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Age, Experience, and NMDA Antagonism: Dynamic Regulation of Parvalbumin Expression in Normal and Chronically Ketamine-Treated Rats

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A marked and selective decrease in the overall expression of the calcium-binding protein, parvalbumin (PV), has been a consistent postmortem finding in patients with schizophrenia. The documented reduction in calcium-binding protein immunoreactivity is specific to PV, and is observed exclusively within the prefrontal cortex and hippocampus (HPC) of patients. Models of schizophreniform cognitive dysfunction induced by chronic NMDA-receptor hypofunction utilizing non-competitive NMDA antagonist drugs such as ketamine (KET), have been shown to show comparable decreases in HPC and prefrontal cortical PV expression. Down-regulation of PV immunoreactivity within these key brain regions associated with cognition is often considered to be a marker for underlying neural pathology. However, contradictory findings are present in the literature, with some groups reporting decreased, increased, or no change in PV expression following comparable NMDA antagonist treatments. Upon examining methodology across studies, it is clear that inconsistencies, particularly with regard to the age of subjects, time of tissue collection, and treatment-induced behavioral impairments as related to PV outcomes. In order to better understand the distinct roles of these factors on PV expression, the present set of studies within this dissertation sought to elucidate upon the following: 1) age-dependent baseline regulation of PV expression; 2) effect of chronic KET treatment (30 mg/kg IP; 14 consecutive days) on cognitive performance in a spatial memory task; 3) effect of

chronic KET treatment on PV expression, and its relationship to observed cognitive impairment; and 4) the interaction between age and KET administration on PV expression. The findings presented herein provide evidence suggesting a dynamic interaction between age and chronic KET treatment on overall PV expression within HPC and neocortical regions. Based on our data, we propose that PV expression is a highly dynamic neuronal marker, which can be modulated based on a myriad of factors (e.g. age, experience, treatment, etc.). Taken together, the findings from the present work suggest that changes in PV expression following NMDA antagonist treatment should be considered in the context of age, and that PV may not be an accurate or translational marker of pathological dysfunction.

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CHAPTER 1

Introduction

1.1 Schizophrenia

Schizophrenia (SCZ) is a debilitating and complex psychiatric illness which affects approximately 1% of the global population. It is often characterized by its positive symptoms including hallucination and delusions, as well as negative symptoms such as an overall flattened affect and deficits in social interactions (Kay et al., 1987; Schultz et al., 2007). Notably, patients with SCZ also suffer from marked and pervasive deficits in both attention and cognition (e.g., working memory, spatial navigation, episodic memory, etc.), which are directly linked to functional outcomes and overall quality of life (Tamminga et al., 1998; Reichenberg, 2010; Carbon & Correll, 2014). As theories regarding the etiology of SCZ have evolved, novel approaches into understanding the developmental trajectory of neuropathological changes within key brain circuits has become a topic of interest (Insel, 2010), especially with regard to neural circuits that exhibit dynamic changes across the lifespan (Swann et al., 1989; Watanabe et al., 1992; Lujan et al., 2005). In order to better understand these impairments, and thus foster development of novel cognitive-targeting interventions, a more comprehensive understanding of the neurobiology of forebrain circuits that support the cognitive processes altered in SCZ is warranted.

The neural pathophysiology underlying symptoms is poorly understood and theories regarding root mechanisms have shifted since the initial dopaminergic hypothesis prevalent in the mid-to-late 20th century to more glutamatergic-centric hypotheses (Carlsson et al., 2001; Sodhi et al., 2008). In support of this advancement in

understanding, emerging research into pathological dysfunction within key brain circuits associated with SCZ cognitive deficits have centered on both glutamatergic (Kantrowitz and Javitt, 2010) and GABAergic (Gonzalez-Burgos et al., 2011) circuitry within the neocortex and hippocampus (HPC). In fact, HPC circuitries, as well as HPC-cingulate cortical circuit systems, are thought to be altered at both macro- (e.g. regional interconnections) and micro- (e.g. local inhibition onto pyramidal cells) levels (Benes, 2000). Afferent and efferent connections between the dorsolateral prefrontal cortex (DLPFC) and HPC are also thought to be altered, thereby contributing to deficits seen during cognitive processes that require the manipulation and temporal and/or spatial encoding of information (Meyer-Lindenberg et al., 2005). Furthermore, local inhibitory circuitry within the HPC has been found to be altered at the synaptic level (e.g. aberrant functionality and synaptic connections) which contributes to widespread cognitive dysfunction (Harrison, 2004). Within these circuits, we are particularly interested in changes seen in GABAergic local circuit networks, as they are critical in regulating cognitive and memory processes (Buzsaki & Chrobak, 1995).

1.1.1 Postmortem neuropathology

Investigations into neuropathology in postmortem human brain tissue of patients with SCZ have revealed abnormalities in structure, as well distinct biological markers, which have given us insight into the possible underlying mechanisms and trajectory of SCZ cognitive dysfunction. Overall, these findings have propelled the advancement of modeling techniques used in rodents to selectively study these putative factors to better understand their role in the perseveration of the SCZ phenotype (Schmitt et al., 2011; Benes, 2015). Studies characterizing postmortem tissue of patients with SCZ have

revealed both gross and focal anatomical changes in cortical circuits, including the HPC and prefrontal cortex that are integral to higher order cognitive processes. Selective reductions of grey matter volume within the prefrontal cortex (Buchanan et al., 1998), as well as within the HPC (Guo et al., 2014) have been reported, and these findings have been linked to cognitive disturbances (Buckley, 2005; Wolf et al., 2008). Researchers have repeatedly characterized significant increases in overall ventricular size, particularly within the lateral ventricles, which coincides with decreased overall temporal lobe volume (for review, see McCarley et al., 1999). White matter tracts have are also impacted in schizophrenic postmortem brains (Davis et al., 2003), indicating a decrease in the overall connectivity between brain regions, thereby severely limiting cooperative transfer of information.

Alterations in activation seen during neuroimaging studies in DLPFC are likely due to dysfunctional neuronal activity. Specifically, a decrease in GABAergic neurotransmission, and consequently decreased inhibition of excitatory pyramidal neurons; conversely, even increased inhibition due to compensatory up-regulation of GABA receptors have been described (Volk & Lewis, 2002; Gonzalez-Burgos & Lewis, 2008; Lisman et al., 2008). Of particular note, the selective decrease of parvalbumin (described in detail later) expression within inhibitory GABAergic neurons, especially within prefrontal cortex and HPC, is thought to play a key role in these observed alterations (Lewis et al., 1999; Hashimoto et al., 2003; Lewis et al., 2004). Disrupted signaling within this circuit is likely the driving force behind deficits, and therefore GABAergic neurons are being considered as targets for possible cognitive-enhancing treatments (Lewis et al., 2005).

In addition to gross anatomical and circuit-level abnormalities, postmortem investigations have also suggested distinct focal changes at the neuronal level: both in neuronal organization, as well as in overall neural morphology (Arnold, 2000; Chana et al., 2003), which may indicate a neurodevelopmental trajectory for the disorder (Fatemi & Folsom, 2009). Within the HPC, there is evidence for altered and disorganized laminar organization of pyramidal neurons (Conrad et al., 1991), which is likely due to disruption in migration during development (Conrad & Scheibel, 1987). Additionally, researchers have observed decreases in HPC (e.g., Arnold et al., 1995), and prefrontal cortical layer 3 (e.g., Pierri et al., 2001) neuron size in SCZ patients. Morphological changes in neurons within the HPC and prefrontal cortex likely stem from early fetal insults (e.g., Meyer & Feldon, 2009), genetic abnormalities (e.g., Kamiya et al., 2005), or even some combination of biological and environmental factors (e.g., Yuii et al., 2007), which disrupt neuronal migration and maturation (Bunney et al., 1995; Lewis & Levitt, 2002).

Interestingly, many of the characteristic morphological changes seen in SCZ neuropathology are associated with subsets of GABAergic inhibitory neurons and glutamatergic pyramidal cells. Such changes include decreases in the level of glutamatergic receptor subunits GluR1 and GluR2 on GABAergic neurons within the prefrontal cortex of patients (Corti et al., 2011); as well as decreases in the overall number of dendritic spines on cortical glutamatergic pyramidal cells (Glantz & Lewis, 2000). Considerable focus has recently been placed on select alterations in GABAergic neurons expressing the calcium-binding protein (CBP) parvalbumin (PV), which is detailed below. Why forebrain PV-expressing GABAergic neurons would be selectively

disrupted is unclear, although evidence presented within this dissertation (specifically, chapters 2 and 5) suggest developmental changes in PV expression that perhaps make these neurons vulnerable to dysfunction.

1.2 Parvalbumin

PV is one of the primary CBPs located in the brain, and is highly expressed in specific subpopulations of GABAergic neurons. In addition to PV, additional CBPs include calretinin, calmodulin, and calbindin (Freund & Buzsaki, 1996; Markram et al., 2004), though particular emphasis is placed on PV-expressing GABAergic neurons due to the selective decrease in this subpopulation, without concomitant changes in other CBP expression, in SCZ postmortem tissue (Reynolds et al., 2004). PV expressing GABAergic inhibitory neurons are located relatively diffusely throughout most, if not all, brain regions but exhibit distinct morphological characteristics as well as specified laminar distribution within the neocortex and HPC (Freund & Buzsaki, 1996; Hof et al., 1999). PV expression can be seen in three primary GABAergic cell types, which include chandelier cells, basket cells, and O-LM cells (which are specifically located in stratum oriens in the HPC; Klausberger, 2009). Chandelier cells are primarily located in cortical layers II-VI, as well as within the HPC (though here they are often referred to as axo-axonic or bistratified cells), and have axonal terminations exclusively on the axon initial segment of excitatory pyramidal neurons (Markram et al, 2004). Though there are relatively few of this cell type compared to other inhibitory interneurons, these are thought to be very powerful with regard to their ability to regulate neural transmission in the brain (Woodruff et al., 2010). Due to their inhibition directly onto the axon initial

segment, chandelier cells also play an important role in regulating pyramidal cell excitatory output (Lewis et al., 2004). The unique ability for a single chandelier cell to target large populations of pyramidal neurons allows for wide-spread synchronization of excitatory transmission (Cobb et al., 1995).

Within the CA1 region of the HPC, basket cells and chandelier cells make up approximately 75% of the total inhibitory neuron population (Szilagyi et al., 2011). The anatomical distribution of PV expressing inhibitory neurons within each sub-region of the HPC (CA1, CA3, dentate gyrus) appears to be consistent in laminar profile across the septotemporal axis, and shows no septotemporal variation in expression within the HPC sub-regions in the rat (Kosaka et al, 1987; Nomura et al, 1997a; 1997b). Basket cells, which are located within the cortex, HPC, and cerebellum (Seto-Ohshima et al., 1989), synapse onto pyramidal cell somata, as well as their proximal dendrites, and are located primarily within the HPC pyramidal and granule cell layers (Nitsch et al., 1990; Freund & Buzsaki, 1996). Similar to chandelier cells, a single PV-containing basket cell has the ability to make a large number of connections onto local pyramidal neurons, as well as synapsing with other PV-expressing neurons (Sik et al, 1995), thereby giving them the ability to strongly modulate postsynaptic excitatory output (Aradi et al., 2002). PV expressing cells are also located outside of the pyramidal and granule cell layers within the hilus, as well as within stratum oriens where these PV-expressing neurons are referred to as O-LM cells (Sloviter, 1989).

A selective decrease in PV immunoreactivity in schizophrenic postmortem tissue has been a robust finding in the literature, particularly within the prefrontal cortex (e.g., Beasley & Reynolds, 1997) and the HPC (e.g., Zhang & Reynolds, 2002). This

decrease in PV expression suggests an overall decrease in the amount of inhibition being provided to excitatory postsynaptic pyramidal cells. This decrease in regulatory inhibition likely puts the system in a state of focal hyperexcitability, which may serve to distort temporal specificity in neuronal interactions and communication, as well as create excess neuronal 'noise' within neocortical and HPC circuits. Overall, PV-containing inhibitory neurons play a key role in the regulation of neuronal communication within and between local circuits in the neocortex and HPC, and disruption of both local and long-range interactions likely lead to impairment the accuracy and efficiency of cognitive processing.

1.2.1 Age-related changes

Age-related changes in PV expression have been found in cortical (e.g., Ouda et al., 2008), as well as subcortical forebrain regions including the HPC (e.g., Vela et al., 2003; Yamada & Jinno, 2013). However, there have been very few studies offering conclusive findings outlining age-dependent changes in HPC PV expression in a systematic way. A paucity of studies have been conducted seeking to directly compare PV expression in young and aged rats in an attempt to characterize CBP distribution as a function of developmental age within the HPC, and the literature reveals conflicting findings. There are findings supporting a decrease in PV expression as a function of age (e.g. Shetty & Turner, 1998;Lee et al., 2008), as well as those indicating no change in PV expression across aging (e.g. Kishimoto et al., 1998; Vela et al., 2003; Stanley et al., 2012). Lee and colleagues (2008) provide evidence for a decrease in PV expression from young (3 months) to aged (6 months), with reduced levels staying stable through 24 months of age. Shetty and Turner (1998) also describe significant decreases in PV

immunoreactivity within HPC CA1, CA3, and hilus between 5- and 24-month old rats, while Kishimoto and colleagues (1998) describe no change in PV expression within the HPC as a function of age. Vela and colleagues (2003), as well as Stanley and colleagues (2012), also describe a lack of age-related effects, though their analyses only focused on PV expression in adult and aged rats and lacked an adolescent comparison. The latter studies indicating no change in PV expression as a function of aging are only exposing a small portion of the lifespan, and it is likely that, had they included an adolescent time point in their analyses, they would have seen age-dependent changes in PV expression in lined with findings from the former studies.

The aforementioned findings, particularly those described in Lee et al. (2008) describing age-related decreases in PV expression, are in agreement with observation in our own lab describing an age-related decline in HPC PV expression (Corriveau, Master's Thesis, 2013), in addition to findings presented in this dissertation (see Chapter 2).The lack of systematically conducted normative baseline data regarding PV distribution across the lifespan is concerning, especially considering that rodent studies examining PV expression rarely account for the age of the animals used. Furthermore, the paucity of baseline data makes it difficult to make generalizations based on data derived from experiments utilizing drug manipulations, as PV immunoreactivity may be dynamically altered based on a variety of endogenous and exogenous factors including, but certainly not limited to, age and experience.

1.2.2 Experience-related changes

Experience-related changes in the expression of neuronal and cellular markers (e.g., BrdU, NeuN, BDNF, apoptotic markers, etc.) are a well-established phenomenon in the

literature (van Praag et al., 2000; Hannan, 2014; Sale et al., 2014). A multitude of experiences are capability of modulating brain structure and function, including (but not limited to): maze training (Van der Borght et al., 2005), social interaction (Woolley & Doupe, 2008); physical stimulation (e.g. stimulation of whiskers; Knott et al., 2002); sexual experience (Leuner et al., 2010); stress (Zoladz et al., 2012); and environmental enrichment (Harati et al., 2011). There are few studies which have explicitly examined the relationship between enrichment and PV expression, but those that have been reported indicate clear enrichment and/or deprivation-related alterations within sensory cortices (e.g., Jiao et al., 2006) and HPC (e.g., Iuvone et al., 1996). Iuvone and colleagues (1996) provide clear evidence of experience-related increases in PV expression across all HPC sub-regions, which is consistent with our findings in extensively trained rats in previous studies conducted in our lab (Corriveau, Master's Thesis, 2013), in addition to the present work outlined in Chapter 4 of this dissertation.

1.2.3 NMDA antagonist induced changes

Of particular relevance to the selective decrease in PV expression within SCZ postmortem tissue have been studies employing acute and/or chronic NMDA-antagonist drug regimens in rodents (e.g. Kittelberger et al., 2012). The administration of pharmacological substances blocking NMDA receptors (e.g. ketamine (KET); PCP; MK-801) has been shown to significantly impact both human and rodent performance in cognitive tasks involving working (e.g., Driesen et al., 2013), episodic (e.g., Nakazawa et al., 2003), and spatial memory systems (e.g., Butelman, 1989), with some rodent studies noting concomitant decreases in PV expression in line with observed behavioral impairments (e.g. Kittelberger et al., 2012). While studies generally report decreases in

overall PV expression as a result of NMDA-antagonist administration (e.g. Romon et al., 2011; Wang et al., 2008; Braun et al., 2007; Keilhoff et al., 2004), there are some labs with evidence suggesting no change in PV-immunoreactivity following acute or chronic NMDA-antagonist pharmacological manipulations (e.g. Benneyworth et al., 2011; Zhang et al., 2008). Adding another level of complexity, there are some reports of increased cortical PV expression following NMDA-antagonist treatments (Abdul-Monim et al., 2007; Sabbagh et al., 2013). These conflicting findings that evidence increases (Abdul-Monim et al., 2007), decreases (Keilhoff et al., 2004; Behrens et al., 2007; Braun et al., 2007; Wang et al., 2008; Romon et al., 2011; Kittelberger et al., 2012), and no significant effects (Zhang et al., 2008; Benneyworth et al., 2011) following NMDA antagonist (e.g. KET) administration, raise questions regarding the methodological preparations employed, as well as the translational validity of the findings. Upon examination of the aforementioned literature, it becomes clear that age is not controlled for as a possible factor influencing PV expression. Rather, many of these studies seem to assume stability in PV expression over the lifespan. A major rationale for the current set of studies was to examine age-related differences in PV expression, as well as other possible mediating factors (e.g. cognitive-behavioral experience; tissue collection timeline) in order to provide clarity to the existing literature and inform future studies centering on NMDA-antagonist induced changes in neuropathology.

