ ).

***AID LIII branch order and Sholl analyses.*** Fear conditioning produced a decrease in AID layer III apical and basilar branch order in the no-extinction group whereas the extinction group showed a decline in basilar dendritic branching only (Figure 4.1.12a). Neither condition produced any apparent changes in dendritic length, however (Figure 4.1.12b). ANOVA's of branch order showed a significant effect of experience on apical [ $F(2,23) = 3.59, p = 0.04$ ] and basilar [ $F(2,23) = 19.85, p < 0.001$ ]. Although both the extinction and no-extinction group exhibited a decline in basilar branch order ( $p$ 's  $< 0.001$ ), the effect on apical branch order was significant in the no-extinction, but not the extinction group ( $p = 0.02$ ;  $p = 0.08$ , respectively). The Sholl analyses revealed no apparent influence of experience on apical [ $F(2,23) = 0.01, p = 0.99$ ], or basilar [ $F(2,23) = 0.84, p = 0.45$ ] dendritic length.

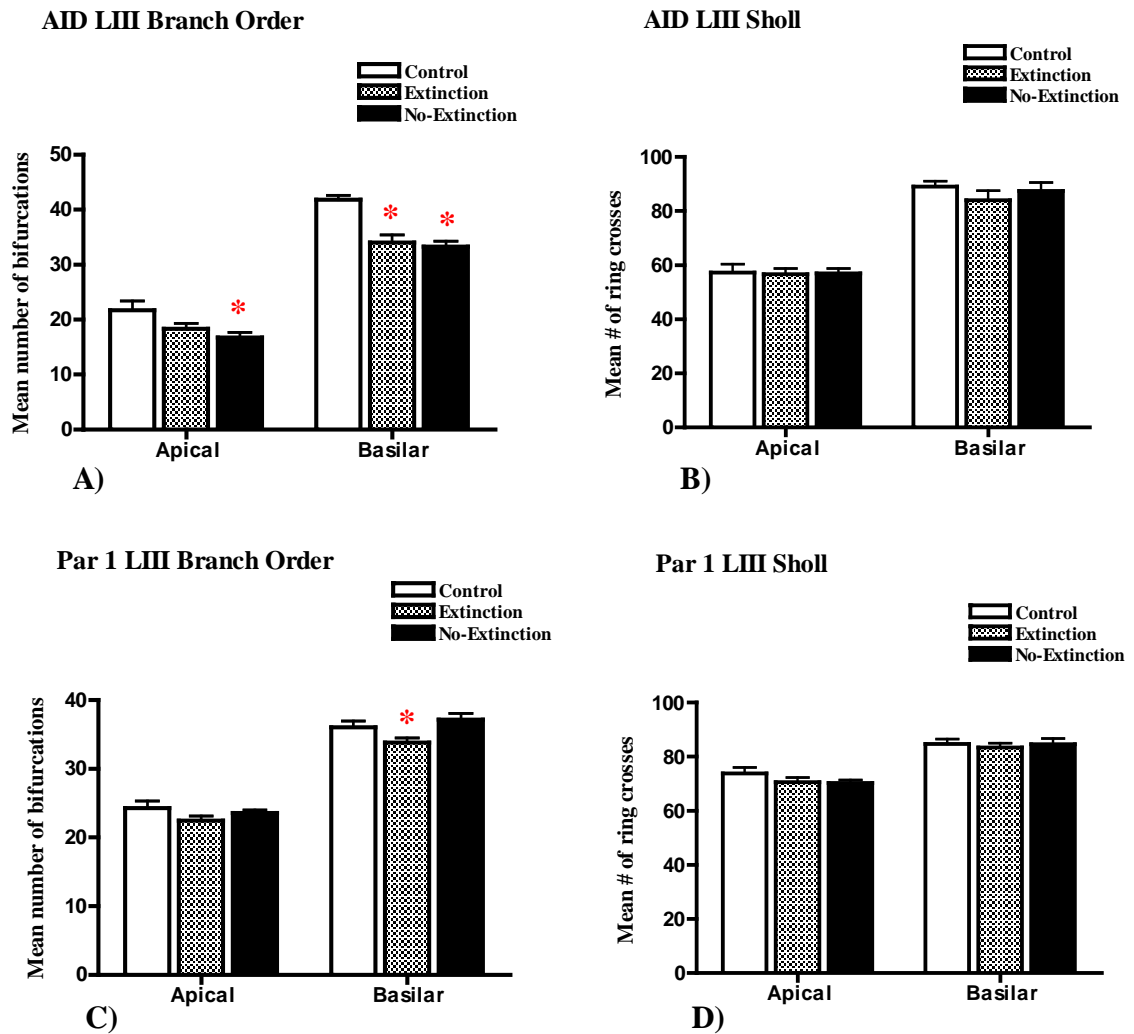
***Par 1 LIII branch order and Sholl analyses.*** Extinction of fear conditioning produced a decrease in basilar branch order in the Par 1 (Figure 4.1.12c). No other differences in branch order or dendritic length were found between groups (Figure 4.1.12d). ANOVA's of branch order showed a significant effect of experience on basilar [ $F(2,27) = 4.41, p = 0.02$ ], but not apical [ $F(2,27) = 1.64, p = 0.21$ ] branch order. The post hoc analysis revealed that the extinction group had a significantly lower mean basilar branch order relative to the no-extinction and control groups ( $p = 0.009$ ;  $p = 0.05$ , respectively). The Sholl analyses showed no group differences in either apical [ $F(2,27) = 1.07, p = 0.36$ ], or basilar [ $F(2,27) = 0.15, p = 0.86$ ] dendritic length.



**Figure 4.1.11.** Fear conditioning, under both extinction and no-extinction paradigms increased basilar branching in Cg3 LV pyramidal neurons (a) but had no apparent effect on dendritic length (b). In contrast, although both extinction and no-extinction decreased apical branching in layer III Cg3 neurons (c), only the no-extinction group decreased basilar branching (c) and length, as well as apical length (d) of layer III cells.

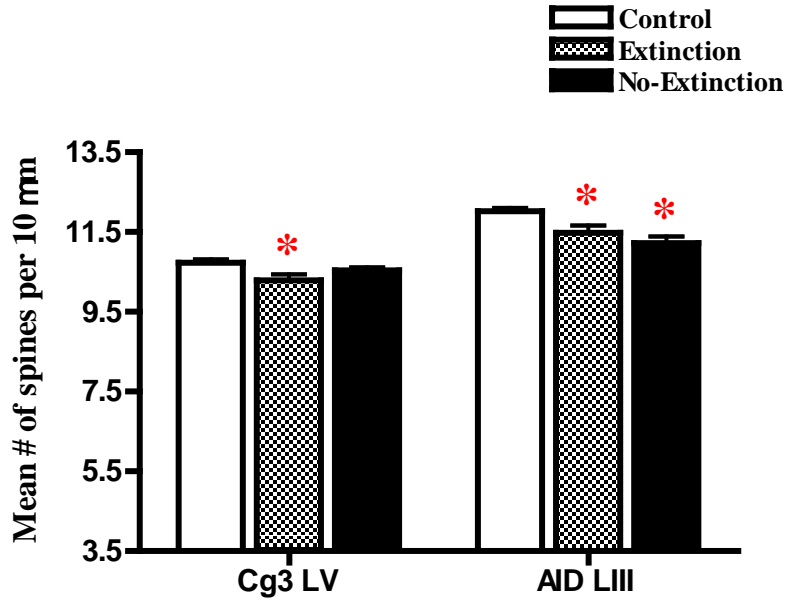
**Cg3 LV and AID LIII basilar spine density.** Fear extinction produced a reduction in Cg3 LV and AID LIII basilar spines, whereas the no-extinction group showed only a decrease in AID LIII spines (Figure 4.1.13). An ANOVA of Cg3 LV spines showed a significant effect of experience on spine density [ $F(2,27) = 3.87, p = 0.03$ ]. The post hoc revealed

that the effect was significant only in the extinction group relative to controls ( $p = 0.01$ ). An ANOVA of AID LIII also showed a significant effect of experience on basilar spine density [ $F(2,27) = 6.31, p = 0.006$ ]. In contrast to Cg3 LV spine density, both the extinction and no-extinction group had significantly lower mean spine density on basilar dendrites of AID LIII neurons relative to controls ( $p = 0.02$ ;  $p = 0.002$ , respectively).