1.3 Dissertation Purpose

The purpose of the present dissertation was threefold: 1) to provide characterization of baseline PV expression as a function of age and behavioral experience; 2) to provide evidence of the dynamic interaction of chronic KET treatment with age and behavioral experience on cognitive performance on PV expression; and 3) to systematically describe the mediation of PV expression by interactions between age, time of sacrifice, and KET administration. Four distinct studies were conducted in order to better understand and characterize the role of glutamatergic hypofunction on age- and experience-dependent changes in PV expression that have been previously observed in our lab. Chapter 1 of this dissertation details the normative developmental distribution of PV within the HPC, striatum, and neocortex across key milestones in the rat: young (1 month), adult (6 months), and aged (12 months), indicating an overall decrease in expression with age. Chapter 2 provides evidence for chronic KET-induced disruption of spatial memory processes in the absence of concomitant changes in HPC and cortical PV expression. Chapter 3 specifically characterizes impact of KET administration in both behaviorally naïve and trained rats, indicating a dynamic effect of treatment based on experience. Finally, chapter 4 provides evidence for an opposing effect of KET treatment on young (1 month) compared to adult (6 months) rats, in addition to providing compelling evidence for a recovery of phenotype based on time of sacrifice. Together, the studies presented herein provide insight into the dynamic nature of PV expression and its relationship to cognitive performance.

CHAPTER 2

Evidence for age-dependent and brain region specific changes in parvalbumin protein expression

2.1 Abstract

A selective decrease in parvalbumin (PV) immunoreactivity is seen in post-mortem schizophrenic hippocampus and prefrontal cortex. Animal models of schizophreniform dysfunction following acute and/or chronic ketamine (KET) treatment show comparable PV decreases. However, there exist conflicting reports with respect to PV immunoreactivity following sub-chronic KET treatment; calling into question the efficacy of using PV as a pathological marker. Upon examination of methodology across studies, it is clear that there are differences in protocols, particularly with regard to age during treatment and/or sacrifice. Furthermore, the literature lacks a sufficient description of baseline/ normative PV expression in drug- and behaviorally-naïve tissue. In order to understand the putative role of PV in pathology, systematic characterization of normative distribution across ages is warranted. The present study examined PV expression across the septotemporal axis of the rat hippocampus and in neocortical and striatal regions in 1, 6, and 12 month old rats. Our findings suggest that hippocampal PV expression in untreated naïve rats decreases from 1 to 6 months of age, with levels remaining stable through minimally 12 months of age. Furthermore, we provide evidence that age-related changes in PV are specific to the hippocampus, as PV expression remained stable in all cortical and striatal areas examined. Based on our data, we propose that PV expression is a dynamic marker, and that changes in expression based on age should be considered when modeling pathology.

2.2 Introduction

In reviewing the literature, it is clear that there is a glaring discrepancy in the ages of rodents used across studies aiming to explore the impact of acute and/or chronic ketamine (KET) administration on parvalbumin (PV) expression and cognitive functioning. Despite using similar pharmacological preparations and methods, Benneyworth and colleagues (2011) found no change in PV expression following KET administration in young rats, whereas Kittelberger and colleagues (2012) found significant changes in PV expression following a similar KET protocol. This oversight in the age of experimental subjects is problematic, as there is evidence of a developmental shift in NMDA receptor expression and composition across the lifespan (Law et al., 2003; Cull-Candy et al., 2001; Monyer et al., 1994; Watanabe et al., 1992). This developmental shift in the pattern of subunit composition likely plays a role in how impactful NMDA antagonist treatment is, both on underlying biology and on behavior. This is evidenced in the ability for NMDA antagonists (e.g. MK-801) to block neurotoxicity in young, but not old, rats (Zhou & Baudry, 2006).

Within normally developing organisms, there is some evidence suggesting that PV protein expression and distribution may vary as a function of developmental age in monkeys (Akil and Lewis, 1992) and rodents (Gao et al., 2000; Sanchez et al., 1992). The findings with respect to PV expression across the lifespan is varied, and evidence also suggests that there may be species- and strain-related differences in the baseline levels of PV immunoreactivity across brain regions (Hof et al., 1999 ; Ouda et al., 2008, respectively). Despite some insight into PV distribution in normally aging animals, developmental changes in baseline PV expression within normally developing behaviorally- and pharmacologically-naïve rats have not yet been *systematically* studied

across the lifespan. Notably, characterization of PV expression with respect to distinct HPC sub-regions and across the septotemporal extent, as well as in cortical regions, across development is warranted., This baseline characterization is needed to address 1) the gap in the literature regarding normative distribution; 2) the lack of understanding of the role of PV in development and cognition; and 3) to inform subsequent studies utilizing PV as a marker of pathology as it relates to psychiatric disorders (i.e. SCZ). To that end, the current study sought to elucidates upon the expression of PV across the lifespan of the rat, and herein we provide evidence for a dynamic interaction between PV expression and developmental age, with particular reference to the HPC formation and across its septotemporal axis.

2.3 Methods

2.3.1 Subjects

The current study utilized eighteen male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) spanning three distinct ages: adolescent (approximate age P30; referred to as 1 month old; *n* = 6), young adult (approximate age P120-180; referred to as 6 months old; *n* = 6), and adult (approximate age of 12 months old; *n* = 6). Adult rats were single-housed, while young rats were pair-housed, in clear polycarbonate caging with access to food and water *ad libitum*. All rats were experimentally and pharmacologically naïve and experienced normal husbandry procedures within a humidity and temperature controlled vivarium with a 12-hour light/dark cycle (lights on at 0800h) and arrived to the colony approximately 3 weeks prior to sacrifice and tissue collection. All housing and experimental procedures were in

accordance with, and approved by, the University of Connecticut Institutional Animal Care and Use Committee.

2.3.2 Immunohistochemistry

Rats were anesthetized with Euthasol (pentobarbital sodium solution; 0.5 – 1mL via intraperitoneal (IP) injections) and transcardially perfused with ice-cold 0.9% physiological saline solution immediately followed by ice-cold 3.7% paraformaldehyde solution. Following perfusions, brains were removed and individually stored in scintillation vials filled with 3.7% paraformaldehyde solution for 1 week. Prior to sectioning, brains were transferred into a 30% sucrose solution for cryoprotection. All brains were sliced into 60µm serial coronal sections on a cryostat and stored in phosphate-buffered saline (PBS)-containing well-plates for storage prior to immunohustochemical procedures.

One series of tissue sections from each brain was selected to process PV immunoreactivity, with sections encompassing tissue approximately -2.5mm through -6mm relative to Bregma (Paxinos & Watson, 1997) to include the septotemporal extent of the HPC, and sections from +2.5mm through +0.25mm relative to Bregma to encompass PFC (anterior cingulate) and caudate regions (see Figure 2.1). Free floating sections were initially blocked in 5% normal goat serum (NGS; Jackson Immuno), 0.1% triton-X, and PBS solution for 1 hour, followed by 3 five-minute rinses in PBS. Sections were then transferred into primary antibody solution (1:4000 anti-rabbit parvalbumin polyclonal antibody (Calbiochem), 0.1% triton-X, and PBS) for a 24 hour incubation period at room temperature. Sections were then rinsed in PBS 3 times at five minutes each, and transferred to secondary solution (horseradish peroxidase (HRP) labeled polymer anti-rabbit; DAKO; Carpinteria, CA) for a 2 hour incubation at room

temperature. Following a final set of 3 five-minute rinses in PBS, sections were transferred into a diaminobenzidine (DAB) chromogen solution (DAKO) for ten minutes to develop the stain. Sections were mounted and dried on glass slides, then cover-slipped for microscopy using DPX. Adjacent sections were processed for Nissl body staining to assist with precise identification of brain regions.

2.3.3 Quantification of PV expression

Slides containing tissue processed for PV immunoreactivity was analyzed using a Nikon Eclipse E600 (Melville, NY) upright microscope equipped with an Insight SPOT digital camera (Diagnostic Instruments, Inc.). Photomicrographs of regions of interest included: prefrontal cortex (anterior cingulate), HPC (including the CA1, CA3, and DG sub-divisions), auditory cortex, caudate, retrosplenial cortex, and somatosensory barrel fields (see Figure 2.2). For each rat, 6 photomicrographs were taken per region, with 3 representing the left hemisphere, and 3 representing the right hemisphere. Photomicrographs for all regions were stored digitally for analysis by a researcher blind to experimental condition. To ensure precision in the quantification of PV-expressing cells within each region of interest, photomicrographs were taken at either 20x magnification (CA1, CA3) or 10x magnification (cortical areas, caudate, DG).

Sections were chosen for analysis based on their anatomical location with reference to Bregma. Prefrontal cortical (anterior cingulate) and caudate representative sections were taken from approximately +2.5 to +0.25mm relative to Bregma (see Figure 2.1A). Representative photomicrographs containing septal HPC, retrosplenial, and somatosensory regions were taken from levels corresponding to -2.45mm to -3.9mm relative to Bregma (Figure 2.1B). Finally, midseptotemporal and temporal HPC

samples were taken from levels corresponding to -5.0mm to -6.0mm relative to Bregma (Figure 2.1C). Photomicrograph samples were analyzed using ImageJ software (NIH), and the number of PV-immunoreactive cells was quantified using macros written to automate particle counting within each region. An example of the ImageJ analysis can be seen in Figure 2.3.

2.3.4 Statistical Analysis

In order to explore differences in PV-immunoreactivity within each region, the number of PV-expressing cells, as measured using ImageJ, was averaged for each rat within each brain region. This average was calculated using 3-6 representative photomicrographs per region, and data was collapsed across hemispheres as there were no significant hemispheric differences in PV expression. Two-way ANOVAs were conducted for each HPC sub-region (CA1, CA3, DG) to determine the relationship between age (1, 6, 12 months) and septotemporal location (septal, MST, temporal). Appropriate post-hoc analyses were conducted to explore significant main effects. Regional differences in the average expression of PV as a function of age were assessed using one-way ANOVAs on each region of interest (e.g. prefrontal cortex, septal CA1), followed by post-hoc analyses where appropriate (Kirk, 2012).

2.4 Results

2.4.1 Summary of previous and current findings

In previous studies, we noted a significant decrease in PV expression across the long axis of the HPC which varied with age (Corriveau, Master's Thesis, 2013). We observed a roughly 10-20% decrease in PV expression in MST and temporal regions of CA1 in 1 month old rats compared to septal regions of CA1. This septotemporal decrease along

the HPC axis was much more prominent (over 50%) in MST and temporal – compared to septal – CA1 in 6 month old rats. These observations were confirmed in the present study and extended to include 12 month old rats, where there was approximately a 50% decrease in MST and temporal CA1 compared to septal CA1. Similarly, we observed a small decrease in PV expression along the long axis (between septal and MST regions) within the DG, and found a significant decrease in PV expression in 6 month old rats as compared to 1 month old rats. No obvious changes were observed in the CA3 region in our previous work. Thus, it appears PV expression is highest in young (1 month old Sprague-Dawley) rats with minimal septotemporal difference across the long axis of the HPC. Age-related decreases are observed in 6 month and 12 month old Sprague-Dawley rats and the decline in PV expression is most prominent at more temporal regions along the septotemporal axis.

Given the prior observations of significant changes in CA1 and the DG, our analyses focused primarily on these two regions. In brief, the major finding of an age-related decline in PV expression from 1 to 6 month olds was most prominent at the temporal regions of CA1; septal and temporal regions of CA3; and both septal and MST regions of the DG. These findings are in line with previous observations seen in our lab. This observed decline was very similar in 12 month old compared to 1 month old rats, with no evidence of further reductions as compared to the 6 month old group observed.

2.4.2 Cortical PV expression remains stable across lifespan

The density of PV positive neurons varied dramatically in different forebrain regions of interest in the present study. The highest density of PV expressing cells was observed in the somatosensory cortex (barrel fields; approximately 150 cells/1600µm^2) as compared to approximately 60-80 PV expressing cells per 1600µm^2 in associative

neocortical areas (prefrontal and retrosplenial cortices). Fewer PV positive cells were located in primary auditory cortex as well as the subcortical caudate nucleus. Despite these regional differences, no significance changes were observed across the three age groups. Thus, one-way ANOVA analyses revealed that there were no significant differences in the number of PV expressing neurons within the somatosensory cortex barrel field region ($F(2, 17) = 0.44$, $p = 0.65$) or within the prefrontal cortex ($F(2, 17) = 2.32$, $p = 0.13$) regardless of developmental age. The same pattern was seen in other cortical regions examined, with no significant age-related changes in PV expression within the caudate ($F(2, 17) = 2.28$, $p = 0.14$), retrosplenial cortex ($F(2, 17) = 0.21$, $p = 0.82$), or auditory cortex ($F(2, 17) = 0.81$, $p = 0.47$). Summary of findings can be seen in Figure 2.4, and representative photomicrographs of each region across age can be seen in Figure 2.5.

2.4.3 Septotemporal variation in HPC PV expression as a function of age

Consistent with our preliminary analysis of age and septotemporal variation in the HPC, the density of PV positive neurons was generally less in each HPC sub-region (CA1, CA3, DG) across all longitudinal positions (septal, MST, temporal) in 6 month old rats as compared to 1 month old rats (see Figure 2.6). The degree of decrease was most apparent in those areas exhibiting the highest number of PV positive neurons at 1 month of age. Two-way ANOVA analyses revealed a significant main effect of age in CA1 ($F(2,45) = 3.75$, $p < 0.05$), CA3 ($F(2,45) = 4.47$, $p < 0.05$), and DG ($F(2,30) = 9.17$, $p < 0.001$). A main effect of septotemporal location was also seen in HPC CA1 ($F(2,45) = 5.19$, $p < 0.01$) and DG ($F(2,30) = 12.72$, $p < 0.001$), but not in CA3 ($p > 0.10$). There were no significant interactions between age and septotemporal location in any HPC sub-region (all p's > 0.10).

Post-hoc analyses conducted to explore the significant main effect of age revealed that 1 month old rats had significantly higher PV expression than 6 and 12 month old rats in CA1(*p*'s < 0.05). In CA3, 1 month old rats had significantly higher PV expression than 6 month and 12 month old rats ($p > 0.01$ and $p = 0.05$, respectively). IN DG, 1 month old rats had significantly higher expression than 6 ($p < 0.001$) and 12 ($p < 0.01$) month old rats. PV expression in 6 and 12 month old rats did not significantly differ from one another. See Figure 2.6 for summary of findings.

Post-hoc analyses conducted to explore the significant main effect of septotemporal location revealed that septal CA1 had significantly higher PV expression than temporal regions ($p < 0.01$), with a trend toward significance in MST ($p = 0.08$). Septal CA3 had significantly more PV expression than temporal CA3 ($p < 0.01$), with a trend toward significance in MST ($p = 0.053$). In HPC DG, septal PV expression was significantly higher than MST regions ($p < 0.001$). In CA1 and CA3, there were no significant differences between 6 and 12 month old rats (*p*'s > 0.10). Summary of findings can be seen in Figure 2.7, and descriptive statistics (M±SEM) can be found in Table 2.1.

2.4.4 HPC PV expression decreases across the lifespan

Our data revealed a significant effect of PV expression as a function of age within each HPC sub-region (CA1, CA3, DG). One-way ANOVA analyses indicated a significant effect of developmental age on PV expression within septal HPC regions of CA3 ($F(2,17) = 4.95$, $p < 0.05$) and DG ($F(2,17) = 5.87$, $p = 0.05$). Within the MST HPC, a significant effect of age was observed in the DG ($F(2,17) = 6.82$, $p < 0.01$). In temporal aspects of the HPC, significant changes in PV expression as a function of age were seen in CA1 ($F(2.17) = 4.72$, $p < 0.05$) and a trend toward significance in CA3 ($F(2,17)$

= 2.96, p = 0.08). In contrast, there were no significant changes in PV expression as a function of age in septal and MST CA1 (p's > 0.05) or in MST or temporal CA3 (all p's > 0.05). See Figure 2.6.

In the event that there was a main effect of age, post-hoc analyses were conducted to explore the relationship between PV expression and developmental age. Within CA1, LSD post-hoc analyses revealed a selective age-related change in PV expression within temporal aspects of the region indicating that 1 month old rats had significantly higher PV expression than 6 or 12 month old rats (p's < 0.05). There was no significant difference in PV expression between 6 and 12 month olds in CA1.

Septal CA3 revealed an effect of age, such that 1 month old rats had significantly higher levels of PV-expressing cells than either 6 or 12 month old rats (p's < 0.05), with no difference in expression between 6 and 12 month olds. Post-hoc analysis of temporal CA3 revealed a significant difference between 1 and 6 month old animals, such that 1 month olds had higher PV expression than 6 month olds (p < 0.05).

Post-hoc LSD analyses within septal DG indicated a significant effect of age such that 1 month old rats had higher PV expression than 6 month olds (p < 0.05), with a trend toward significant between 1 and 12 month olds (p = 0.08). Within MST DG, 1 month old animals had significantly higher PV expression than 6 and 12 month old animals (p's ≤ 0.01); with no significant differences between 6 and 12 month old rats. Descriptive statistics (M±SEM) for all of HPC regions can be seen in Table 2.1, and summary of findings can be found in Figure 2.6, and representative photomicrographs for each region can be found in Figures 2.8 – 10.

2.5 Discussion

Our data provide support for a growing body of evidence describing an age-dependent decline in PV expression within the HPC. The present work also provides compelling evidence for the stable expression of PV across ages within many neocortical areas including auditory, somatosensory, and retrosplenial cortices – at least within the age ranges observed in the present work – which is consistent with findings across aging in human populations (Bu et al., 2003). However, it is possible that PV expression in these cortical regions may mature at an earlier developmental time point not included in the current study. In contrast, there were no significant changes across neocortical tissue and no age-related changes in PV expression within the striatum across groups, consistent with previous findings indicating stability in PV expression as a function of age in the caudate region (Bae et al., 2015). The present data from the medial prefrontal cortical area (specifically the anterior cingulate cortex) revealed a trend toward statistical significance such that as age increased, as did the relative density of PV expression (see Figure 2.5). This finding mirrors human data suggesting an increase in PV expression commiserate with age (e.g., Fung et al., 2010; Reynolds & Beasley, 2001), which likely underlies normal age-dependent maturation of prefrontal inhibitory circuitry (Lewis et al., 2006). In summary, it appears that dynamic changes in PV expression may mirror the delayed development of higher-order associative cortices including the HPC and prefrontal cortical areas (CITE).

The most prominent changes were evidenced within the HPC, where we observed a significant decline in PV expression as a result of age in CA1, CA3, and DG regions. 1 month old rats generally express higher levels of PV immunoreactivity than 6 and 12 month old rats, suggesting that expression may be developmentally dynamic.

These findings are in agreement with previous studies reporting age-related decreases in PV expression within the HPC (Lee et al., 2013; Shetty & Turner, 1998; de Jong et al., 1996; Lolova & Davidoff, 1992), though these findings are primarily in reference to septal regions. Importantly, our data argues against findings indicating an absence of age-related decline (e.g. Hwang et al., 2008; Potier et al., 2006; Vela et al., 2003; Kishimoto et al., 1998; Miettinen et al., 1993) or the presence of age-related increases (e.g., Choi et al., 2010) in HPC PV expression.

Findings from Nomura and colleagues (1997) indicate a high degree of PV expression within young (5 week old) rats that is in line with our findings of high levels of PV expression coinciding with a lack of HPC septotemporal PV gradient in 1 month old rats. In the current study, we found that there were notable septotemporal variations in PV immunoreactivity within the HPC formation in 6 and 12 month old rats. Specifically, we observed a significant decrease in PV expression from septal to MST and temporal CA1, with no difference in expression between MST and temporal CA1 locations in 6 and 12 month old rats. This pattern of HPC septotemporal gradient in PV expression was selective to CA1 regions, with CA3 regions showing consistent PV expression across the long axis in both 6 and 12 month old rats. However, it is worth noting that the DG region of the HPC trends toward a statistically significant septotemporal gradient from septal to MST regions in both 6 and 12 month old rats – a finding which is in agreement with previous investigations (Corriveau, Master's Thesis, 2013).

The lack of septotemporal difference within the CA3 region in all age groups might suggest the possibility that PV expressing neurons in this region may be more resilient to the effects of aging, however previous work looking at age-related effects of

calcium dysregulation suggest that all HPC sub-regions are negatively impacted by aging (Mattson & Magnus, 2006). CA1 and DG regions may exhibit increased vulnerability to the adverse effects of aging, as evidenced in cognitive-behavioral tasks and imaging studies (e.g., Small et al., 2004; Barnes et al., 1997; for review see Burke & Barnes, 2006). Overall, these findings indicate a dynamic interaction between aging, HPC sub-region (CA1, CA3, DG), and septotemporal location within the HPC on overall immunohustochemical visualization of the calcium-binding protein PV. The present study is the first to systematically characterize the expression of PV immunoreactivity across the septotemporal axis of the HPC within three distinct developmental time points.

2.6 Conclusion

Overall, we present compelling evidence in support of age-dependent decreases in PV protein expression within CA1, CA3 and DG regions of the HPC from 1- to 6-month old rats, a finding which is sustained for minimally up to 12 months old age. A decrease in PV expression across the septotemporal extent of the HPC was evident in the CA1 regions of 6 and 12 month old rats, with preservation of PV levels across the long axis in all regions within 1 month old rats. Evidence for a dynamic interaction between PV protein expression within the HPC and neocortical and striatal regions as a function of developmental age suggest that PV expression is differentially regulated depending on brain location. The present findings provide a normative developmental framework to inform future studies looking at both normal and pathological distribution of PV protein expression in rodents. Specifically, the regional and age-related differences presently

shown here can shape future experiments seeking to understand the acute and long-term impact of pharmaceutical agents (e.g. NMDA-antagonists) and their relationship to PV-related neuropathology.

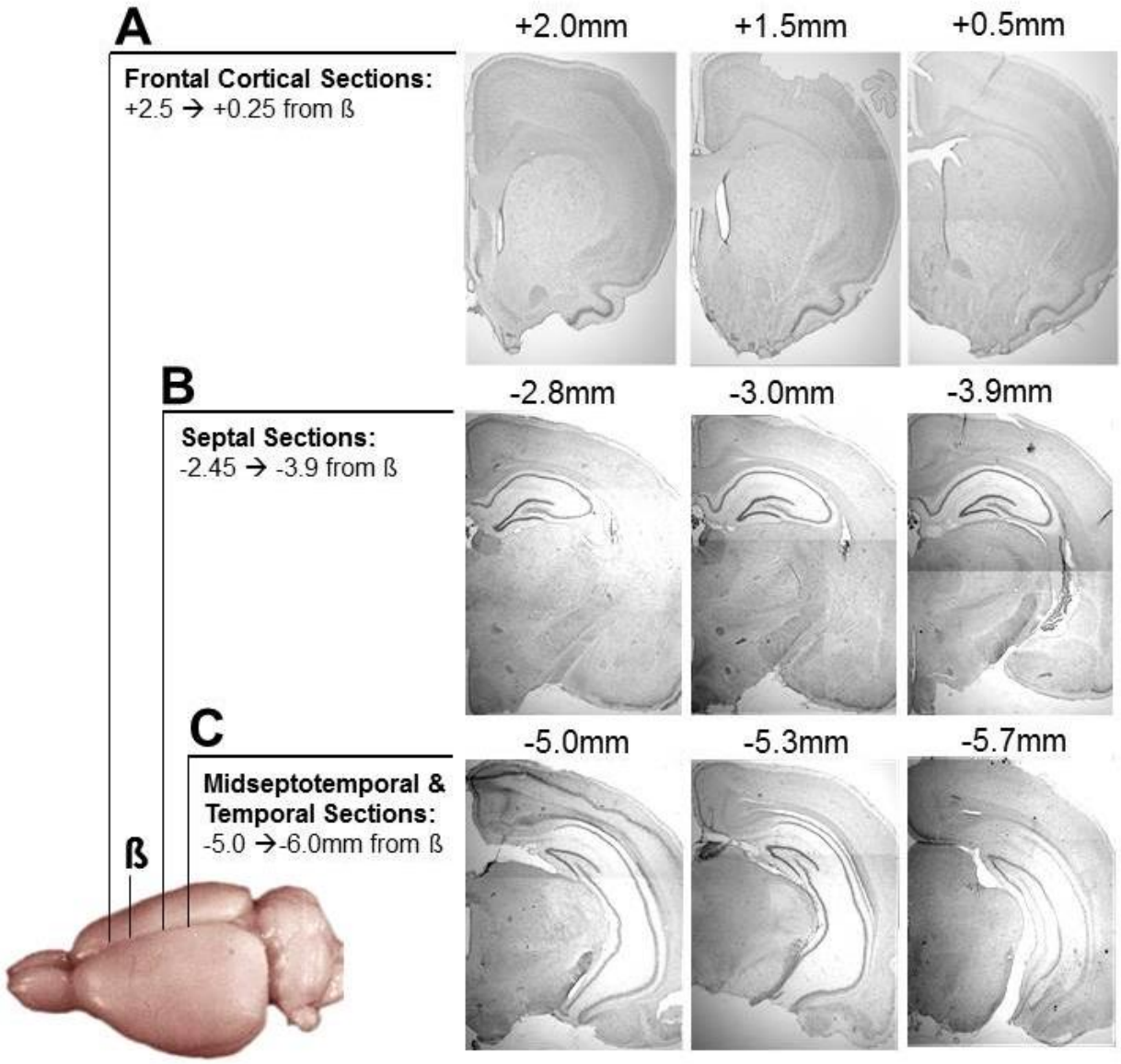

Figure 2.1: Location of representative sections relative to Bregma (ß)

Sections processed for PV immunoreactivity were selected for analysis of PV expression across the long axis of the HPC, as well as within the prefrontal cortex (anterior cingulate), caudate, somatosensory barrel fields, and retrosplenial cortex. Anterior cingulate and caudate samples were taken from sections between +2.5mm and +0.5mm relative to Bregma **(A)**. Septal HPC regions (CA1, CA3, DG), somatosensory barrel fields, and retrosplenial cortex samples were taken from sections between -2.45mm and -3.9mm relative to Bregma **(B)**. Midseptotemporal (CA1, CA3, DG) and temporal (CA1, CA3) HPC samples were taken from sections between -5.0mm and -6.0mm relative to Bregma **(C)**. This figure serves as an example of how each brain was processed; note that photomicrographs were taken in both hemispheres of the sampled sections, and that sections were roughly 240µm apart from one another.

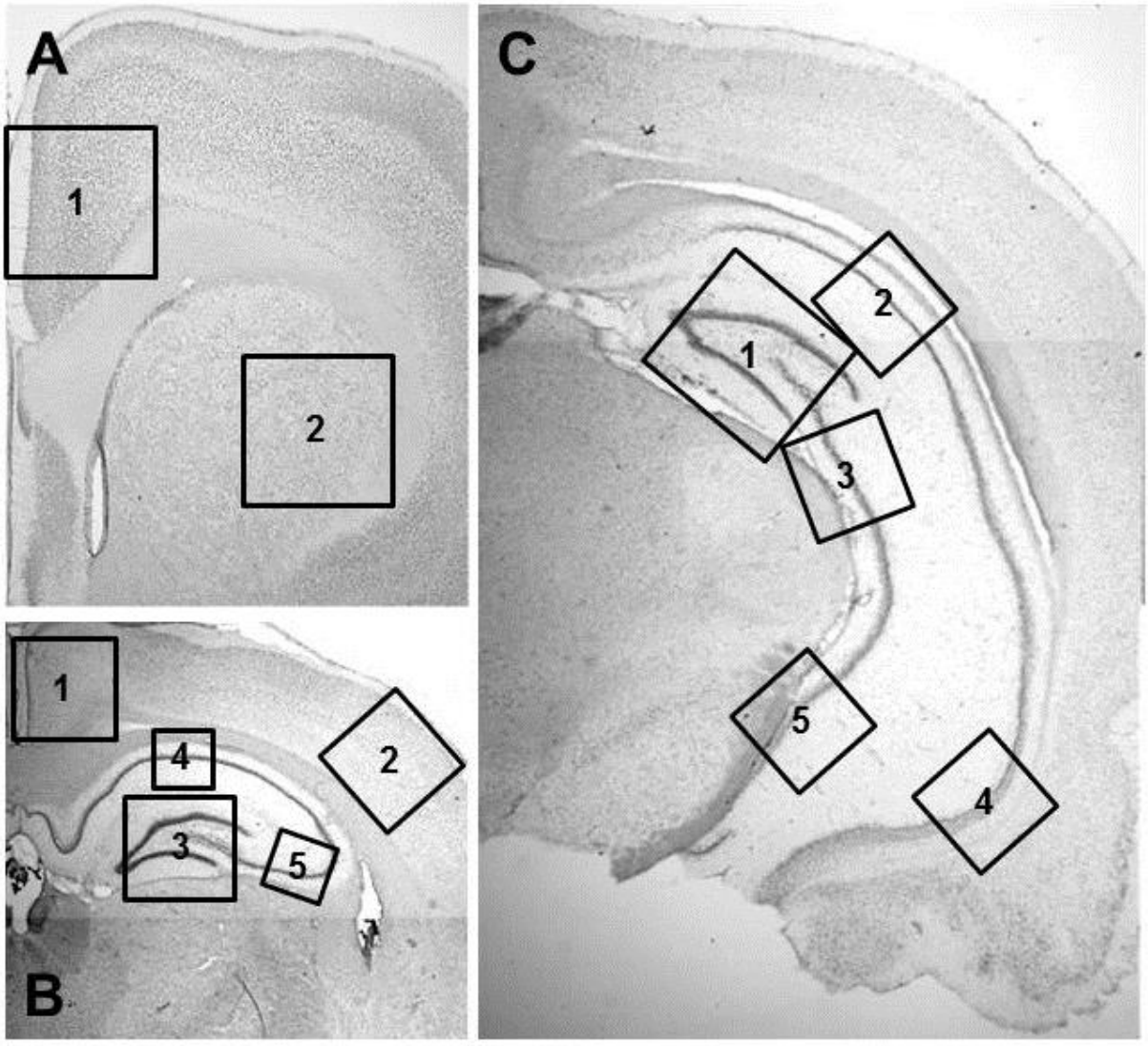

Figure 2.2: Anatomical locations of photomicrographs samples taken for PV analysis in hippocampal and cortical regions of interest.

Photomicrographs of frontal cortical (anterior cingulate cortex) **(A.1)** and caudate **(A.2)** were sampled between +2.5 through +0.25mm relative to Bregma. Retrosplenial cortex **(B.1)**, somatosensory barrel fields **(B.2)**, and septal regions of HPC – including DG **(B.3)**, CA1 **(B.4)**, and CA3 **(B.5)** – were sampled between -2.45 through -3.9mm relative to Bregma. Midseptotemporal DG **(C.1)**, CA1 **(C.2)**, and CA3 **(C.3)** – as well as temporal CA1 **(C.4)** and CA3 **(C.5)** – were sampled between -5.0 through -6.0mm relative to Bregma. All cortical regions, as well as all DG regions, were photographed at a magnification of 10x, while all CA1 and CA3 regions were photographed at a magnification of 20x. Photomicrographs of analyzed samples were taken within the boxed regions of interest as outlined in the figure.

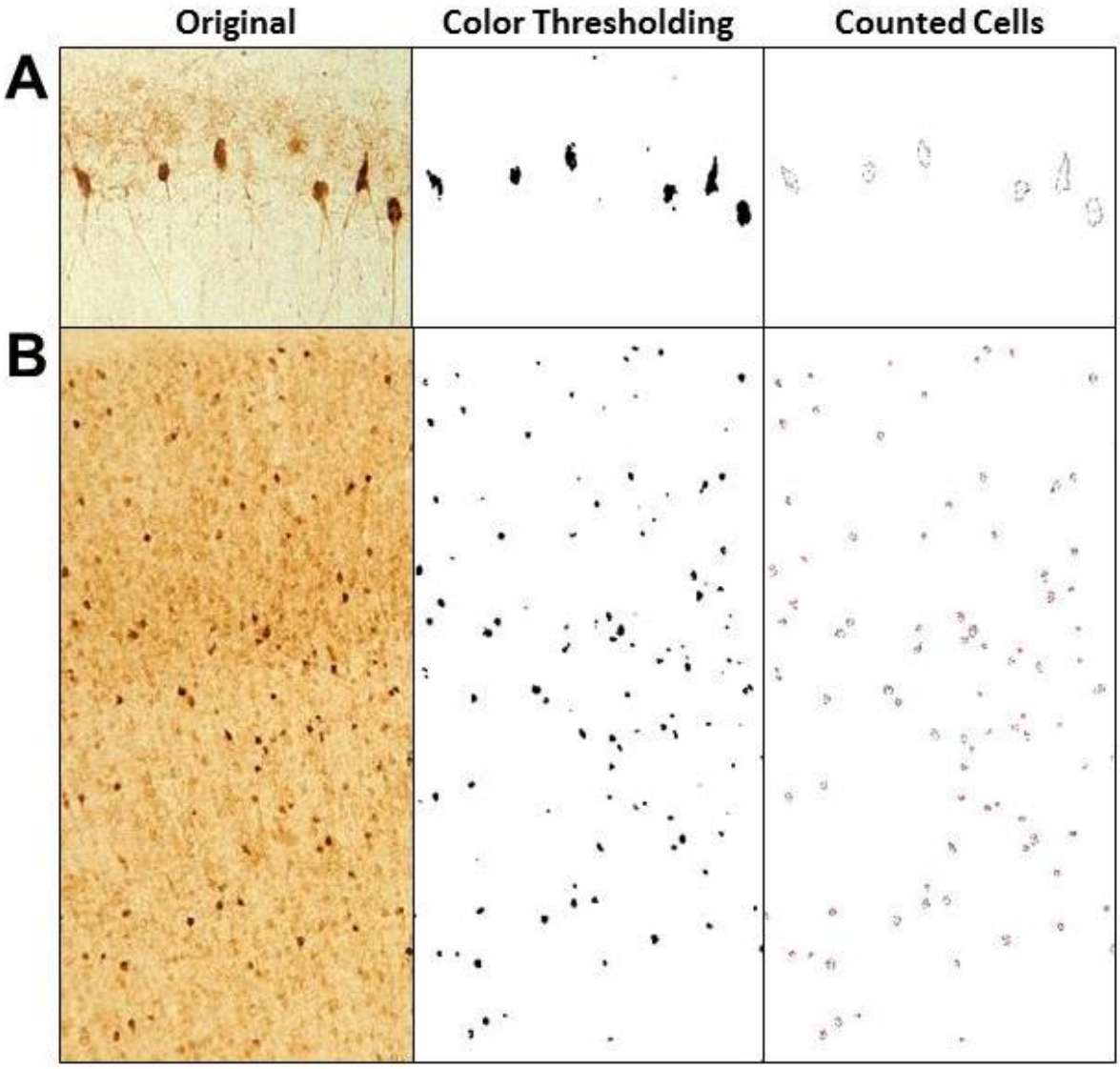

Figure 2.3: Example of quantification of PV-expressing cells using ImageJ Color-Based Thresholding.