**Figure 4.1.12.** Fear conditioning, under both extinction and no-extinction paradigms decreased basilar branching in AID LIII pyramidal neurons (a), but the no-extinction group alone also decreased apical branching. Neither condition influence dendritic length in the AID (b). Aside from a decrease in basilar branching of Par 1 neurons in the extinction group (c), fear extinction and no-extinction did not influence dendritic arborization in layer III Par 1 cells (d).

### Basilar spine density



**Figure 4.1.13.** Although the fear extinction group displayed a decrease in both Cg3 LV and AID LIII basilar spine density, fear conditioning with no extinction decreased only AID basilar spine density.

## Summary

Owing to the sheer volume of anatomical data in the current set of studies, a summary table was created to highlight the findings (Table 4.1.1).

	Cg3 LV			Cg3 LIH		AID LIH			Par 1	
	Branch	Length	Spine	Branch	Length	Branch	Length	Spine	Branch	Length
<b>Enriched</b>	Ü A,B	—	—	ÜB	ÜA,B	ÜA	ÜA	Ü	ÜB	—
4										
8	—	—	Ü	ÜA,B	ÜA,B	ÜA	ÜA	Ü	—	ÜA
16	—	—	Ü	ÜA,B	ÜA,B	—	—	Ü	ÜA,B	ÜB
<b>T-maze</b>										
train	—	—	—	ÜA	ÜA,B	ÜB	ÜB	Ü	ÜB	—
yoked	ÜA	ÜA	—	ÜA	ÜA	ÜB	—	Ü	ÜB	—
<b>Grice</b>										
train	—	—	—	ÜA,B	ÜA,B	n/a	n/a	Ü	—	—
yoked	ÜB	—	Ü	ÜA,B	ÜA,B	n/a	n/a	Ü	—	—
<b>Fear</b>										
extinction	ÜB	—	Ü	ÜA	—	ÜB	—	Ü	ÜB	—
no-extinction	ÜB	—	—	ÜA,B	ÜA,B	ÜA,B	—	Ü	—	—

**Table 4.1.1.** A summary of the anatomical findings from all four studies conducted. Abbreviations: (A) = apical; (B) = basilar; (—) = no change; (ÜÜ) = decrease/increase relative to control.

## Discussion

There is compelling evidence that learning produces alterations in cortical morphology, and these changes are most profound in the regions that support the specific task requirements (for reviews see [6,17,26]). For example, visual discrimination tasks produce alterations in the visual cortex (e.g.,[4]). Thus, for the most part research has focused on sensory and motor cortices when investigating neuronal changes as a wide

range of learning paradigms require some component of sensory and/or motor activity. The prefrontal cortex on the other hand, has received little attention, with only two known studies that have examined learning-related changes in dendritic morphology of the PFC [21,24].

The goal of the current study was to investigate experience-induced changes in cortical neuronal morphology based on the assumption that structural alterations reflect changes in cognitive function [18], implying that learning tasks that involve prefrontal cortical circuitry should also promote changes in dendritic morphology in the PFC. This prediction was tested in the current study by evaluating the cortical dendritic morphology in the PFC, as well as the Par 1 following completion of one of four learning paradigms. The results of these studies revealed a number of novel findings. First, the structural morphology of pyramidal neurons in the PFC was indeed altered in all of the learning paradigms examined. Second, at least some of the changes in the PFC are transient. Third, all learning paradigms showed changes in opposite directions in subregions of the PFC (namely, the Cg3 and OFC). Each of these findings will be discussed separately.

***Learning produces changes in the morphology of pyramidal neurons in the PFC.***

Experience-induced changes in the morphology of PFC neurons were evident in all learning paradigms assessed in the current study. A consistent finding under all paradigms was the decrease in layer III neurons in the Cg3. These results were apparent in all groups regardless of the duration (4 or 16 days of complex housing) or whether the animals were in the trained or yoked groups (T-maze and Grice box tasks). Moreover, in most cases the decrease was found in branch order and length of both apical and basilar

dendrites. Layer III neurons of the PFC receive numerous inputs from other cortical and subcortical regions and synapse with apical dendrites of layer V neurons as well, perhaps modulating layer V output [35]. The lack of learning-specific changes in layer III would indicate the processing of sensory information may be less selective in these neurons, as suggested by Withers and Greenough [35] in reference to non-lateralized changes in layer III neurons in the motor cortex as a result of reach training. It should be noted here however; that there were instances where training resulted in slightly different patterns of dendritic change in layer III neurons (e.g., in the T-maze training alone showed decreases in basilar dendritic length). More prominent differences were also found between the extinction and non-extinction groups following fear conditioning, with extinction producing a decrease in apical branch order only, in contrast to the decreases in branch order and length in both apical and basilar dendrites in the non-extinction group. Therefore, training may indeed produce selective changes in layer III neurons, albeit subtle ones that are task dependent.

It is not unreasonable to conclude that only subtle differences would exist between those animals that were involved in ‘active’ training and those rats that were included in comparable experiences with the exception of formal training. After all, yoked animals were undoubtedly ‘learning’ as they investigated their surroundings, and they received non-contingent rewards for their efforts. Thus, it might also be expected that yoked animals would exhibit alterations in brain circuitry compared to naïve animals, as was the case in the current study. It was rather surprising that the yoked but not trained rats showed alterations in Cg3 LV neurons in the T-maze and Grice box, however. One explanation for the absence of selective training-induced changes in the frontal cortex

may be that once a 'rule' is learned and the task acquired, activation of the PFC is not longer required to perform the task. It may follow then that the increased dendritic field of the PFC in the yoked but not trained rats in the current study was due to the absence of any predictable rules with non-contingent rewards. Thus, the PFC would continue to show training-related activation, as would be the case with the yoked animals in the current study.

Alternatively, it could be argued that the changes found were unrelated to training per se and merely a consequence of incidental learning. This is unlikely however, as it would be expected then that both training and yoked rats would show comparable changes, which was not the case.

Alterations in layer V of the Cg3 in the fear conditioning paradigm requires separate consideration. Interestingly, both the extinction and non-extinction groups showed a similar increase in basilar branching that was unique to this learning paradigm. Although training-induced changes have not previously been reported in the PFC, there have been reports of stress-related alterations in PFC neuron morphology, showing reduced dendritic fields in layer II/III (e.g., [25,29]). Therefore, it could be argued that the unique changes in these groups may be related to stress alone and not learning per se. The findings of Quirk and colleagues [28] demonstrate that the PFC is involved in extinction learning however, supporting the notion that the changes found are indeed related to learning itself.