ImageJ software was used to automize PV+ cell counting using region- and magnification-specific macros to ensure unbiased, consistent quantification within each area of interest. Examples of threshold-based quantification from septal HPC CA1 **(A)** and somatosensory barrel fields **(B)** can be seen above. The left set of panels show original photomicrographs with darkly stained PV+ cells clearly evident. The center panel shows the same photomicrograph thresholded based on color density to isolate the most darkly stained cells. A macro was run to quantify the number of PV+ cells based on previously set parameters to exclude any thresholded particles (e.g. non-cell artifacts) which did not meet criteria for inclusion (e.g. size, sphericity). The right panel shows the output from the macro identifying counted PV+ cells.

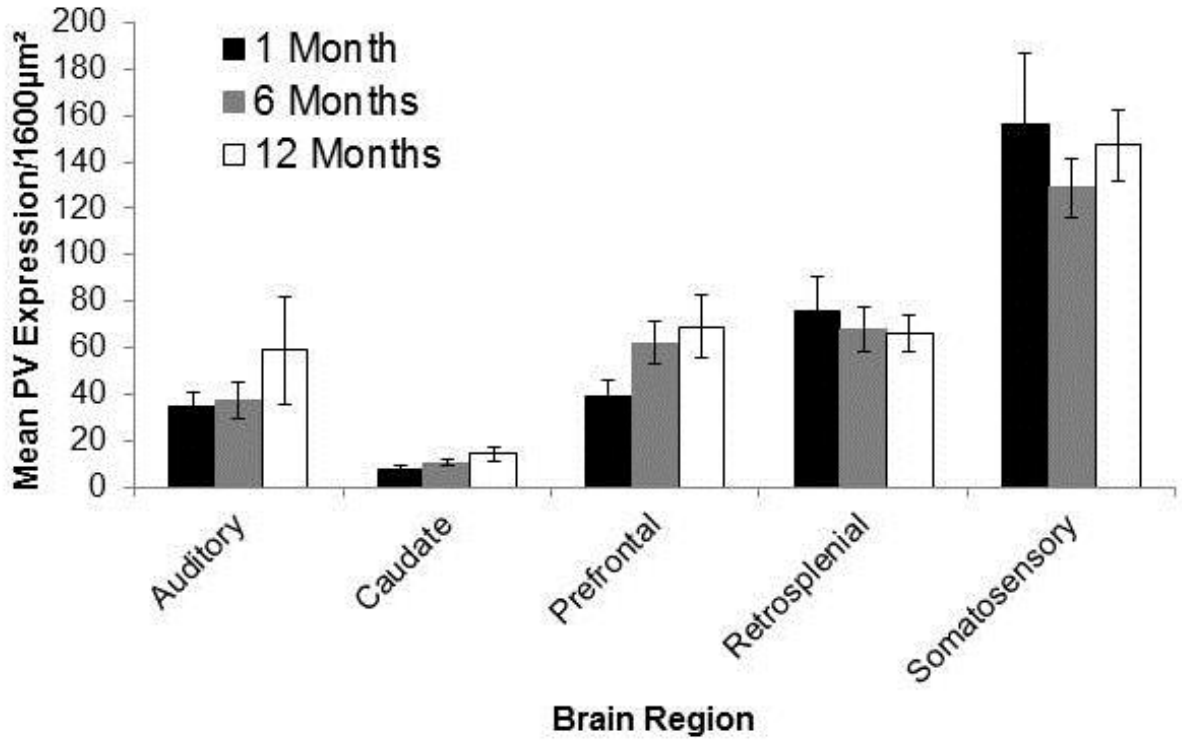

Figure 2.4: Parvalbumin expression remains stable across the lifespan within the neocortex and striatum.

Analysis of PV expressing neurons within the neocortex and striatum revealed no significant changes in expression between 1, 6, and 12 month old rats within neocortical areas (auditory, frontal (anterior cingulate), retrosplenial, and somatosensory cortices), or the striatum (caudate). All *p*'s > 0.05.

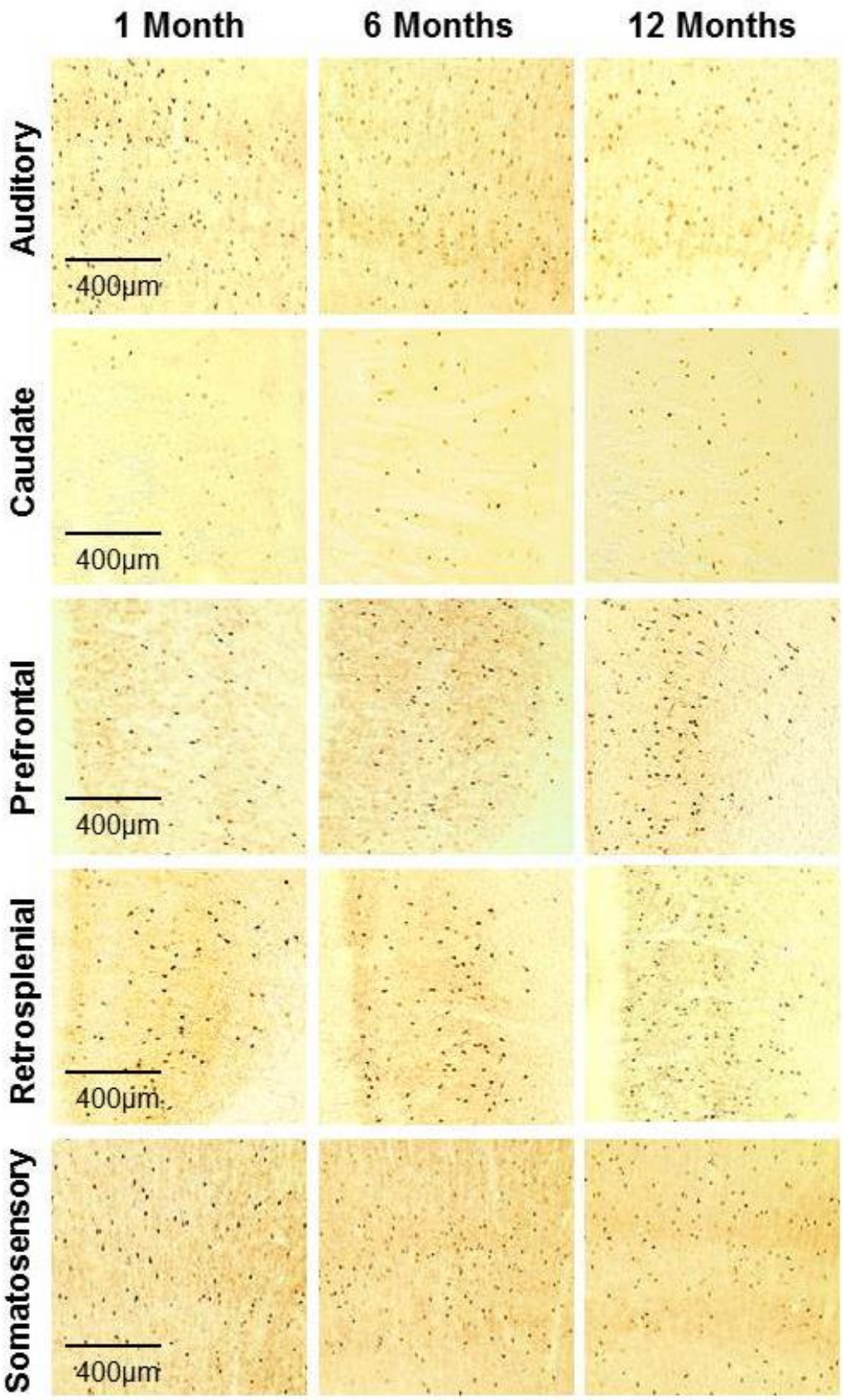
1 Month
6 Months
12 Months
Auditory
Caudate
Prefrontal
Retrosplenial
Somatosensory
400µm
400µm
400µm
400µm
400µm

Figure 2.5: PV expression as a function of age and brain region in the neocortex and striatum

Representative photomicrographs of neocortical and striatal regions of interest included (from top of figure to bottom): rat auditory cortex, caudate, prefrontal cortex (anterior cingulate region), retrosplenial cortex, and somatosensory cortex (barrel fields). There were no significant changes observed in PV expression within these regions. However, there was a trend toward significance in the prefrontal cortex suggesting that PV expression may increase as a function of age. Photomicrographs were taken at 10x magnification.

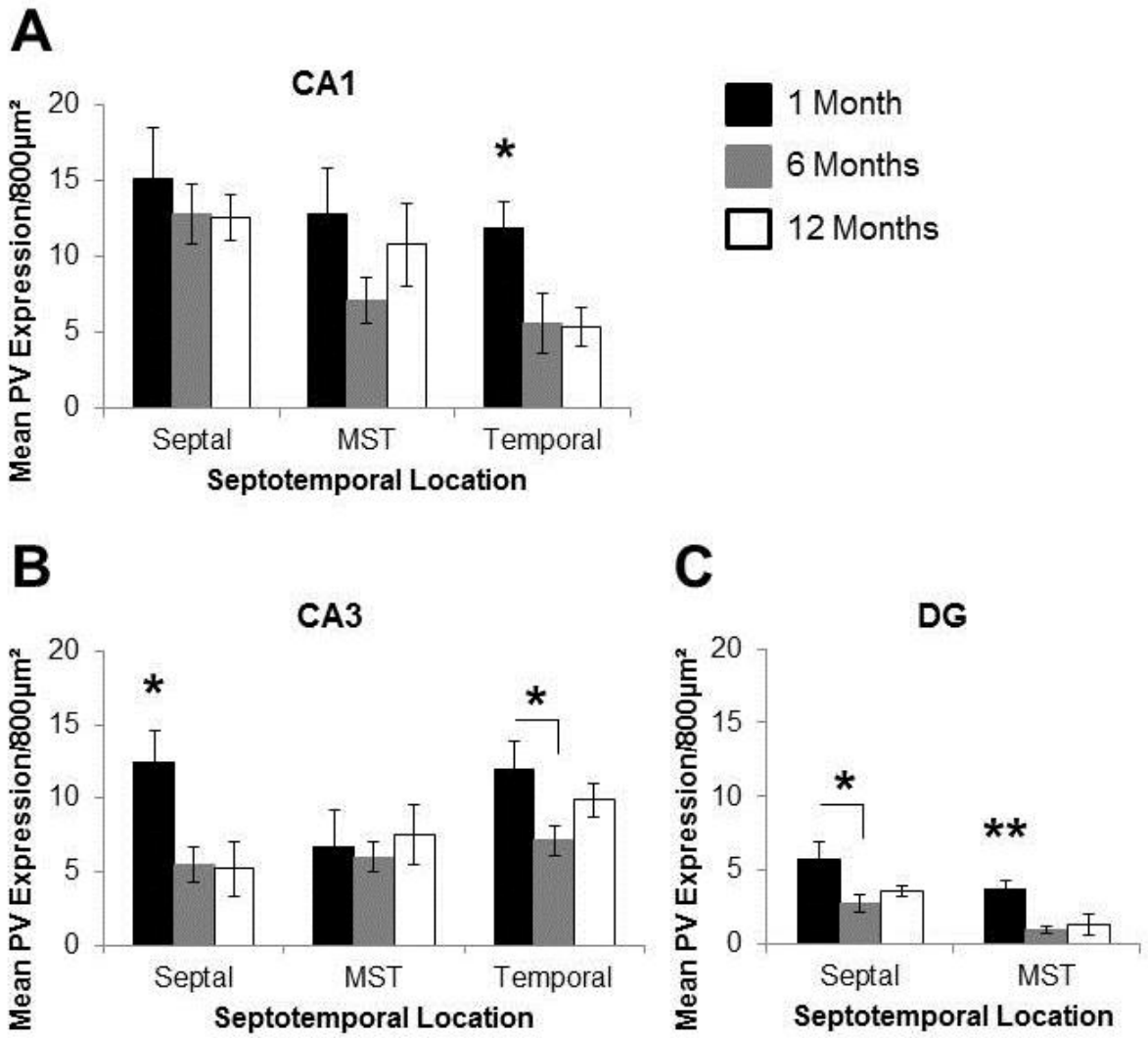

Figure 2.6: Age-dependent changes in PV expression as a function of hippocampal region and septotemporal location.

There were significant differences in all HPC sub-regions (CA1, CA3, DG) as a function of age, such that there was a general pattern of more PV expressing cells in 1 month old rats compared to 6 and 12 month old rats. Specifically, in the temporal region of CA1 **(A)**, 1 month old rats had significantly higher PV expression than both 6 and 12 month old rats. In CA3 **(B)**, 1 month old rats had significantly higher PV expression in septal regions than 6 and 12 month olds; and 1 month olds had significantly higher PV expression than 6 month olds in temporal CA3. Within the DG **(C)**, 1 month old rats had significantly higher PV expression than 6 month olds in septal regions, and significantly higher than 6 and 12 month olds in midseptotemporal regions.
*****$p < 0.05$, ******$p < 0.01$

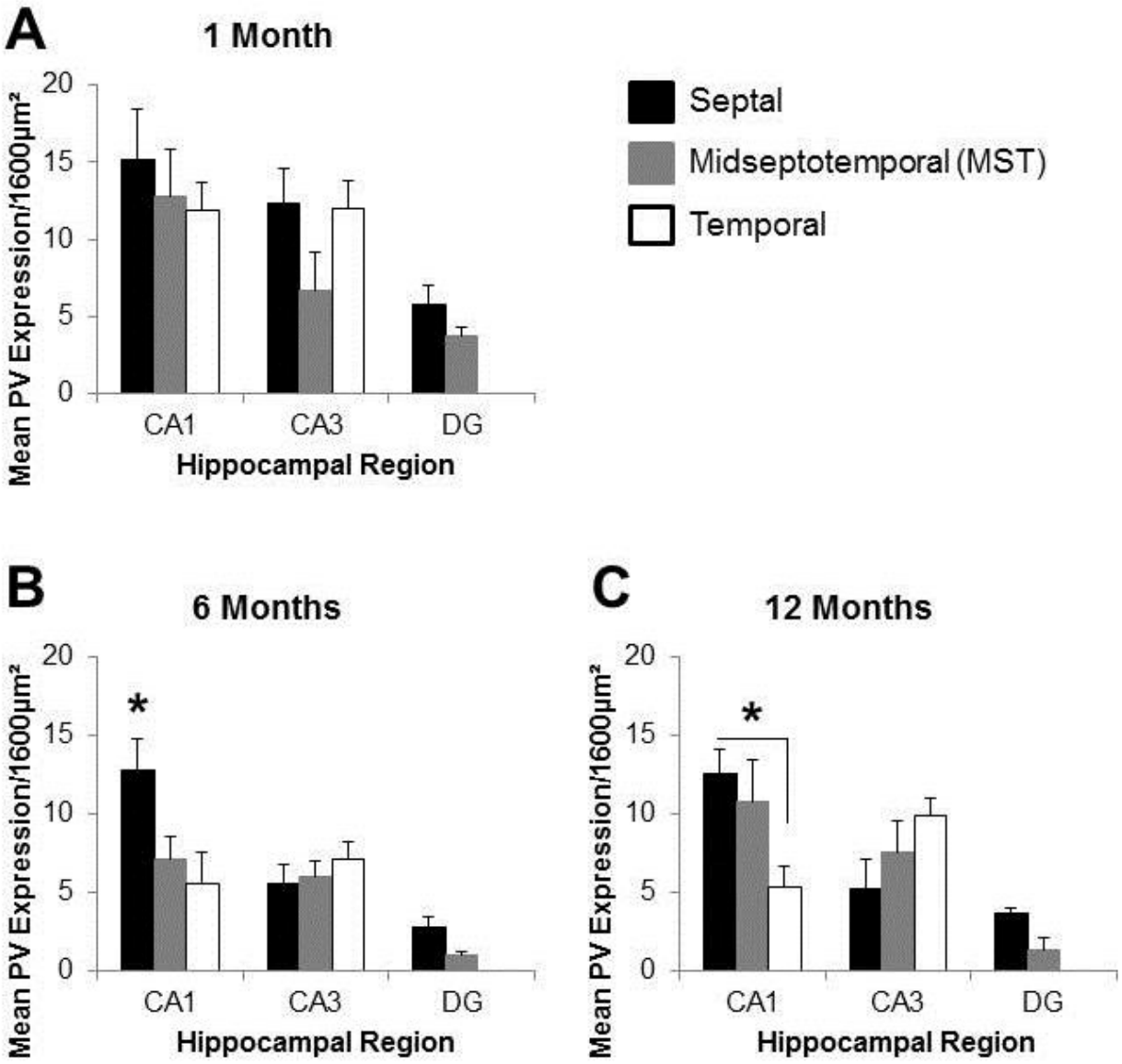

Figure 2.7: PV expression varies across the septotemporal axis in CA1 in 6 and 12 month old rats.