***Altered structural morphology is transient following complex housing.***

The findings of the current study would suggest that the absence of dendritic changes in LV PFC neurons in the earlier study of Kolb and colleagues [21] was related

to the duration of the experience. Here we have found that 4 days of complex housing decreased apical and basilar branch order in Cg3 LV neurons, whereas no changes were apparent after either 8 or 16 days. The results of the Par 1 dendritic analysis are in keeping with the earlier study, but again were dependent on the duration of the experience. So, whereas 4 days of complex housing decreased Par basilar branch order 16 days of housing increased both apical and basilar branch order and increased basilar dendritic length as well.

It should be noted here that the animals in the other three learning paradigms were sacrificed immediately following completion of the tasks, and as such it is not possible to conclude if these changes were persistent or transient at this time.

Finally, whether or not the current findings are in keeping with the recent study of Kozorovitskiy and colleagues [24] who have shown housing-induced increases in layer II/III PFC neurons in primates, is more difficult to determine. Although at first glance it would appear that the results of the current study are in contrast to those of Kozorovitskiy and colleagues, it may simply be that the discrepancy is related to differential effects of experience in subregions of the PFC. Kozorovitskiy and colleagues [24], measured the dendritic field of neurons in the FD of the primate cortex, whereas in the current study measurements were taken from Cg3 and OFC in the cortex. Given that we have found areal-specific changes in the rat, it seems likely that this will also be true of primates.

***Opposite direction of learning-related changes in the Cg3 and OFC.***

An interesting finding in the current study was that experience produced opposing changes in LV of the Cg3 and LIII of the OFC (AID). The phenomenon of opposing effects of experience on subregions of the PFC is not novel. A similar

phenomenon has been found following other experiences, including psychostimulant use [5], morphine exposure (see [23], and stress [25]. Of interest is that although all experiences cited produce opposing effects in the mPFC and OFC, the direction differs depending on the experience. So for example, whereas psychostimulant use increases the dendritic field of Cg3 LV and decreases dendritic fields in layer III of the OFC, the opposite is the true of morphine and stress, with decreases in the Cg3 and increases in the OFC. In the current study, the effects of complex housing were in line with morphine and stress-induced alterations, whereas the other three learning paradigms were more comparable to changes produced with psychostimulant use, with increases in Cg3 and decreases in the OFC dendritic fields.

This is the first study that has shown that complex housing produces opposing effects on neurons of the Cg3 and OFC, as well as the first to show that the areal differences are not restricted to any one form of experience, and indeed may occur with all learning paradigm tested. The significance of these opposing effects remain to be elucidated but one might predict that increases in the dendritic fields of the OFC, a region associated with emotion and stimulus valence, may translate into an over representation of the OFC within the interconnected PFC circuitry, a theory that has been applied to psychostimulant use but perhaps also applicable to other experiences as well [15]. Future studies will be needed to determine whether these changes are indeed persistent and if so the behavioural correlates of these changes will need to be elucidated.

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**I. Summary:** There are a lot of data to be considered both in the current set of studies and related studies conducted in the Kolb laboratory. In order to simply the discussion of the results, I have provided a series of tables that summarized past and current findings.

**A. Previous Research**

		Basilar					Apical					FL	Basilar		Apical		Author
EXPERIENCE	Sacrifice	mPFC LIII	OFC LV	Par1 LIII	Oc1 LIII	mPFC LIII	OFC LV	Par1 LIII	Oc1 LIII/ LV	FL LIII/ LV	mPFC Spine LV	OFC Spine LIII	mPFC Spine LV	OFC Spine LIII			
<b>Adult</b>	<b>Drugs</b>																
	Amphet	delay									h	i	h	i	[13]		
		delay		∅	∅	∅	h		∅		∅		h		[51]		
		delay		∅			hh				h		h		[52]		
	Cocaine	delay		hh			hh				h		h	∅	[52]		
		delay									h	∅	h	∅	[15]		
	Nicotine	delay		hh		∅		∅		∅	h		h		[22]		
					∅					∅					[9]		
	THC	delay	∅	hh	∅	∅	hh	∅	h						[29]		
	Morphine	delay		i		ii		ii		i		i	h	i	h	[53]	
		delay													[50]		
	Complex	immed		∅		hh		∅		hh		h		h	[30]		
	90 + days	immed				hh				∅					[22]		
		immed				h				h					[28]		
	immed				hh	hh			hh					[27]			
<b>Train</b>																	
visual	delay				h				hh					[26]			
									/h					[11]			
motor	delay									h				[26]			
										h/h				[20,60]			
<b>Drug +</b>																	
Complex	immed				ii				∅					[22]			
Complex	immed				∅				∅					[28]			
Train	immed									h				[19]			

		Basilar					Apical					Basilar		Apical		Author	
EXPERIENCE	Sacrifice	mPFC		OFC	Par1	Oc1	mPFC		OFC	Par1	Oc1	mPFC	OFC	mPFC	OFC		
		LIII	LV	LIII	LIII	LIII	LIII	LV	LIII	LIII	LIII	Spine LV	Spine LIII	Spine LV	Spine LIII		
Young	<b>Drugs</b>																
	Amphet.																
	P22-34	delay		hh				∅				h		h		[14]	
	Caffeine																
	P1-13	delay	hh													[25]	
	<b>Complex</b>																
	P10-21	immed					hh									[57]	
	P22-120	immed			hh	hh				hh							[27]
	P22-P60	immed				h					h					[58]	
P31-P35	immed				hh										[59]		

**Table 5.1.1.** The table depicts the results of previous studies that have evaluated experience-dependent changes in cortical dendritic morphology and spine density in both adult and young rats. These studies illustrate that 1) experience produces regionally specific alterations, 2) subregions of the PFC (mPFC & OFC) often respond differently to experience, 3) psychostimulant use has the capacity to alter subsequent experience-induced plasticity, and 4) there has been little focus on the experience-dependent changes in the PFC.

**Abbreviations:** PFC (prefrontal cortex); OFC (orbital prefrontal cortex); Par 1(primary somatosensory cortex); Oc1 (visual cortex); FL (forelimb regions of the frontal cortex). The red arrows are used to depict changes in branch order, whereas the blue arrows depict changes in dendritic length (Sholl analysis), and the symbol ‘∅’ depicts no change. Blank cells indicate that the perspective region was not examined in that particular study.

**B. Current studies.**

		Basilar				Apical				Spine (basilar)		
		mPFC		OFC	Par1	mPFC		OFC	Par1	mPFC	OFC	
STIMULANTS	Sacrifice	LIII	LV	LIII	LIII	LIII	LV	LIII	LIII	LV	LIII	
Young	<b>Ritalin</b>											
	short (P22)	immediate	i	h	∅	i	i	∅	∅	i	h	∅
	short (P22)	delay	∅	∅	∅	h	i	∅	∅	∅	∅	h
	long (P22)	delay	h h	∅	∅	∅	h	∅	∅	∅	∅	i
Adult	<b>Ritalin</b>											
	short (~P60)	immediate	∅	∅	n/a	∅	∅	∅	n/a	∅	∅	∅
	short (~P60)	delay	∅	h	n/a	∅	∅	h	n/a	∅	∅	∅

**Table 5.1.2.** The table summarizes the anatomical correlates of stimulant use in young and adult rats. Analyses were conducted on animals sacrificed either immediately following a short (4-5 day) drug free period or in adulthood after an extended drug wash out (delay) period. The anatomical results indicate that the pattern of altered circuitry is much different when measured immediately following the drug treatment phase versus after a delay period. Moreover, the response pattern varies between early and adult Ritalin exposure. For example, there is an increase in Cg3 LV and a decrease in Par 1 basilar branch order immediately following the drug-treatment period whereas, there is an absence of Ritalin-induced in Cg3 LV and an increase in Par 1 branch order after a drug-free delay period in young animals. In contrast, adult Ritalin use produces no immediate effects in dendritic morphology but an increase in Cg3 LV basilar and apical dendritic arborization after a delay period. Additionally, prolonged use (long = 38 days) also results in a unique pattern of connectivity relative to short-term (short = 12 days) use with increases in Cg3 LIII apical and basilar neurons after prolonged exposure and decreases with short (12 day) exposure.

**Abbreviations:** PFC (prefrontal cortex); OFC (orbital prefrontal cortex); Par 1(primary somatosensory cortex); Oc1 (visual cortex); FL (forelimb regions of the frontal cortex). The **red arrows** are used to depict changes in branch order, whereas the **blue arrows** depict changes in dendritic length (Sholl analysis), and the symbol '∅' depicts no change. Blank cells indicate that the perspective region was not examined in that particular study.

		Basilar				Apical				Spine (basilar)		
		mPFC		OFC	Par1	mPFC		OFC	Par1	mPFC	OFC	
COMPLEX		Sacrifice	LIII	LV	LIII	LIII	LIII	LV	LIII	LIII	LV	LIII
Young	short (4)	young	∅	∅	∅	∅	∅	∅	∅	h	h	h
	long (30)	adult	∅	h	∅	h h	∅	∅	∅	∅	∅	∅
Adult	Short (4)	immediate	i	i	∅	i	i i	i	h h	∅	∅	i
	Long (16)	immediate	i i	∅	∅	h h	i i	∅	∅	h	i	i

**Table 5.1.3.** The table summarizes the effects of short and long term complex housing on dendritic morphology in the young and adult brain. The results illustrate that the young and mature brain responds very differently to complex housing, producing opposite effects in the spine density as well as layer LV basilar branch order. Moreover, whereas long-term complex housing produces changes in dendritic arborization of LV Cg3 neurons in young animals, only short-term complex housing produces changes in LV Cg3 neurons in adulthood. Moreover, basilar spine density in mPFC and OFC neurons appears to operate in the opposite manner, showing decreases as a result of long-term housing in adult animals, whereas juvenile rats show an increase in spine density following short-term housing.

			Basilar				Apical				Spine (basilar)		
TRAIN		Sacrifice	mPFC	OFC	Par1	mPFC	OFC	Par1	mPFC	OFC	Par1	mPFC	OFC
			LIII	LV	LIII	LIII	LIII	LV	LIII	LIII	LV	LIII	LIII
Young	T-maze+	adult	h	∅	∅	∅	∅	i	∅	∅	∅	∅	h
Adult	T-maze +	immediate	∅	∅	n/a	∅	i	∅	n/a	∅	∅	∅	h
	T-maze	immediate	i	∅	i i	h	i i	∅	∅	∅	∅	∅	h
	Grice	immediate	i i	∅	n/a	∅	i i	∅	n/a	∅	∅	∅	i
	Fear	immediate	i i	h	i	i	i i	∅	i	∅	∅	∅	i

**Table 5.1.4.** A summary of anatomical measures taken from animals following training in a number of learning paradigms. Animals in the T-maze + group were also involved in additional behavioural testing prior to the final T-maze task. All animals were sacrificed immediately after completion of the task. The results indicate that early training produces a much different response to subsequent training in the T-maze relative to pretraining in adulthood (T-maze + young versus adult). Additionally, different learning paradigms produce distinct alterations in brain circuitry that would suggest that the effects are not a simply a by product of increased activity.

		Basilar				Apical				Spine (basilar)		Behaviour	
		mPFC	OFC	Par1	mPFC	OFC	Par1	mPFC	OFC		Tmaze		
RITALIN +		LIII	LV	LIII	LIII	LIII	LV	LIII	LIII	LV	LIII		Errors
Young	Complex (4)	immediate	∅	∅	∅	∅	∅	i	i	∅	∅		
	Complex (30)	immediate	∅	∅	∅	∅	∅	∅	∅	∅	∅		
	Train +												
	Short (P22)	immediate	i h	∅	i	∅	h	∅	∅	h	∅	∅	h
	Short (P29)	delay	∅	h	∅	∅	i	∅	h	∅	i	i	h
	Long (P22)	immediate	∅	h	∅	∅	∅	h	∅	∅	h	∅	h
	Caffeine	delay	∅	h	∅	∅	∅	i	∅	∅	i	h	i *
Adult													
	Train +	immediate	∅	∅	n/a	∅	h	h h	n/a	∅	∅	∅	∅
CAFFEINE +		Sacrifice	LIII	LV	LIII	LIII	LIII	LV	LIII	LIII	LV	LIII	Errors
Young	Train +	delay	∅	h	∅	∅	∅	i i	∅	∅	∅	∅	∅

**Table 5.1.5.** A summary of anatomical changes and behavioural correlates of Ritalin use in young and adult animals exposed to additional experiences either during (caffeine) or following the drug treatment period. The results show that the brain responds very differently to various forms of learning (e.g., complex housing versus training) following Ritalin use. Moreover, the effects are mediated by other factors such as age at time of experience (P22, P29, or adulthood), duration of both the initial drug experience (short = 12 days; long = 38 days) and the subsequent experience (4 vs 30 days of complex housing) and the interaction with other stimulants (in this case caffeine).

The term Train + indicates that the animals were enlisted in a number of behavioural tests (e.g., activity and play behaviour) prior to training in the final task (T-maze). The ‘\*’ signifies a significant decrease in T-maze errors relative to Ritalin alone, although not significantly different than saline-treated rats.

## ***II. Discussion***

The beneficial effects of enriched/complex environments have been well documented, beginning with the first studies of Hebb [23], which found improved subsequent cognitive performance in rats following complex housing. Decades of follow-up research on Hebb's original findings have convincingly demonstrated the influence of experience-induced alterations on brain chemistry and structural morphology. Surprisingly, although there has been nearly 40 years of study of the effects of experience on the sensory and motor systems, it is only in the past few years that studies have begun to examine experience-dependent changes in the prefrontal cortex [30,34]. One might have predicted that given the intense interest in prefrontal activity in ongoing behavior, such as measured in unit recordings and noninvasive imaging studies (e.g., Fuster, [17] ; Posner et al.,[46]), that there would have been a parallel interest in the plastic response of the prefrontal cortex to experience but this has not been the case.

The goal of the current set of studies therefore was to focus on experience-dependent changes in the developing and adult prefrontal cortex. I chose to use drug, learning, and enriched housing as tools to induce changes in neuronal morphology and their behavioural correlates. The results of these studies produced a number of novel findings that begin to open the door to understanding how the prefrontal cortex is affected by a wide range of experiences. I will itemize the main conclusions from the studies beginning with the behavioural results.