No significant changes in PV expression were seen across the septotemporal axis of the HPC in 1 month old rats **(A)** in any of the HPC sub-regions (CA1, CA3, DG). In 6 month old rats **(B)**, there was a significant decline in PV expression from septal to MST and temporal areas, with no significant changes across the long axis in CA3 or DG. Similarly, 12 month old rats **(C)** had significantly higher PV expression in septal compared to temporal CA1, with no significant differences across the long axis in CA3 or DG.
***p* < 0.05

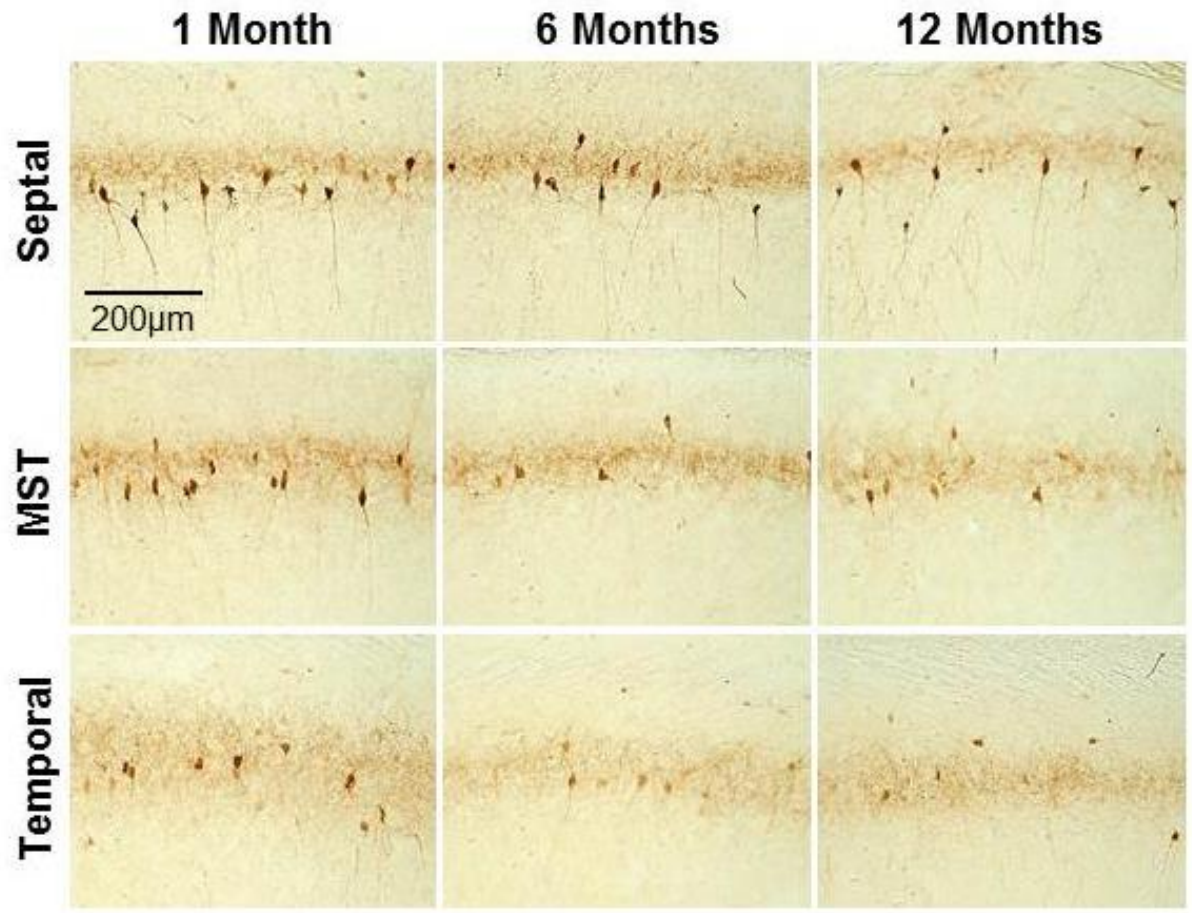

Figure 2.8: PV expression in HPC CA1 as a function of age and septotemporal location

Representative photomicrographs visualizing PV expression in CA1 as a function of developmental age (1, 6, 12 months) and septotemporal location (septal, midseptotemporal (MST), and temporal). PV expression remained stable across ages in septal and MST regions, but was significantly reduced in 6 and 12 month old rats compared to 1 month old rats in temporal regions. In 1 month old rats there was no effect of septotemporal location on PV expression, whereas 6 and 12 month old rats had significant decreases in expression along the septotemporal axis from septal to temporal regions. Photomicrographs were taken at 20x magnification.

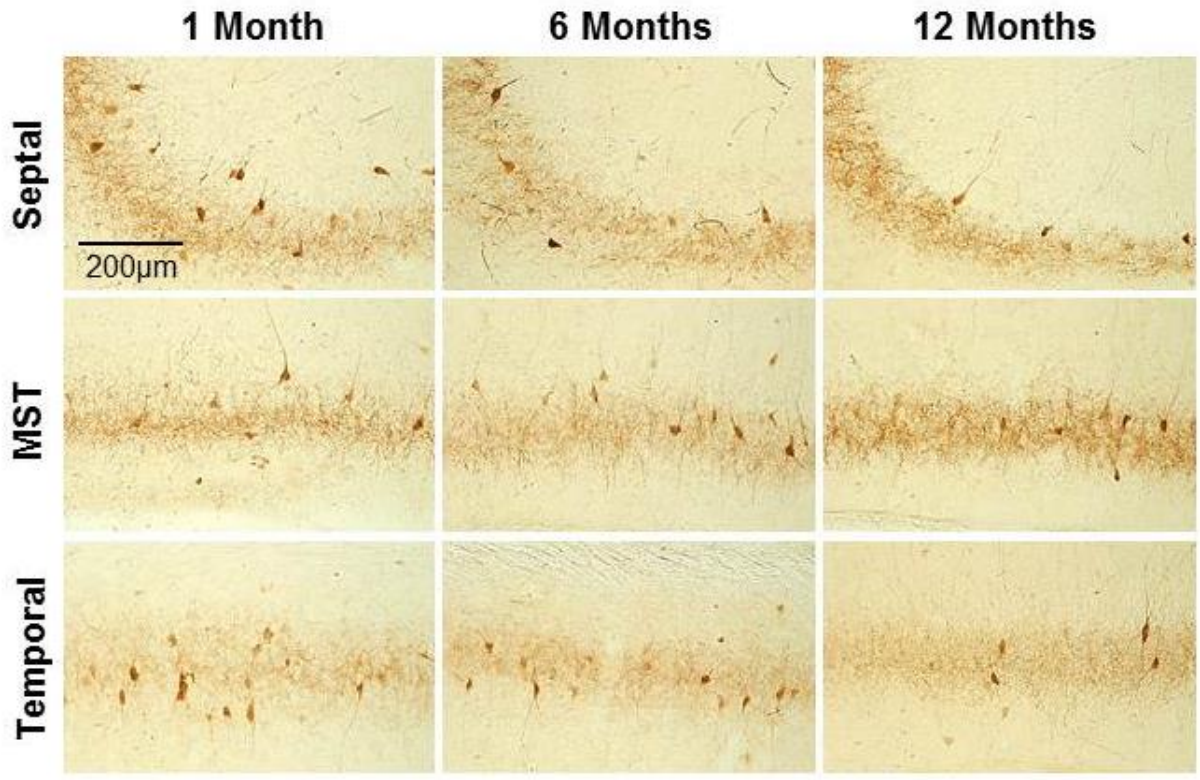

Figure 2.9: PV expression in HPC CA3 as a function of age and septotemporal location

Representative photomicrographs visualizing PV expression in CA3 as a function of developmental age (1, 6, 12 months) and septotemporal location (septal, midseptotemporal (MST), and temporal). PV expression remained stable across ages in MST regions, but was markedly reduced in 6 and 12 month old rats compared to 1 month old rats in septal and temporal regions. There were no significant changes in PV expression across the septotemporal axis in this region. Photomicrographs were taken at 20x magnification.

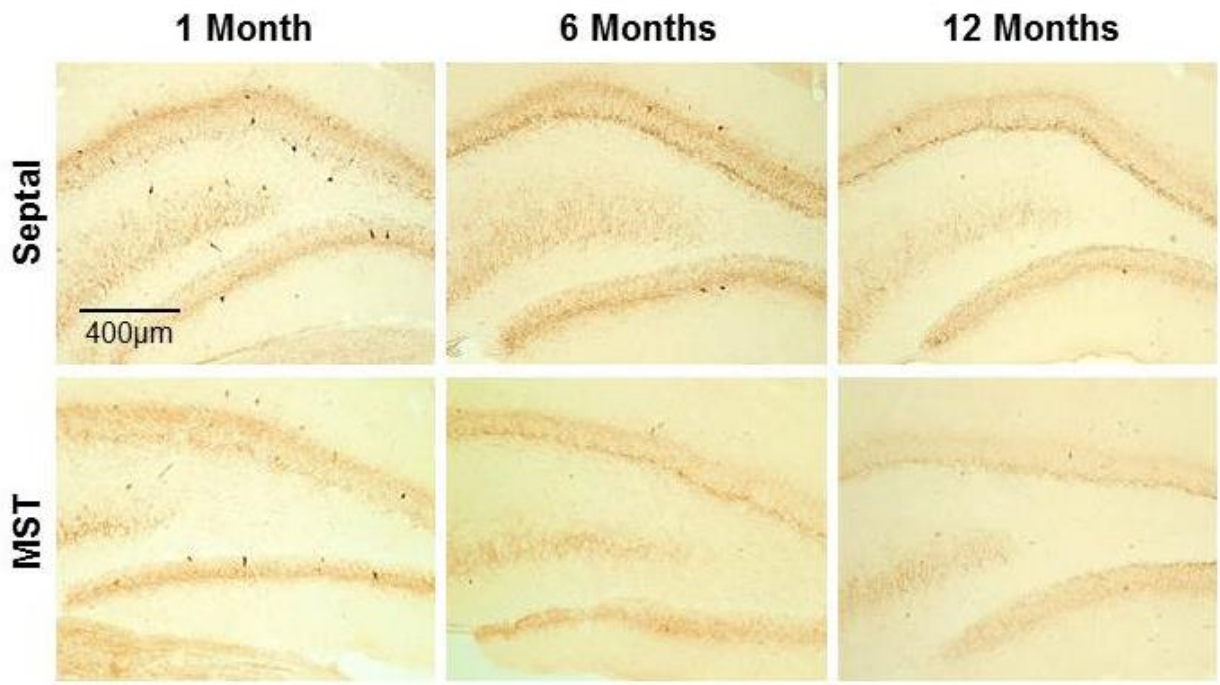

Figure 2.10: PV expression in HPC DG as a function of age and septotemporal location

Representative photomicrographs visualizing PV expression in DG as a function of developmental age (1, 6, 12 months) and septotemporal location (septal and midseptotemporal (MST)). The pattern of PV expression shows a general decrease across the septotemporal axis, with less expression within the MST as compared to septal regions. 1 month old rats generally had higher PV expression within the DG than both 6 and 12 month old rats. Photomicrographs were taken at 10x magnification.

Table 2.1: Descriptive Data

			1 Month	6 Months	12 Months
Auditory			34.5 ± 6.0	37.3 ± 7.7	58.7 ± 23.6
Caudate			7.8 ± 1.6	10.4 ± 1.4	14.1 ± 2.9
Prefrontal (Cingulate)			38.6 ± 7.1	61.9 ± 9.4	69.0 ± 13.7
Retrosplenial			75.6 ± 15.0	67.8 ± 9.4	65.9 ± 8.1
Somatosensory			156.4 ± 30.7	128.8 ± 12.7	147.3 ± 15.5
Hippocampus					
	CA1	Septal	15.1 ± 3.4	12.8 ± 2.0	12.6 ± 1.5
		MST	12.8 ± 3.1	7.1 ± 1.5	10.8 ± 2.7
		Temporal	11.9 ± 1.8	5.6 ± 2.0*	5.3 ± 1.3*
	CA3	Septal	12.4 ± 2.2	5.5 ± 1.2*	5.2 ± 1.9*
		MST	6.7 ± 2.4	6.0 ± 1.0	7.5 ± 2.0
		Temporal	11.9 ± 1.9	7.1 ± 1.1*	9.9 ± 1.1
	DG	Septal	5.8 ± 1.2	2.8 ± 0.6*	3.6 ± 0.4
		MST	3.7 ± 0.6	1.0 ± 0.2*	1.3 ± 0.7*

Descriptive data in the above table is expressed as M ± S.E.M. and represents the average number of PV expressing cells per 1600µm² for each region (n's = 6*).*

Within the HPC, an asterisk (*) following the M±SEM indicates that the PV expression is significantly different from 1 month old rats at the p < 0.05 level

CHAPTER 3

Chronic ketamine administration impairs cognitive performance in a DMTS water maze task without concomitant changes in parvalbumin expression

3.1 Abstract

A selective decrease in parvalbumin (PV) immunoreactivity is a robust finding in postmortem schizophrenic hippocampus (HPC) and prefrontal cortex. Rodent models of schizophreniform cognitive dysfunction following acute and/or chronic ketamine (KET) treatments show comparable decreases in PV expression, and it is often considered a maker of neuropathology. However, the literature detailing KET-induced cognitive-behavioral impairment reveals inconsistent – and often contradictory – findings with regard to concomitant changes in PV expression within key brain regions implicated in cognition. In order to provide clarity to this growing body of literature, the present study sought to explore the short- and long-term impact of chronic KET administration (30mg/kg for 14 consecutive days) compared to saline controls on cognitive performance in a delayed matching-to-sample radial water maze task. Additionally, the present work sought to elucidate upon the relationship between chronic KET-induced cognitive impairment and levels of PV immunoreactivity within the HPC, prefrontal/cingulate cortex, and retrosplenial cortex of adult male Sprague-Dawley rats. Our data suggests that, while chronic administration of KET does, indeed, impair cognitive performance in a spatial navigation task, it does not significantly alter PV immunoreactivity within any of the brain regions observed. These findings indicate that chronic KET-induced cognitive impairment may be occurring independently of PV expression within inhibitory GABAergic neurons in these regions. Furthermore, these

findings provide compelling evidence that PV immunoreactivity may not be the underlying neural mechanism of KET-induced cognitive impairments, and therefore may not be a suitable marker of schizophreniform pathology.

3.2 Introduction

Schizophrenia (SCZ) is a complex psychological disorder characterized by marked impairments in prefrontal and medial temporal lobe supported cognitive processes (Eisenberg & Berman, 2010). The exact etiology of these impairments remain unclear, however it is thought that NMDA receptor hypofunction , in addition to downstream consequences, may underlie impairments in cognitive functioning (Anticevic et al., 2012; Moghaddam & Javitt, 2012; Snyder & Gao, 2013).

A number of investigators have used acute and chronic treatment with the NMDA antagonist, ketamine (KET), to induce cognitive, behavioral, and neuropathological features of SCZ (Frohlich & Van Horn, 2014; Steeds et al., 2015). Within the human literature, there is evidence for acute KET-induced disturbances in memory and executive function comparable to those seen in SCZ patients (e.g. Parwani et al., 2005; Honey et al., 2006; Lofwall et al., 2006). A review of the literature by Morgan and Curran (2006) suggests that acute administration in humans significantly impairs working memory performance, while chronic use may create long-lasting KET-independent impairments in the formation of episodic memories (Curran & Monaghan, 2001). Furthermore, chronic KET use in humans is correlated with deficits in prefrontal and medial temporal lobe driven cognitive processes such as visual recognition memory (Chan et al., 2013) and spatial working memory (Morgan et al., 2010), which mirrors dysfunction seen in patient populations.. Comparable to the human literature, administration of acute and/or chronic sub-anesthetic doses of KET have been shown to produce significant impairments in working and reference memory (Enomoto & Floresco, 2009; Rushforth et al., 2011), episodic-like spatial memory (Chrobak et al.,

2008), and spatial alternation (Verma & Moghaddam, 1996), as evidenced in behavioral tasks.

Postmortem findings point to a selective decrease in the calcium-binding protein (CBP), parvalbumin (PV), within prefrontal cortical (Beasley & Reynolds, 1997) and hippocampal (HPC; Zhang & Reynolds, 2002) GABAergic inhibitory neurons. Acute and chronic KET administration has been shown to produce pathological changes in the expression and distribution of the PV within neural circuits in key brain regions such as the prefrontal cortex and HPC (e.g., Keilhoff et al., 2004; Behrens et al., 2007; Kittelberger et al., 2012; Zhou et al., 2015). However, there exists conflicting findings suggesting the possibility that KET administration may actually serve to increase PV expression (e.g. Sabbagh et al., 2013), or that it may have no discernable impact on levels of PV immunoreactivity (e.g. Benneyworth et al., 2011). Overall, the literature to date on this phenomenon is limited and is in need of further characterization to clarify the relationship between chronic NMDA antagonism, cognitive-behavioral performance, and concomitant changes in PV immunoreactivity.

In order to characterize the relationship between these variables, the goals of the current study were two-fold, and aimed to investigate: 1) the acute and long-term impact of chronic KET administration on performance in a delayed matching-to-sample radial water maze task (DMTS-RWM); and 2) the effect of chronic KET treatment on neuropathology, with specific emphasis on PV immunoreactivity within the HPC and prefrontal cortex. Our findings suggest that there may not be a clear relationship between cognitive dysfunction in a DMTS-RWM cognitive-behavioral task and PV expression as a result of chronic KET exposure in adult rats.

3.3 Methods

3.3.1 Subjects

Twenty adult (approximate age1- 6 months during behavioral training) male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used in the current study. All rats were individually housed in clear polycarbonate caging with free access to food and water until rats weighed ~450 grams, At ~4 months of age eat rat received approximately 20-24 grams of rat chow per fay with free access to water. Normal husbandry procedures were followed for all rats in a temperature and humidity controlled vivarium with a 12-hour light/dark cycle (lights on at 0800h). Rats were behaviorally trained in a DMTS-RWM task (see Chrobak et al., 2008 for detailed methods) beginning at approximately 1 month of age and continued until rats were sacrificed and their brain tissue was collected (approximately 6-7 months of age). At approximately 6 months of age, when rats were well-trained on the maze task, half of the rats received a chronic regimen of either KET (30 mg/kg IP; *n* = 10) or a comparable intraperitoneal (IP) injection of physiological saline (*n* = 10). All housing and experimental procedures were performed in accordance with, and approved by, the University of Connecticut Institutional Animal Care and Use Committee.