### ***1. Altered motor activity but not behavioural sensitization after chronic Ritalin use***

Although Ritalin was used in the current studies, as discussed the focus was on the anatomical and behavioural influence of experience in the developing and mature

brain. As such, the dose and drug regimens used were more in keeping with the clinical use of Ritalin and less relevant to the study of addiction. Nonetheless, owing to the interest in the potential increased risk for developing addiction as a consequence of pre-exposure to Ritalin measures of behavioural sensitization and cross-sensitization were taken to evaluate the behavioural effects of chronic Ritalin exposure in both developing and mature rats.

Put simply, behavioural sensitization refers to an augmentation of the motor activating effects with repeated drug administration. One of the strengths of behavioural sensitization as a model for addiction is that the behavioural effects are enduring, lasting well beyond the drug treatment period. Thus, behavioural sensitization is considered by some to be a marker of drug-induced neural plasticity that emulates what may be permanent alterations in behaviour found with clinical addiction (see [49]). There are a number of ways that behavioural sensitization can be assessed, however. For example, whereas some studies determine behavioural sensitization based on only escalating motor response during or after the treatment period (e.g., [3]), others ascertain behavioural sensitization based on the motor response after a drug-washout period, or drug 'challenge' (e.g., [40]). In the latter case, the time between the last injection and the challenge may vary and stimulants other than Ritalin are often used to determine increased sensitivity to psychostimulants in general (cross-sensitization). In the current set of studies the sensitizing capability of Ritalin was assessed through: 1) the ongoing assessment of motor activity over the treatment period; 2) the motor response to caffeine after a short wash-out period (usually 3 to 5 days); and, 3) the motor response to amphetamine following a prolonged drug-free period.

The current studies showed that although Ritalin did not induce behavioural sensitization during the treatment period, early Ritalin use also did not produce the habituation in activity measured over time that is characteristic of saline-treated rats. So, for example whereas all animals regardless of treatment group showed a comparable initial motor response in the activity chambers, over the 20 minute testing period the Ritalin treated groups did not reduce their activity levels to the same degree shown in Saline-treated rats. Additionally, 12 days of chronic Ritalin use failed to produce cross-sensitization to either the early caffeine, or later amphetamine challenge. Moreover, the retarded habituation response and absence of cross-sensitization was evident following both early and adult Ritalin treatment, with one exception. Prolonged, chronic Ritalin use initiated during development was the only treatment group to show an augmented motor response in the caffeine challenge. In the latter case the activity pattern was very similar to that which occurred during the treatment period in that Ritalin-treated rats failed to show the same habituation-induced decline of activity over the testing period.

It should be noted here that the capacity of early Ritalin use to produce behavioural sensitization is controversial. For example, whereas some studies have found increased motor activating effects of Ritalin (e.g.,[18,40]), or enhanced response to a later cocaine challenge [1,56], others have shown decreased Ritalin-induced activity (e.g.,[61]) and a depressed or absent response to subsequent cocaine exposure [21]. At least some the discrepancies can be attributed to methodological issues, such as drug dose. A number of studies have shown dose-dependent induction of behavioural sensitization in both early [40,47] and adult Ritalin use [5]. For example, Prieto-Gomez and colleagues [47] found that administering Ritalin at doses of 0.6, 2.5, and 10 mg/kg produced a

differential motor response in that whereas 2.5 and 10 mg/kg doses produced increased motor activity, the lower 0.6 mg/kg dose did not. Thus, the current results are in keeping with the findings of Prieto-Gomez and colleagues [47], Berridge and colleagues [5], as well as McDougall and colleagues [40], all of whom have shown that low doses of Ritalin (0.5-0.6 mg/kg) during development or in adulthood are insufficient to induce behavioural sensitization. Nonetheless, the augmented motor activity over the treatment period during early and adult exposure as well as that produced by prolonged Ritalin use in the caffeine challenge indicates that even a low dose has the capacity to alter motor behaviour, albeit short-term.

A possible explanation for the Ritalin-induced motor effects found in the current studies may be taken from the study of Andersen and colleagues [10] who found that rats treated with Ritalin (2.0 mg/kg) maintained the initial response to the novel environment whereas saline treated rats showed a decline. Interestingly, the researchers proposed that the effect may be due to attentional deficits. The argument would be that reduced attention would inhibit the development of familiarity to novel events/locations. Based on the activity data alone it would be impossible to determine if this was indeed the case in the current studies. The results from the cognitive tasks that follow show an enduring cognitive deficit as a consequence of early Ritalin however, which may be interpreted in part as an attention deficit, and as such would certainly support the argument.

In summary then, **chronic low dose Ritalin use alters the motor response in a novel environment in the developing and mature animal but does not produce behavioural sensitization although the effect is augmented further by prolonged, chronic use.**

***2. Early drug experience influences play behaviour and adult cognitive functions subserved by the prefrontal cortex (PFC).***

A novel finding in the current studies was that early Ritalin use produced enduring cognitive deficits into adulthood. So, rats exposed to Ritalin during development showed deficits in performance in the T-maze task in adulthood. That cognitive deficits as a consequence of early exposure to such a low dose of Ritalin could endure into adulthood was extraordinary. Key to these findings was that the Ritalin-induced cognitive deficits were consistent across studies that varied in timing of drug administration (postnatal day (P) 22 and P29) and duration of treatment (12 days and 37 days), suggesting a period of increased ‘sensitivity’ rather than a disruption in development during a ‘critical’ period. It is of interest that the timing of drug administration in the current studies parallels the developmental timing of synapse over-production in the developing brain (see [2]). Correlations between cognitive development and brain structure such as synapse production and pruning have been inferred in both human [33] and non-human [32] studies. One might speculate then that the deficits following early drug exposure might at least in part be related to drug-induced alterations in the patterns of connectivity. Such alterations would have the ability to permanently alter the organism’s response to experiential factors.

In addition to producing deficits in T-maze performance, Ritalin-treated rats also displayed altered social behaviour during the course of the drug treatment period as well as after a short washout period. The results of these studies showed that the effect of early drug exposure on social behaviour was correlated with the individual’s responsiveness to

a psychomotor stimulant. Thus rats that showed a heightened motor response during the drug-treatment period displayed the greatest impact on subsequent social behaviour, seemingly withdrawing from social interactions. In one of the few studies that have investigated the long term effects of early Ritalin use on behaviour, Bolanos and colleagues [6] found reduced response to natural rewards such as sucrose and sex in adulthood following early, chronic Ritalin (2.0mg/kg) use, as well as increased anxiety to novel environments in these animals. Based on the results of the initial experiment in the current studies and Bolanos findings, one might conclude that the reduced initiation of play displayed here was due to a reduction in what would typically be considered the rewarding expression of play [44]. Yet, the second paper in the current studies measured play behaviour in a slightly different way, assessing the ‘desirability’ of one play partner over another, offering an alternative explanation for the drug-induced decrease in play initiation.. In this latter study, animals were placed in triads. The findings were that Saline-treated rats would initiate play less often with Ritalin-treated rats and Ritalin-treated rats were more likely to avoid play attempts from Saline-treated rats, although initiating play equally with both Saline and Ritalin-treated play partners. The results suggest that there are subtle behavioural differences in Ritalin-treated rats that make them less desirable as play partners. On the other hand, Ritalin-treated rats may have difficulty perceiving social nuances such as play and attack in ‘normal’ rats.

***Ritalin’s behavioral influence is age-dependent.***

Interestingly, adult Ritalin exposure, although producing similar motor activating effects to that of early Ritalin exposure, appeared not to generate the behavioural deficits that were evident in adult rats exposed to Ritalin during development. For example,

whereas performance in the T-maze task was impaired following early Ritalin exposure, performance was unaffected by adult Ritalin use. A tenable explanation for the continued cognitive deficits following early but not adult Ritalin exposure may be due to decreased dopamine transporters (DAT) in the striatum. These transporters, located on the presynaptic terminals, are one of the main mechanisms for the removal of surplus dopamine from the synaptic cleft. Moll [42] has shown that 7 days of 2.0 mg/kg Ritalin beginning at P25 but not P50 produced a reduction in the density of striatal DAT but not midbrain DAT. Further the decrease continued between post treatment day 6 and day 32. These results might suggest that Ritalin alters the still developing DA system, perhaps by reducing axonal outgrowth/DA synapse formation [42].

In addition to the absence of cognitive deficits following chronic Ritalin use in adulthood, adult exposure also did not produce any apparent social abnormalities such as those found following early Ritalin exposure. The age-dependent effect of Ritalin on social behaviour was especially interesting given the suggested role that the OFC plays in social behaviour and the fact that bilateral removal of the OFC in the perinatal period or in adulthood produce enduring abnormalities in social behaviour [31,43]. It would appear then that chronic drug use does not result in an overall dysfunction of regions such as the OFC that subserve social behaviour but perhaps instead early Ritalin use produced more subtle changes in the still developing neural circuitry. Thus, the adult brain with its mature circuitry in place would be more able to cope and adapt to experience-induced alterations in circuitry.

In summary, **the early but not adult Ritalin-induced alterations in social and cognitive behaviour may reflect permanent alterations in developing neural circuitry.**

### ***3. The PFC shows experience-dependent plasticity.***

Previous studies had shown that mPFC and OFC neurons, particularly pyramidal neurons of the Cg3 and AID, changed in response to both drugs and hormones but there was little evidence of neuronal modification in response to either training or sensorimotor experience. The current set of studies confirmed that a psychomotor stimulant (Ritalin) alters mPFC and OFC neuronal morphology but, in addition, showed that these cells also respond to training in both developing and mature brain.

OFC. The finding of OFC neuronal changes in a variety of paradigms is consistent with other unpublished parallel work showing that social experiences also alter dendritic fields in the OFC. Thus, when adult animals are given new social partners every other day there is an increase in dendritic fields (Hamilton et al., SFN abstract), whereas when juvenile animals are housed with siblings versus adults there is either an increase (siblings) or decrease (adults) in dendritic fields (Bell et al., SFN 2007 abstract). It is clear that OFC neurons do respond to experience but it is not yet clear what determines whether there is an increase or decrease in synaptic space or how such changes relate to other behavioral changes.

Nonetheless, selective examination of OFC spine density in the current studies revealed a pattern of change that may help to provide insight into the functional significance of at least some of these changes. For example, *short-term* exposure to Ritalin, complex housing, and T-maze training all *increased* OFC spine density in young

animals, whereas in contrast young animals with *prolonged* Ritalin use exhibited a *decreased* OFC spine density. The functional significance of increased OFC spine density across such a wide variety of experiences is not immediately clear, Together with the additional finding that adult animals trained in the T-maze task also showed increased OFC spine density in the current studies, however, would indicate that some aspect(s) of the task requirements, such as attention, maintenance of motivationally relevant information during the delay component and/or response inhibition [12,55], activated a plastic response in the OFC. The cases mentioned above show that the OFC is altered by experience. A question that needed to be addressed was the relevance of altered connectivity on subsequent behaviour and learning.

What was particularly intriguing here was that experience-induced alterations in OFC spine also caused changes in the level of performance of the T-maze task, a task believed to be subserved by PFC circuitry, including the OFC. So groups with Ritalin use plus T-maze training showed a *decrease* in OFC spine density and *deficits* in T-maze performance, whereas in contrast, groups with a combination of Ritalin and caffeine exposure showed an *increase* in OFC spine density and *enhanced* performance in the T-maze task relative to Ritalin treatment alone. Furthermore, early caffeine exposure and subsequent T-maze training produced no change in OFC spine density and performance in the T-maze task was comparable to that of controls. Thus it appeared that the behavioural outcome of these altered patterns of OFC spine density was in keeping with the proposed function of the OFC in executive functions such as incentive value and short-term information storage during delay tasks such as the T-maze [55]. Put another way, increased OFC spine density was synonymous with T-maze learning.

**Thus, decreased OFC spine density was correlated with behavioural deficits in OFC- mediated tasks.**

*Cg 3 LIII.* A curious finding in the current set of studies was the experience-related decreased arborization in LIII neurons as a result of adult experience. The decrease was found following complex housing as well as training in various learning paradigms under study but not following stimulant use. Moreover, contrary to adult experience, early experience produced an increase in LIII neuron arborization. Although it is unclear why learning would result in a laminae-specific decrease in arborization the consistency of learning-related decreases would indicate that these changes play an important role. One possibility, however, is that the decrease represents a mechanism by which the signal to noise ratio is enhanced thereby minimizing attention to irrelevant stimuli. Although there is no direct evidence to support the argument, indirect evidence comes from the findings of Mehta and colleagues [41] during an investigation of the mechanism by which acute Ritalin exposure may facilitate spatial working memory in humans. Using positron emission technique (PET) to measure regional cerebral blood flow (rCBF) during a working memory task, Mehta et al., [41] found that patients exhibited a decreased rCBF in the dorsolateral prefrontal cortex (DLPFC), an area believed to play an important role in working memory. At first the finding appears to be counterintuitive but the authors argued that the reduced rCBF may be indicative of an increased efficiency whereby the signal to noise ratio was reduced through selective attention to relevant stimuli while suppressing irrelevant information.

Applying Mehta and colleagues' [41] conclusions to the current studies, it might be argued that the decreased arborization of layer III neurons in the PFC is a learning-

related mechanism that allows for increased attention to task-related information and a decreased attention to distracters. Based on the purported lack of inhibitory control in the young brain, under this explanation it would follow that such a mechanism may not be in place until the later stages of PFC development. Indeed, in the current studies layer III Cg3 neurons show an opposite, if any, learning-induced plastic response to environmental influences. Furthermore, stimulant use altered the pattern of change in these neurons, which might suggest that stimulant use caused deficits in filtering out irrelevant information.

In summary, **learning-specific changes that occur in LIII neurons are age-dependent and may act to modify LV output.**

Cg 3 LV. Relative to OFC neurons, adult experience had relatively little influence on layer V Cg3 neuronal spine density. In fact, a decrease in spine density after prolonged housing in a complex environment was the only change found in the current studies. As with previous studies however, alterations in spine density and arborization are not highly correlated and in fact may occur in opposite directions [26,30]. So, whereas adult experience-induced changes in spine density were limited, Cg3 LV neuronal arborization was altered under a variety of conditions, albeit differently. For example, whereas complex housing decreased dendritic arborization, fear conditioning and Ritalin use increased LV neuron arborization.