3.3.2 Behavioral testing – delayed matching to sample radial water maze task

Rats were trained and d tested in the delayed matching-to-sample radial water maze (DMTS-RWM) cognitive-behavioral task to assess the impact of chronic KET administration on cognitive performance. The delayed matching-to-sample radial water maze task (DMS-RWM) is comprised of a 140cm diameter black fiberglass pool with a depth of 40cm. The pool houses eight black removable stainless steel corridors measuring 14cm wide x 36cm long and they extend from a central octagonal hub

measuring 50cm in diameter. The pool is filled with cool water (22 ± 2ºC) with the corridor walls extending 16cm above water level. A removable 10cm in diameter black PVC platform is submerged approximately 6cm below the surface of the water which serves as a hidden escape route from the pool.

Shortly following arrival into the colony rats were given four days of standard Morris water maze (four trials per day from each of four start locations to a fixed goal position; beginning at approximately 1 month of age) training to acclimate them to water escape and the general testing procedure. On the fifth day, the goal location was moved to a different location to test their reversal performance over four trials on that day. The following week, rats were trained on a delayed matching-to-sample radial water maze (DMTS-RWM) task with control trials and standard protocols implemented as outlined previously (Chrobak et al.., 2008). Briefly, rats were initially trained with a single 'forced-choice' sample session followed by a single thirty minute delayed test trial each day for eight weeks. Subsequently, rats were trained on a series of delay trials during which the sample-test interval were systematically varied between 5 minutes to 8 hours. After the completion of delay trial testing (~6 weeks), rats were tested on a series of two sample-test trials each day where the interval between each sample-test and the interval between sample-test sessions were systematically varied to examine the degree of interference exhibited across the two sample-test sessions within a day (see Figure 3.1 for example).

After five months of training and testing with either a single, or two, sample-test trials each day rats were tested with a fixed series of two sample-test trials as indicated in Figure 3.1. Morning trials consisted of a forced sample, followed by a 30 minute

delayed test (see Figure 3.1A), while afternoon trials consisted of a forced sample, followed by a 30 minute delayed second sample, and then a 5 minute delayed test trial (see Figure 3.1B). Rats were tested in this manner for 8 days prior to KET treatment (see Figure 3.2) as well as throughout the duration of chronic KET treatment (14 consecutive days) and were additionally tested for 10 days subsequent to the 14-day KET regimen. Importantly, KET treatments were administered between 4-6pm each evening approximately 2 hours after the completion of the behavioral training. Thus, none of the behavioral data was collected with KET "on-board" but minimally 12-14 hours post KET treatment.

3.3.3 Drug administration

Following extensive behavioral training in the DMS-RWM, rats began a chronic KET treatment regimen to induce hypoactivation of NMDA receptors within the brain. Rats were randomly assigned to one of two experimental groups consisting of either 1) chronic KET (n = 10) or 2) comparable saline (*n* = 10). Rats in the chronic KET group received a 30 mg/kg dose delivered IP once per day, while the control group received a comparable IP injection of physiological saline for 14 consecutive days. Injections were given at the same time each day (between 4-6pm) for the duration of the treatment, and were administered subsequent to any of the DMTS-RWM testing, ensuring that the treatment would not interfere with task performance. To explore any long-term impact of KET treatment on cognitive performance, we continued to test rats 10 days post drug-cessation, after which rats were perfused and brain tissue collected.

3.3.4 Immunohistochemistry

Rats were anesthetized with Euthasol (pentobarbital sodium solution; 1mL via IP

injection) and transcardially perfused with ice-cold 0.9% physiological saline solution immediately followed by ice-cold 3.7% paraformaldehyde solution. Following perfusions, brains were removed and individually stored in scintillation vials filled with 3.7% paraformaldehyde solution for 1 week. Prior to sectioning, brains were transferred into a 30% sucrose solution for cryoprotection. Brains were sliced into 60µm serial coronal sections on a cryostat and were stored in phosphate-buffered saline (PBS)-containing well-plates for storage prior to immunohustochemical procedures.

One series of tissue sections from each brain was selected to examine PV immunoreactivity, with sections encompassing tissue approximately -2.5mm through -6mm relative to Bregma (Paxinos & Watson, 1997) to include the septotemporal extent of the HPC, as well as retrosplenial and somatosensory cortices. Additionally, sections encompassing tissue ranging from approximately +2.5mm through +0.25mm relative to Bregma were collected to examine PV expression within the anterior cingulate cortex. Refer to Figure 2.1 for section selection specifications. Free floating sections were initially blocked in 5% normal goat serum (NGS; Jackson Immuno), 0.1% triton-X and PBS solution for 1 hour, followed by 3 five-minute rinses in PBS. Sections were then transferred into primary antibody solution (1:16000 anti-rabbit PV polyclonal antibody (ABCAM), 0.1% triton-X, and PBS) for a 1 hour incubation period at room temperature. Sections were then rinsed in PBS 3 times and transferred to secondary solution (horseradish peroxidase (HRP) labeled polymer anti-rabbit; DAKO) for a 1 hour incubation at room temperature. Following a final set of rinses in PBS, sections were transferred into a diaminobenzidine (DAB) chromogen solution (DAKO) for ten minutes to develop the stain. Sections were mounted and dried on glass slides, then cover-

slipped for microscopy using DPX.

3.3.5 Quantification of PV expression

Glass microscopy slides containing tissue processed for PV immunoreactivity was analyzed using a Nikon Eclipse E600 (Melville, NY) upright microscope equipped with an Insight SPOT digital camera (Diagnostic Instruments, Inc.). Photomicrographs of regions of interest included: anterior cingulate cortex, HPC (including the CA1, CA3, and DG sub-divisions; as sampled in Stuellet et al., 2010 and Nomura et al., 1997), retrosplenial cortex, and barrel field cortex were compiled and stored digitally for analysis by a researcher blind to treatment condition. To ensure precision in the quantification of PV-expressing cells within each region of interest, photomicrographs were taken at either 20x magnification (CA1, CA3) or 10x magnification (cortical areas; DG). Refer to Figure 2.2 for photomicrograph sampling locations for each region of interest.

Digital photographs were analyzed using ImageJ software (NIH), and the number of PV-immunoreactive cells quantified using macros written to automate particle counting within each region of interest (for an example of color-thresholding analysis using ImageJ software, refer to Figure 2.3). The sizes of the analyzed regions were as follows: CA1/CA3, 800μm²; DG and cortical areas, 1600μm². HPC CA1 and CA3 averages were normalized to represent the average number of PV reactive cells per 1600μm² for cross regional comparisons and consistency across data sets.

3.3.6 Statistical Analysis

3.3.6.1 Analysis of DMTS-RWM performance

Performance measures for the DMTS-RWM task include: 1) mean number of errors (the

number of incorrect corridor entries during test trials); 2) latency per choice (total time to navigate to the goal corridor divided by the number of choices made); and 3) first choice latency (time to navigate to the first corridor during the test trial, regardless of choice). For the purpose of the current analyses, only mean error during test trials is presented.

In order to determine whether chronic KET administration significantly impaired cognition in the DMTS-RWM task compared to saline-treated controls, split-plot two-way repeated measures analyses of variance (RMANOVAs; Wickens & Keppel, 1983; Kirk, 1982) for treatment, trials (analyzed in 2 day blocks), and treatment by day interactions. Separate RMANOVA's were conducted on performance 1) during 4 x 2-day blocks (8 days) prior to treatment (pretreatment), 2) during7 x 2-day blocks (14 days) during KET treatment, and 3) during3 x 2-day blocks (6 days) subsequent treatment (post-treatment; to determine the effect of treatment on mean errors following KET). Where appropriate, significant RMANOVA's were followed up with Tukey's HSD post-hoc analyses (Kirk, 1982) to compare differences between saline controls and KET treatment on specific 2-day blocks.

3.3.6.2 Analysis of PV expression

In order to explore differences in PV-immunoreactivity within each region, the number of PV-expressing cells within each brain region, as measured using ImageJ, was averaged for each rat and then group means were computed for each age. The average for each brain region was calculated using 3-6 representative photomicrographs per region (2-3 photomicrographs for each hemisphere), and was collapsed across hemispheres as there were no significant differences in PV expression observed. This was done to determine whether there were differences across HPC regions (CA1, CA3, DG) at all

septotemporal locations (septal, MST, temporal), in addition to possible differences within/across neocortical areas as a result of KET treatment. Averages for each HPC region were calculated such that each rat contributed data points for each region at each septotemporal location. Differences in the distribution of PV expression as a function of KET treatment within all regions of interest (e.g. anterior cingulate; CA1) were assessed using one-way Analysis of Variance (ANOVA), followed by post-hoc analyses where appropriate (Kirk, 2012).

3.4 Results

3.4.1 DMTS-RWM performance is impaired by chronic KET treatment

Consistent with prior preliminary data in a 24 month old group of well-trained rats, we observed no impairment in performance following repeated KET administration during performance of a single daily sample-test session. In this case, the delay interval was 30 minutes and both groups of rats exhibited fairly stable performance (approximately, 0.2-1.0 mean errors per day and 2-day block for both groups) with roughly 50% of rats making no errors, and the remaining making 1-2 errors per day on any given day of testing. It should be noted that such performance is typical across delay intervals from 1 minute to several hours (see Chrobak et al., 2008). RMANOVA's for each block of testing (pretreatment, KET treatment, and post-treatment) indicated no significant effect of treatment, day, or treatment by day interactions (see Figure 3.2A; *p*'s > 0.4).

In contrast, well-trained 6 month old rats exhibited marked memory deficits by day 7-8 of KET treatment on the "high" interference trials of the afternoon session (see Figure 3.2B). Notably, this group of rats was well trained to perform the DMTS-RWM task with one, two, or three forced sample-test pairs within the same day. It should also

be noted, that we do not typically test rats on multiple sample-test pairs each day for many days; rather, such multiple same-test pairs are parsed between single sample-test pairs (e.g., every other or third day) as performance during such high interference trials improves over consecutive daily testing trials.

Performance in both groups was similar during the pretreatment blocks (see Figure 3.2A; *p*'s > 0.5) with both groups making 0.9-1.5 errors per day (or 2-day blocks). Notably, KET treated rats began exhibiting progressively impaired performance as compared to controls by day 7-8 of testing through the last 13-14 days of testing. A RMANOVA during the KET treatment period indicated a significant trial by treatment effect ($F(1,9) = 11.4$, $p < 0.01$), a significant trial effect ($F(6,54) = 8.22$, $p < 0.01$), and a significant treatment interaction ($F(6,54) = 5.44$, $p < 0.01$). Post-hoc analyses indicated significant differences during the 7-8 day block and all subsequent testing during the treatment period.

Upon the cessation of treatment, KET treated rats did not exhibit a sustained impairment; rather, performance improved to control levels by day 3-4 post-treatment. A RMANOVA on the post-treatment period indicated a significant difference ($p < 0.01$) on only the first 2 days (48 and 72 hours) post KET treatment.

3.4.2 No effect of chronic KET treatment on PV expression within the cortex

Within the cortical regions of interest, one-way ANOVA analyses revealed that there were no significant differences in the average number of PV immunoreactive neurons as a function of treatment. There was no significant effect of chronic KET administration as compared to saline controls within the anterior cingulate cortex ($F(1,19) = 1.53$, $p = 0.23$), the retrosplenial cortex ($F(1,19) = 1.35$, $p = 0.26$), or within the somatosensory barrel fields ($F(1,19) = 0.12$, $p = 0.73$). A summary of findings can be seen in Figure 3.3,

with representative photomicrographs of these regions in Figure 3.4. Descriptive summarization of data can be seen in Table 3.1.

3.4.3 No effect of chronic KET treatment on PV expression within the HPC

Overall, there were no significant effects of chronic KET administration on average levels of PV immunoreactivity within the HPC. This lack of significance was seen in CA1 ($F(1,59) = 1.05$, $p = 0.31$), CA3 ($F(1,59) = 0.29$, $p = 0.59$), and DG ($F(1,39) = 0.75$, $p = 0.39$).Summary of data can be seen in Figure 3.5. These findings indicate that chronic KET administration did not concomitantly alter PV immunoreactivity in these HPC regions as compared to saline-treated controls. Representative photomicrographs of these regions at all septotemporal locations can be seen in Figure 3.6.

3.5 Discussion

The present findings provide support to a small body of research indicating no change in PV immunoreactivity following chronic KET administration in adult rats. Our data is in agreement with previous work by Benneyworth and colleagues (2011) which indicates no effect of repeated KET treatment on PV immunoreactivity in mice or rats. Furthermore, they report no change in the HPC or prefrontal cortex as a result of chronic KET treatment, which is in line with the present findings. A growing number of groups utilizing other non-competitive NMDA antagonist drugs (e.g. MK-801 or PCP) have also reported the absence of chronic NMDA hypofunction-induced changes in PV immunoreactivity in these brain regions (e.g., Braun et al., 2007), though the aggregate impact of these findings is underwhelming when compared to the number of published studies indicating clear decreases in PV expression following similar pharmacological

treatments (e.g. Keilhoff et al., 2004; Kittelberger et al., 2012; for review see Jaaro-Peled et al., 2010).

Alternatively, it is possible that the absence of KET-induced changes in PV immunoreactivity may be due to the behavioral and/or cognitive training that the rats in the current study experienced. Our lab has previously observed marked up-regulation in PV immunoreactivity in 24 month old rats that had undergone extensive behavioral training in the DMTS-RWM task for approximately 1.5 years. Interestingly, this increase in expression was seen within all observed brain regions (e.g. neocortex; HPC) when compared to younger, behaviorally-naïve rats (Corriveau, Master's Thesis, 2013). Previous research has linked environmental enrichment to increases in PV immunoreactivity (e.g., Iuvone et al., 1996; Urakawa et al., 2013). Komitova and colleagues (2013) found that environmental enrichment was able to attenuate hypoxia-induced PV-expression abnormalities. This 'rescue' effect of PV phenotype driven by enrichment may explain the lack of significant changes in PV expression following chronic KET administration, as the sustained behavioral training impacts the system in a similar, albeit perhaps more robust, manner as environmental enrichment. Other labs have reported comparable results, with behavioral experience and/or cognitive training recovering insult-induced loss of PV reactivity in the rodent brain (e.g. de Villers-Sidani et al., 2010; Zhou et al., 2015).

In the present study, we describe chronic KET-induced deficits in DMTS-RWM performance when compared to saline-treated controls. However, due to a lack of effect of KET treatment on PV expression, it is difficult to determine whether observed cognitive-behavioral deficits were driven by impairment in the PV-expressing

GABAergic inhibitory circuitry. The absence of a relationship between these two factors could be explained by: 1) a KET-induced cognitive impairment which is independent of aberrations in PV immunoreactivity; 2) the sustained cognitive-behavioral training before and during chronic KET treatment, thereby buffering the deleterious impact of KET on overall PV expression; or 3) the timeline of tissue collection for PV immunohustochemical examination. As PV has been inextricably linked to the coordination of neuronal ensembles integral for sustained and efficient cognitive processes, it is unlikely that the impairments described herein are not driven, in some way, by KET-induced dysfunction in PV function (Carlen et al., 2012; Gonzalez-Burgos et al., 2015). This disconnect is more likely influenced by the behavioral training and/or the delay between KET treatment-cessation in tissue collection. Since brain tissue was collected 10 days post-cessation, it is possible that this delay may have provided ample time for PV immunoreactivity to normalize. Behrens and colleagues (2008) show a two-fold increase in PV expression 10 days post KET cessation when compared to 1 day post-cessation, indicating a recovery of PV phenotype. This argument becomes stronger when the recovery of cognition comparable to control performance in the DMTS-RWM task is seen 2-3 days following the end of the chronic KET regimen, and could therefore be indicative of a concomitant recovery of both cognitive performance and PV phenotype.

3.6 Conclusion

Overall, the present study provides compelling evidence for schizophrenic-like cognitive impairment in a DMTS-RWM task in rats chronically treated with KET as compared to

controls. Furthermore, we provide evidence for a prolonged effect of chronic KET exposure on cognitive dysfunction, which eventually returns to baseline control performance 2-4 days post-cessation. An effect of treatment on PV immunoreactivity within the neocortex and HPC was not observed, and is likely a consequence of the cognitive-behavioral training mediating the deleterious effects of the KET regimen, or a time-dependent recovery of PV phenotype following cessation; or perhaps some combination of the two. These findings suggest the possibility that cognitive-behavioral experience/training may serve to buffer the anatomical effects imposed by chronic KET administration, which provides support for behavioral therapies in mediating cognitive deficits in schizophrenic patient populations. We propose that additional studies be conducted to conclude whether behavioral training and/or timeline of tissue collection significantly impact PV immunoreactivity following chronic KET protocols.