The latter finding was in keeping with previous studies that have shown that Cg3 LV neurons are altered by previous drug experience in both the juvenile [14] and adult (for review [54]) rat. Specifically amphetamine, cocaine, and nicotine have all been shown to produce increases in Cg3 LV neuronal arborization and/or spine density. What

was rather surprising here, however, was that owing to the relatively low dose of Ritalin used in the current studies (0.5 mg/kg), and the absence of behavioural sensitization, one might not have expected to find drug-induced changes in the PFC of adult rats.

The effects of experience on LV neurons were age-dependent. So for example, early exposure to a complex environment increased LV spine density and arborization, opposite to the effects of adult exposure reported above. Moreover, whereas early Ritalin use increased the dendritic field of LV neurons as reported with adult animals, the alterations in neuron morphology were only evident in young animals sacrificed shortly after the drug treatment period, whereas adult animals showed altered dendritic morphology only after a delay period.

Relative to the OFC and Cg3 LIII neurons, the patterns of experienced-induced connectivity in Cg3 LV neurons varied considerably across the various experience paradigms, making it difficult to make inferences related to the functional significance of the findings. One thing that should be noted, however, was that whereas early drug exposure had very little influence on LV neuronal circuitry, the combination of the early Ritalin exposure and later training produced considerable change in both spine density and arborization. These findings suggest that early drug experience produced some fundamental change in the way that the brain responded to later experience such as learning. Moreover, as with all other PFC areas studied, early Ritalin use inhibited complex environment-induced changes in LV neurons as well. The functional significance of this effect was not investigated in the current set of studies. Nonetheless, it would be expected that blocking housing-induced changes would also block the cognitive benefits of complex housing as well. Although the effects of complex housing

on PFC circuitry has not been studied in the context of neuron morphology in the past, complex-environments have been considered to be beneficial, enhancing cognitive performance and cortical morphology in other regions such as the visual and sensory cortices. Thus it would be expected that housing-induced changes in the PFC would also be beneficial.

**In summary, LV neurons of the mPFC selectively respond to experiential factors, retracting during sustained experience with two exceptions: 1) Permanent alterations occurred with drug use; and, 2) early enrichment. These two effects likely reflect relatively permanent changes in behaviour.**

#### ***4. Plastic changes in PFC circuitry vary with both age and duration of experience.***

When thinking about the brain's plastic response to experience there is often a tendency to concentrate on the consistencies of change. Equally important, however are the inconsistencies as they emphasize the variability in the brain's response to experience.

In the current set of studies we found time-dependent changes in layer V neurons of the Cg3. For example, whereas the immature brain showed no effect of short-term complex environments (4 days) on dendritic length or branch order in the PFC, the mature brain showed extensive changes in layers III and V of the Cg3 as well as layer III of the OFC. In contrast, prolonged exposure to complex housing during development (30 days) resulted in an increase in LV Cg3 neurons, whereas the adult brain showed no changes. Thus, in the adult brain changes that occur with initiation of experience are transient, retracting with prolonged exposure. In contrast, the immature brain retains the experience-induced changes, perhaps even permanently. The implications are that early

experience may leave a lasting imprint on adult PFC connectivity patterns, whereas adult experience-induced changes may be incorporated into existing patterns or circuitry.

So how would this relate to the learning-induced changes found with adult experience in the current studies? The most likely explanation would be that the effects of training are also transient. In the current studies the animals were sacrificed immediately after completion of the tasks which would explain the experience-related changes in PFC dendritic morphology. The caveat here was that although PFC neuron morphology was also influenced by adult drug exposure, the effects were evident only after a delay and not immediately after treatment ended. So, whereas there were no changes in LV arborization in animals sacrificed shortly after 12 days of Ritalin use, there was an increase in both apical basilar dendrites after a delay period (> 30 days). The drug-related effects however may follow different rules as would be suggested by the permanency of anatomical and behavioural abnormalities following chronic psychostimulant use [48].

Duration of the drug treatment period in the current studies also resulted in a unique pattern of connectivity. So, prolonged Ritalin use (37 days) increased arborization of LIII Cg3 neurons and decreased OFC spine density, a pattern that was opposite that of short-term chronic exposure (12 days), which showed decreases in layer III arborization and increases in OFC spine density. It might be expected that increasing the duration of drug use would amplify-induced alterations in circuitry as would be suggested by the study of Porrino and colleagues [45]. Porrino and colleagues found that the affect of short-term (5 day) self-administration decreased functional activity in restricted regions of the striatum (mostly ventral). When self-administration continued for 100 days the decrease in functional activity spread to include most of the dorsal striatum and putamen

as well [45]. So why would prolonged Ritalin exposure produce opposite, rather than augmented effects instead? Although hardly an explanation the only answer that would seem to fit at this point is that there are fundamental differences in the way that the young brain responds to experience relative to the mature brain. Indeed, a previous study by Kolb, Gibb & Gorny [27] showed just this in young and adult animals placed in complex environments.

***5. The prefrontal cortex follows a different set of rules than the rest of cerebral cortex.***

A perplexing phenomenon first raised by Kolb and colleagues [30] was that subregions within the PFC do not respond to experience in the same manner. For example, whereas psychomotor stimulants produce increased dendritic fields in Cg3 Layer V neurons, there are decreased dendritic fields in the OFC (e.g.[13]). Follow up studies with morphine, another psychoactive drug, have shown opposite, but again directional differences in mPFC and OFC neurons [31] such that the Cg3 neurons were atrophied and the OFC neurons were hypertrophied. Other experiences have also been shown to have differential effects on dendritic morphology of mPFC and OFC neurons, including chronic stress [38] and gonadal hormones [16]. Areal differences in PFC response are also evident in stimulant-induced activation of mPFC and OFC neurons. Homayoun and Moghaddam [24] found that repeated injections of amphetamine produced opposing effects on the activity of Cg3 and OFC neurons. Whereas repeated amphetamine use produced an increased inhibition of the Cg3 neurons, there was an increased excitation in OFC neurons. The prediction was that over time the changing physiological responses of the cells would lead to behavioural changes and there would

be a progressive decline in PFC control over behaviour and a progressive increase in the OFC control.

Why different regions of the PFC would respond differently to experience is especially puzzling given that the two regions have such similar patterns of inputs and outputs. One possible explanation, however, would be that variable changes in these regions represent a response to some experience-induced imbalance in the homeostatic relationship between the mPFC and OFC. Thus under conditions that over activate the OFC, the PFC may respond by retracting synapses through modifications in spine density and arborization, thereby reducing excitation and maintaining homeostasis within PFC circuitry. This is purely conjecture at this point but provides a direction for future research.

#### ***6. Learning produces transient patterns of plasticity in the PFC that are laminae and areal specific***

The current findings demonstrate that the PFC exhibits structural changes following both juvenile and adult housing in complex environments. Additionally, formal training alters PFC circuitry in adult rats as well. The persistence of these training-induced changes in the PFC have yet to be determined, however, as in the current studies the animals were all sacrificed shortly after completing the cognitive tasks. Based on the complex housing data, however, and the fact that training and complex housing are similar in their influence on neuronal structure, it would be reasonable to predict that the effects of training would also be transient in the PFC. The question remains as to why the PFC would show only transient structural changes when persistent learning-induced changes are found in other cortical and subcortical areas. A plausible explanation for this

disparity is that the PFC is a highly plastic region, enabling it to generate and revise new plans of action in response to incoming information. In the case of novel experience or stimuli, once the response or rules have been fine-tuned to deliver the most beneficial outcome the PFC is no longer required to carry out the actions and the learned response is now stored in engrams (neuronal networks) within regions more directly involved in the behavioural output. Based on this explanation, it would be reasonable to suggest that experiences that promote enduring effects on PFC circuitry, such as psychomotor stimulants may also interfere with PFC plasticity, reducing the ability to influence later learning, memory and subsequently behaviour.