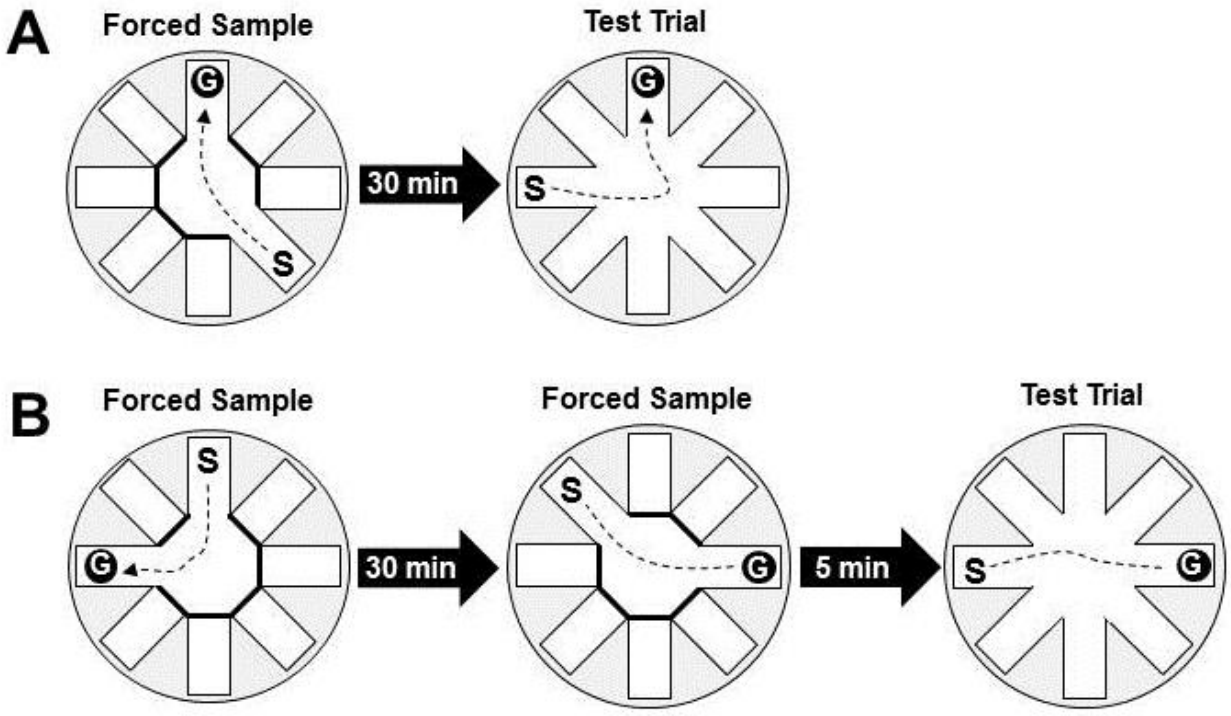

Figure 3.1: Delayed matching-to-sample radial water maze (DMTS-RWM) trial procedure

Beginning 1 week pre-treatment, rats were given two trial sessions per day consisting of AM trials (**A**) and PM trials (**B**). AM trials consisted of an initial forced sample, where rats were started at a randomized start ("S") location with all corridors except the goal ("G") blocked. Rats were then given a 30 minute retention interval before being placed back in the pool at a new start location and all corridors open to determine whether the rat remembered the most recent goal location. During PM trials (**B**), rats were placed in a random start ("S") location with all corridors but the goal ("G") blocked, then given a 30 minute retention interval. Rats were then returned to the pool for a second forced sample trail, with the goal in a new location, and upon locating the new goal location, were given a 5 minute retention interval. Rats were returned to the pool from a new start location with all corridors open for a test trial in order to determine whether rats could locate the most recent goal representation. If rats remembered the most recent goal location, they showed no impairment. However, if rats went to either the first forced sample goal location from the PM trials, or the goal location from the AM trials, it was considered an error imposed by proactive interference from a previous, and now incorrect, goal representation.

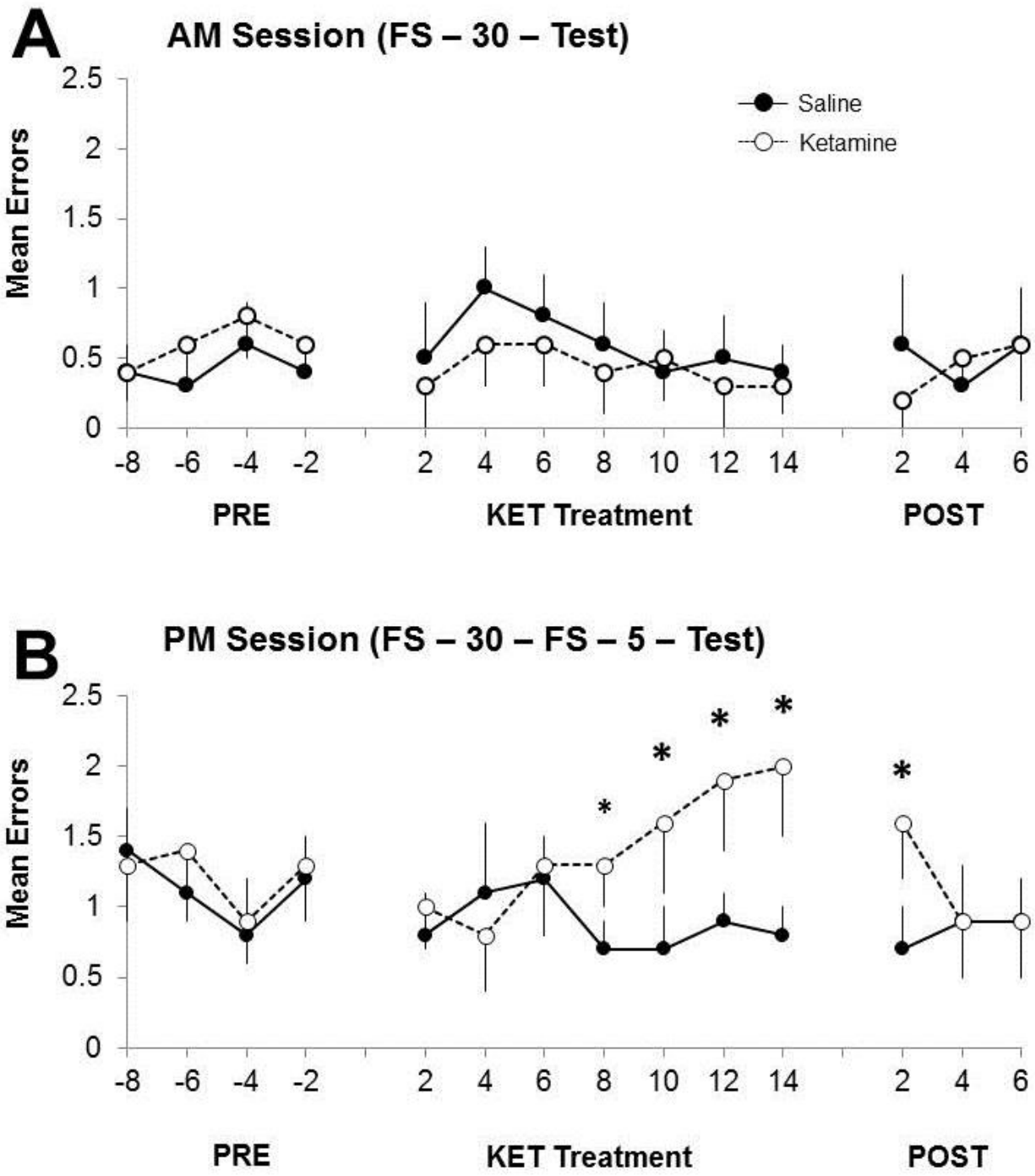
A
AM Session (FS – 30 – Test)
Mean Errors
2.5
2
1.5
1
0.5
0
Saline
Ketamine
-8 -6 -4 -2 2 4 6 8 10 12 14 2 4 6
PRE
KET Treatment
POST
B
PM Session (FS – 30 – FS – 5 – Test)
Mean Errors
2.5
2
1.5
1
0.5
0
*
-8 -6 -4 -2 2 4 6 8 10 12 14 2 4 6
PRE
KET Treatment
POST

Figure 3.2: KET significantly impairs DMTS-RWM performance compared to Saline

DMTS-RWM training took place twice per day and included: AM trials (**A**) and PM trials (**B**). In the AM trials (**A**), rats received a forced sample trial (FS) to learn the location of a hidden platform goal (indicated by "G") from a randomized start (indicated by "S") location. The FS was followed by a 30 minute retention interval, after which the rats were returned to the pool to a new start location to find the previously learned goal. In the PM trials (**B**), rats were given a FS, followed by a 30 minute retention interval, then a second FS with a new goal location. This new FS trial was followed by a 5 minute retention interval, after which the rats were returned to the pool to a new start location. In order to make a correct first choice and escape the pool, rats had to go to the most recent goal location (as indicated during FS). During AM trials, chronic KET treatment did not impact pre-treatment (PRE), during treatment (KET Treatment), or post-treatment (POST) cognitive performance (**A**). However, during the proactive interference trials in the PM trials, chronically KET treated rats had significantly more errors that saline control during KET treatment beginning at day 8 and continuing 2 days post-treatment (**B**). There was no effect of KET during PRE trials or during POST trials (after 4 days post-KET).
*$p < 0.05$

**$p < 0.01$

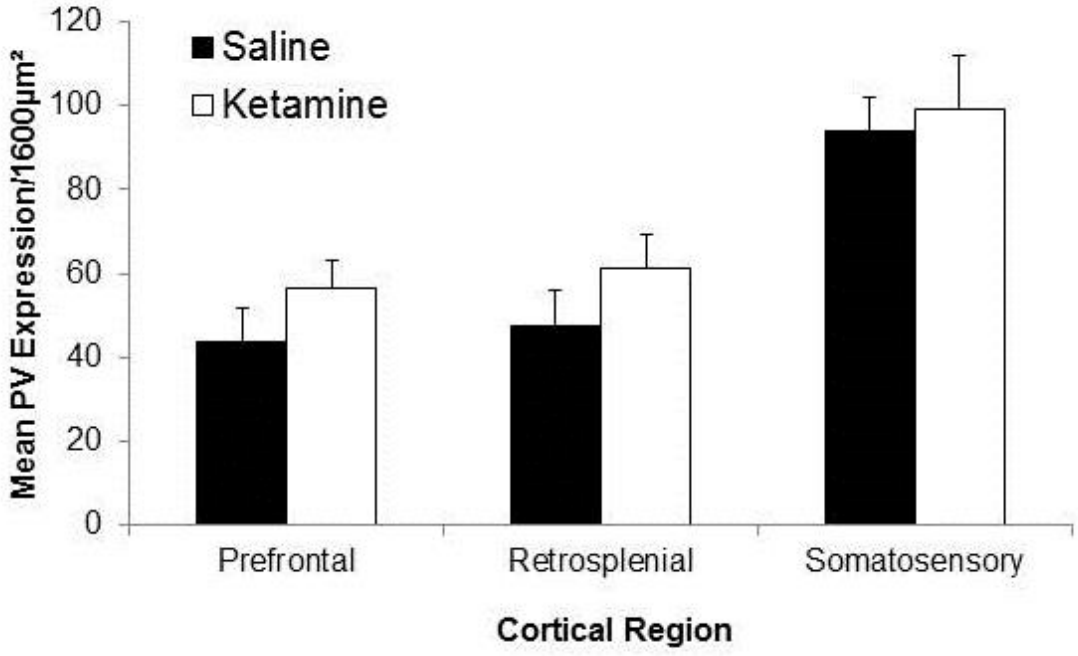

Figure 3.3 Chronic KET administration does not alter cortical PV expression

Chronic KET administration (30mg/kg KET for 14 consecutive days) in adult male rats did not impact overall PV expression within the prefrontal (cingulate) cortex, retrosplenial cortex, or somatosensory (barrel field) cortex compare to saline-treated controls.

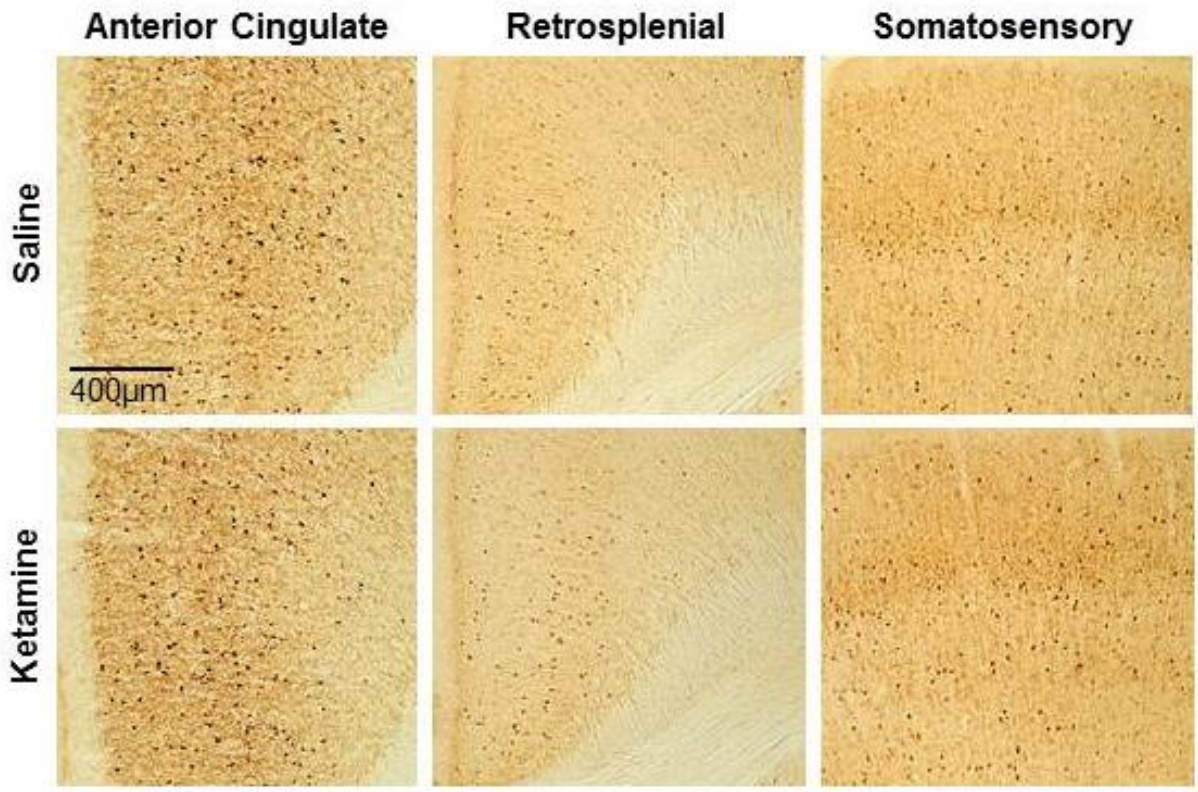

Figure 3.4 Representative photomicrographs: Cortex

Representative photomicrographs taken from prefrontal (cingulate) cortex, retrosplenial cortex, and somatosensory barrel fields. As can be seen, chronic KET administration did not alter overall PV immunoreactivity as seen in saline controls.

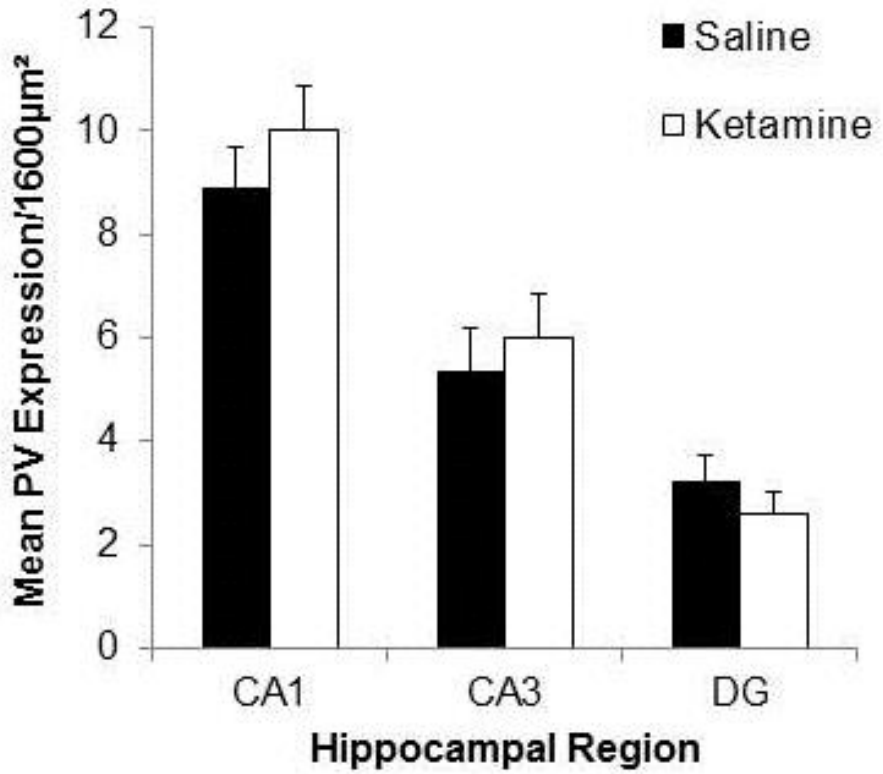

Figure 3.5 Chronic KET administration did not alter hippocampal PV expression

Chronic NMDA-receptor hypofunction induced by chronic KET administration (30 mg/kg IP for 14 consecutive days) did not significantly impact overall PV immunoreactivity within the hippocampal structure compared to saline-treated controls. There were no significant effects of treatment within CA1, CA3, or DG.