Indeed, research has shown that altered PFC circuitry as a consequence of exposure to early stressors (e.g., impoverished maternal care/housing) that persist into adulthood, produce deficits in learning and memory and inhibit plasticity. Similarly, chronic drug exposure as illustrated in both current and previous studies, also results in persistent changes in PFC synaptic connectivity, and as shown in the above data, can produce deficits in later learning as well. For example, Liston and colleagues [38] have shown that stress produces alterations in dendritic morphology of layer II/III neurons in both the mPFC (decreases) and OFC (increases). The altered mPFC circuitry was correlated with poor performance in an alternation task. Interestingly, the increase dendritic field in the OFC did not appear to decrease performance on the reversal trials.

### ***7. Early drug experience alters the capacity of the brain to change under subsequent experimental conditions***

Complex housing is one of the most widely used models of experience-induced plasticity in animal research, producing robust alterations in the visual and sensory

cortices in young, mature and aged animals. Moreover, the current set of studies has shown that the influence of complex housing extends to the PFC as well. Given that complex housing has the capacity to produce robust alterations in brain circuitry at all ages, it was astounding that chronic pretreatment with such a low dose of Ritalin would have the capacity to alter/inhibit these experience-induced changes in brain circuitry. Although both Cocaine and amphetamine have been found to inhibit complex housing-related dendritic changes in Par 1 of adult rats as well [28], the doses used by Kolb and colleagues [28] was meant to mimic the effects of addiction and thus much higher than that used in the current studies. It was rather unexpected then that low doses of Ritalin would have similar effects, especially given the absence of any apparent display of addiction-like behaviour. The implications are that early stimulant exposure has the capacity to alter the trajectory of PFC development thereby changing the way that the brain responds to later experiences.

***8. Low doses of Ritalin do not produce behavioural sensitization or dendritic changes in NAc.***

The absence of behavioural sensitization to Ritalin and the lack of any significant alterations in dendritic morphology of spiny neurons of the NAc in the current studies suggest that it is unlikely that repeated use of Ritalin at such low doses is sufficient to induce addiction. Additional support for the absence of addiction in the current studies comes from the behavioural results of the amphetamine challenges. Neither early nor adult Ritalin use produced any long-lasting sensitivity to amphetamine as inferred from the lack of enhanced motor activating effects during the challenge.

The results of the current set of experiments are consistent with studies that argue a functional significance for altered cell morphology of medium spiny neurons of the NAc. Robinson and colleagues [37,51], contend that these changes in morphology are directly related to the acquisition of behavioural sensitization, a marker of addictive behaviour an argument that is supported by the work of Berlanga and colleagues [4].

Using electron microscopy procedures, Berlanga et al [4] confirmed that repeated cocaine use produced increases in the number of excitatory synapses on medium spiny neurons in the core of the NAc, a region that Robinson suggests is involved in the transition from drug abuse to drug addiction. Further, the functional significance of long-term changes in dendritic morphology of NAc core on subsequent behaviour in addicted rats is supported by the work of Martin and colleagues [39]. In this study they found that cocaine-addicted rats showed repressed plasticity (as shown with inability to induce LTD) after 21 days of abstinence. Though both the shell and core displayed similar responses just 1 day after cocaine abstinence, only the core retained this state after 21 days abstinence.

Thus, aside from providing additional support for the previous studies on addiction, the current results also suggest that the animals within our studies did not show addiction-like behaviour, nor was there any evidence of increased risk for addiction in later life as a consequence of early psychostimulant exposure. This is an important finding as much of the concern over Ritalin use is based on the potential for later drug abuse. From the current results it would appear that the inconsistent reports of increased sensitivity to later drug exposure is likely related to the dose of Ritalin used in these studies.

A large percentage of studies that report enhanced sensitivity to later drug use following early Ritalin exposure use doses that exceed what others have argued are clinically relevant doses. For example, although the studies of both Brandon, Marinelli and White [8] and Adriana et al.[1], found alterations in striatal neuron activity and gene expression, respectively, in both studies 2.0 mg/kg of Ritalin was provided daily via i.p. administration over periods of one week to sixteen days in adolescent rats, which is at least twice as high as doses that produce comparable blood plasma levels to human clinical use[5].

The view that low doses of MPD do not facilitate the development of behavioural sensitization has been discussed in relation to the motor activating effects and the risk for later drug abuse. DA levels were not assessed in the current set of studies. Nonetheless, Kuczenski and Segal [35] have found that low doses of Ritalin do not increase DA nor NE release in the NAc. This finding has been confirmed more recently by Berridge and colleagues [5], in which they also showed that the influence of psychostimulants such as Ritalin on DA and NE release in the NAc are dose-dependent. The interpretation of Kuczenski & Segal, and our own based on the additional results of the amphetamine challenges in the current experiments, has been that these results would suggest that low dose MPD use is unlikely to increase the risk for later drug abuse. As noted by LeBlanc-Duchin and Taukulis [36], it should also be noted that the absence of behavioural sensitization does not mean that Ritalin use is without effects.

## **Conclusion**

The findings in the current studies identify some key characteristics of the PFC's response to experience that were previously undefined. First, it would appear that

learning under many, if not all, learning paradigms has the capacity to alter dendritic arborization and/or spine density in the PFC, albeit differently, in the rat. Interestingly, in contrast to psychostimulant-induced changes which appear to be relatively permanent, it is likely that training or learning-induced alterations are transient in nature. It is reasonable to conclude that these transient changes would occur to facilitate initial learning processes but would be unnecessary once the requirements of the task were well established.

Secondly, these studies point toward an increased susceptibility of the pre- and peri-adolescent brain to environmental factors such as Ritalin use, supporting the notion that adolescence is a 'sensitive' period in development. So, although Ritalin altered circuitry in both the immature and adult brain alike, the age-related differences in the anatomical and behavioural results would indicate that the developing brain responded very differently to the experience, with early exposure resulting in behavioural deficits that were not present following adult exposure. Although age-related differences in response to environmental factors in itself is not novel, the current set of studies are the first to show that such differences are present in the PFC and can be seen in both the effects of complex housing as well as psychostimulant use.

A third and unexpected finding was that such a low dose of Ritalin would have the capacity to produce such enduring changes in brain circuitry and behaviour supported by the PFC. Moreover, the long-term impact of early Ritalin use on later behaviour is especially disconcerting when again taking into consideration the low dose of Ritalin used in the current studies.

The news may not be all bad, however. The results of study four in which caffeine appeared to 'off-set' the later cognitive deficits produced by early Ritalin use are encouraging as they suggest that the negative impact of early drug experience may be reversible. Indeed a recent study by Bolanos and colleagues [7] have shown that antidepressants may be useful in reversing some of the enduring behavioural effects of early Ritalin exposure. Additionally, the anatomical results would suggest that it would be prudent for future investigation to focus on treatment strategies that produce comparable plastic responses in the OFC (increases) and Cg3 LV (decreases) neurons.

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