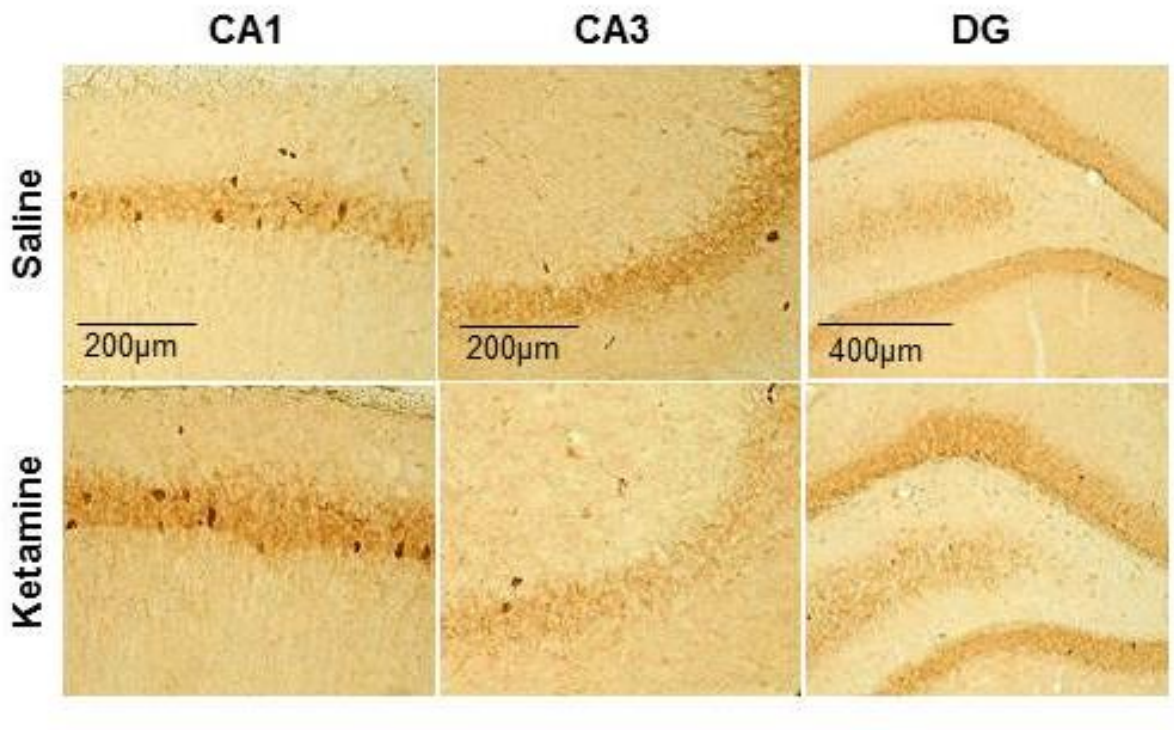

Figure 3.6 Representative photomicrographs: Hippocampus

Representative photomicrographs taken from HPC CA1, CA3, and DG. As can be seen, chronic KET administration, as compared to saline, did not alter overall PV immunoreactivity within these HPC sub-regions. CA1 and CA3 were photographed at 20x magnification, while DG was photographed at 10x magnification.

Table 3.1: Descriptive Data

		Saline	Ketamine
Prefrontal (Cingulate; n = 10)		43.8 ± 7.9	56.6 ± 6.7
Retrosplenial (n = 10)		47.5 ± 8.6	61.1 ± 7.9
Somatosensory (n = 10)		94.1 ± 8.1	99.4 ± 12.5
Hippocampus			
	CA1 (*n* = 30)	4.4 ± 0.4	5.0 ± 0.4
	CA3 (*n* = 30)	2.7 ± 0.4	3.0 ± 0.4
	DG (*n* = 20)	3.2 ± 0.5	2.6 ± 0.4

Descriptive data in the above table is expressed as M ± S.E.M. and represents the average number of PV expressing cells per 1600µm² for each region. There were no significant differences in average PV expression as a function of treatment (KET v. saline).

CHAPTER 4

Behavioral experience and chronic ketamine administration differentially impact parvalbumin expression in rats

4.1 Abstract

Parvalbumin (PV) expression within GABAergic inhibitory neurons is selectively decreased within the prefrontal cortex and hippocampus (HPC) of postmortem tissue from schizophrenic patients. It is generally accepted that aberrations in the overall functionality of PV-containing inhibitory neurons contribute to cognitive impairments characteristic of this disorder. Rodent models of cognitive impairment utilizing non-competitive NMDA antagonists, such as ketamine (KET), are used to induce a state of NMDA hypofunction within neocortical and HPC circuits underlying cognitive processes. The literature exploring this phenomenon with regard to cognitive dysfunction is mixed, with some reports indicating concomitant decreases in PV expression, with others suggesting no role of PV alterations in cognitive phenotypes. The present study provides characterization of PV expression in young (2-3 months) behaviorally trained rats and the relationship between PV expression and cognitive performance. We provide evidence for KET-induced cognitive impairments in a spatial water maze task, in addition to a parallel decrease in PV expression selective to the HPC CA1 sub-region. Based on the present data, we suggest that loss of PV expression in CA1 may underlie KET-induced pathology, and that deficits seen in cognitive performance may be related to decreases in PV expression in the HPC of young rats.

4.2 Introduction

Deficits in cognition are the arguably the most pervasive and difficult to treat impairments seen in schizophrenia (SCZ), yet the underlying neural mechanisms of these deficits are poorly understood (O'Donnell, 2007; Vohringer et al., 2013). A prime candidate underlying cognitive impairment is glutamatergic hypofunction within prefrontal cortex and hippocampal (HPC) brain circuitry integral to these processes (Moghaddam and Javitt, 2012). It is likely that decreased glutamatergic transmission brought on by receptor hypofunction may indirectly impact other neuronal elements within the affected circuitry, thereby giving rise to an array of characteristic symptoms (Harrison and Weinberger, 2005; Lisman, 2012). Models of NMDA receptor hypofunction (e.g. via the administration of NMDA-antagonist drugs) provide clues as to the relationship between NMDA receptor hypoactivation and cognitive impairment, in addition to the putative downstream neuropathological consequences () and have provided insight into the interplay between NMDA receptors and GABAergic circuitry.

Recent findings implicate GABAergic inhibitory neurons in the pathophysiology of SCZ cognitive impairment (Nakazawa et al., 2012; Inan et al., 2013); specifically those expressing the calcium-binding protein (CBP), parvalbumin (PV). In post-mortem SCZ brain tissue, PV expression is found to be selectively and significantly reduced in both the prefrontal cortex (Beasley & Reynolds, 1997) and the HPC (Zhang & Reynolds, 2002). It is thought that the effects of NMDA-receptor hypofunction may actually be localized to PV-containing GABAergic inhibitory neurons (Coyle, 2006; Carlen et al., 2012). Thus, a specific hypofunction of glutamatergic input onto these neurons in key brain circuits may underlie an overall decrease in the inhibition of principal excitatory neurons, whose firing precision is essential to the processing and encoding of relevant

information (Gonzalez-Burgos et al., 2011; Gonzalez-Burgos & Lewis, 2012; Rotaru et al., 2012).

While the evidence in support of a link between NMDA hypofunction, impaired cognition, and concomitant changes in PV expression in GABAergic neurons is compelling, there are conflicts within the rodent literature. While a number of groups have reported observed changes in PV expression following acute or chronic NMDA hypofunction induced cognitive impairment (e.g. Keilhoff et al., 2004; Behrens et al., 2007; Kittelberger et al., 2012), other groups report a lack of concomitant neuropathological changes following similar preparations (e.g. Braun et al., 2007; Benneyworth et al., 2011), or even increases in PV expression (e.g. Sabbagh et al., 2013). Furthermore, it is possible that the training animals, in and of itself, in a cognitive task may serve to modulate PV expression based on the complexity and time-span of training, as indicated by previous observations in our lab (Corriveau, Master's Thesis, 2013). This caveat is supported by compelling findings indicating an increase in PV expression as a result of environmental enrichment (e.g. Iuvone et al., 1996; Komitova et al., 2013). Thus, it is likely that PV may be differentially regulated based on a myriad of factors including cognitive-behavioral training, chronic NMDA antagonism, and developmental age, among others.

Previous work in our lab has outlined an important role for age in the density of PV expression within the HPC (Corriveau, Master's Thesis, 2013). Additionally, work detailed in Chapter 2 of this dissertation provides further evidence of an age-dependent decrease in PV expression from 1 to 6 and 12 month old rats. Data presented in Chapter 3 indicate a chronic KET-induced impairment in cognitive performance in the

absence of KET-related changes in PV expression. We posit that this lack of an effect following KET treatment may be due to the fact that rats in the study were 6 months of age – an age at which we have already observed very low baseline levels of PV. Based on these data, we propose that younger rats e.g. 1-3 months of age) may show chronic KET-induced cognitive impairment with a concomitant decrease in PV expression, particularly because inhibitory circuits are likely still maturing (Monyer et al., 1994; Scheetz & Constantine-Paton, 1994; Yu et al., 2006), and may therefore be more susceptible to the deleterious effects of KET treatment (e.g., Huang et al., 2012). Furthermore, previous research employing NMDA hypofunction models in younger animals have described decreases in HPC PV expression (e.g., Braun et al., 2007).

In order to address the dynamic nature of PV expression based chronic NMDA antagonism in a younger cohort of rats than previously examined in our lab, the present study sought to characterize PV expression within the HPC and neocortex. In the present work, we describe the impact of chronic KET administration on PV expression in a delayed matching-to-sample radial water maze (DMTS-RWM) task, and provide data indicating KET-induced cognitive impairments*.* Additionally, we provide evidence for a significant KET-induced decrease in PV expression within HPC CA1. Taken together, the findings of the present study suggest that cognitive-behavioral training – while not necessarily mediating cognitive outcomes following NMDA antagonism – may serve to buffer KET-induced changes in PV expression, at least within CA3 and DG sub-regions of the HPC, though CA1 appears to be particularly susceptible to KET-induced impairments, and may underlie – to some extent – the cognitive impairment seen in the DMTS-RWM task.

4.3 Methods

4.3.1 Subjects

Sixteen male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) aged approximately 2-3 months were utilized for the current set of experiments. All rats were individually housed in polycarbonate cages with food intake restricted to approximately 12 grams of rat chow per day and free access to water. Rats were housed in a temperature and humidity controlled vivarium with a 12-hour light/dark cycle (lights on at 0800h) and all experienced normal husbandry procedures. Rats in this study experienced behavioral training in a DMTS-RWM task beginning when rats were approximately 1 month of age and continuing until sacrifice and tissue collection (approximately 2.5 months of age). Half of the rats used for the present experiment received a chronic regimen of KET (30 mg/kg IP; *n* = 8) or a comparable injection of physiological saline (*n* = 8). All housing and experimental procedures were performed in accordance with, and approved by, the University of Connecticut Institutional Animal Care and Use Committee.

4.3.2 Behavioral testing – delayed matching-to-sample radial water maze task

Rats were trained and d tested in the delayed matching-to-sample radial water maze (DMTS-RWM) cognitive-behavioral task to assess the impact of chronic KET administration on cognitive performance. The task apparatus is comprised of a 140cm diameter black fiberglass pool with a depth of 40cm. The pool houses eight black removable stainless steel corridors measuring 14cm wide x 36cm long which extend outwards from a central octagonal hub measuring 50cm in diameter. The pool is filled with cool water (22 ± 2ºC) with corridor walls extending 16cm above water level. A

removable 10cm diameter black PVC platform is submerged approximately 6cm below the surface of the water and serves as a hidden escape route (‘goal’) from the pool.

One week after arrival into the colony, rats were given four days of standard Morris water maze (four trials per day from each of four start locations to a fixed goal position; beginning at approximately PD30) training to acclimate them to water escape and the general testing procedure. On the fifth day, the goal location was moved to a different location to test their reversal performance over four trials on that day. The following week, rats were trained on a delayed matching-to-sample radial water maze (DMTS-RWM) task (Chrobak et al., 2008). The current procedure involved one forced sample trial each day followed by two test trials to the same fixed goal position (see Figure 4.6A). The first test was at 5 minutes following the sample, with a second test to the same goal position at 30 minutes following the sample. Rats were trained on this procedure for 25 days.

At 60 days of age, rats began receiving chronic KET or comparable saline treatment. On the first day of KET treatment rats were tested on the sample-test=test procedure as described above (see Figure 4.1A), and were additionally tested on a second set of sample-test trials (see Figure 4.1B). The initiation of the second sample-test trial within a day increased errors which typically manifest as proactive errors to the first sample goal on any given day (see Chrobak et al., 2008). All rats were tested with a 5 minute delay between the sample and test on this second sample-test sequence. Subsequently, all rats were tested for 14 consecutive days over the course of the 14 day KET, or comparable saline, treatment.

4.3.3 Drug administration

Following continuous training in the DMTS-RWM task, rats began chronic KET treatment consisting of 14 consecutive days of 30 mg/kg KET via IP injections (*n* = 8) or a comparable injection of saline (*n* = 8). Injections were given at the same time each day (between 4-6pm) for the duration of the treatment, and were administered subsequent to DMTS-RWM testing, ensuring that the treatment would not directly interfere with task performance.

4.3.4 Immunohistochemistry

Rats were anesthetized with Euthasol (pentobarbital sodium solution; 1mL via IP injection) and transcardially perfused with ice-cold 0.9% physiological saline solution immediately followed by ice-cold 3.7% paraformaldehyde solution. Following perfusion, brains were removed and individually stored in scintillation vials filled with 3.7% paraformaldehyde solution for 1 week. Prior to sectioning, brains were transferred into a 30% sucrose solution for cryoprotection. All brains were sliced into 60µm serial coronal sections on a cryostat and stored in phosphate-buffered saline (PBS)-containing well-plates.

One series of tissue sections from each brain was selected to examine PV immunoreactivity, with sections encompassing tissue approximately -2.5mm through -6mm and +2.5mm through +0.25mm relative to Bregma (Paxinos & Watson, 1997) to include the septotemporal extent of the HPC and prefrontal cortex (anterior cingulate), respectively. Refer to Figure 2.1 for section selection specifications. Free floating sections were initially blocked in 5% normal goat serum (NGS; Jackson Immuno), 0.1% triton-X, and PBS solution for 1 hour, followed by 3 five-minute rinses in PBS. Sections

were then transferred into primary antibody solution (1:16000 anti-rabbit parvalbumin polyclonal antibody (ABCAM), 0.1% triton-X, and PBS) for a 1 hour incubation period at room temperature. Sections were then rinsed in PBS 3 times at five minutes each, and transferred to secondary solution (horseradish peroxidase (HRP) labeled polymer anti-rabbit; DAKO) for a 1 hour incubation at room temperature. Following a final set of 3 five-minute rinses in PBS, sections were transferred into a diaminobenzidine (DAB) chromogen solution (DAKO; Carpinteria, CA) for ten minutes to develop the stain. Sections were mounted and dried on glass slides, then cover-slipped for microscopy using DPX.

4.3.5 Quantification of PV expression

Microscopy slides containing tissue processed for PV protein expression was analyzed using a Nikon Eclipse E600 (Melville, NY) upright microscope equipped with an Insight SPOT digital camera (Diagnostic Instruments, Inc.). Photomicrographs of regions of interest including: prefrontal cortex (anterior cingulate), HPC (including the CA1, CA3, and DG sub-divisions), retrosplenial cortex, and somatosensory barrel field cortex, were compiled and stored digitally for later analysis by a researcher blind to experimental condition. To ensure precision in the quantification of PV-expressing cells within each region of interest, photomicrographs were taken at either 20x magnification (CA1, CA3) or 10x magnification (cortical areas, DG). Refer to Figure 2.2 for photomicrograph sampling locations for each analyzed region.

Digital photographs were analyzed using ImageJ software (NIH), and the number of PV-immunoreactive cells was quantified using macros written to automate particle counting within each region of interest (refer to Figure 2.3 for an example of color-

thresholding analysis performed). The sizes of the analyzed regions were as follows: HPC CA1/CA3, 800µm²; HPC DG and cortical areas, 1600µm². The average number of PV expressing cells for HPC CA1 and CA3 were normalized to represent the average number of PV-positive cells per 1600µm² for cross regional comparisons and consistency across data sets.

4.3.6 Statistical Analysis

4.3.6.1 Analysis of DMTS-RWM cognitive performance

Performance measures for the DMTS-RWM task include: 1) number of errors (average number of incorrect corridor entries during the first test trial); 2) latency per choice (total time to navigate to the goal divided by the number of choices made); and 3) first choice latency (time to navigate to first corridor of test trial, regardless of choice). For the purpose of the current analyses only mean error during test trials is presented.

In order to determine whether chronic KET administration significantly impaired cognition in this task compared to saline-treated controls, split-plot two-way repeated measures analyses of variance (RMANOVA, Wickens & Keppel, 1983; Kirk, 1982) were conducted for treatment, trials (2 day blocks), and treatment by day interactions on the number of errors averaged over 2-day blocks. Separate RMANOVA's were conducted on performance: 1) on the first sample-5 minute delayed test trial (7 x 2 day blocks (14 days)); 2) on the first sample-30 minute delayed test trial; and 3) on the second sample-5 minute delayed test trial. Significant RMANOVA's were subsequently followed by Tukey's HSD post-hoc analyses (Kirk, 1982) to compare the differences between KET or comparable saline treatments on specific 2-day blocks.

4.3.6.2 Analysis of PV expression

In order to explore differences in PV-immunoreactivity within each region, the number of PV-expressing cells, as measured using ImageJ, was averaged for each rat in each rat, and group means were computed for each treatment. This was done to determine whether there were differences across HPC regions (collapsed across septotemporal location), in addition to possible differences within neocortical regions as a function of KET administration. The average number of PV expressing cells for each brain was computed from 3-6 representative photomicrographs per region (2-3 photomicrographs per hemisphere), and was collapsed across hemispheres as there were no significant hemispheric differences in PV expression. Differences in PV expression based on region and treatment were assessed using one-way Analysis of Variance (ANOVA) for each region of interest, followed by post-hoc analyses where appropriate.

4.4 Results

4.4.1 DMTS-RWM performance is impaired by chronic KET treatment

4.4.1.1 First sample-5 minute delayed test

Following 25 days of training on the sample-5 minute delayed test-30 minute delayed test procedure, rats were performing at a fairly high level, making 0.8-1.4 errors per day as a group on the 5-minute delayed test trial and controls performed slightly better on the second test (0.5-1.4 errors per day as a group). We observed no significant impact of chronic KET treatment on performance in this “short” (5-minute) delayed test over 14 consecutive days of treatment as compared to saline controls. RMANOVA’s indicated no treatment, trial (2-day blocks), or treatment by trial interactions (p’s < 0.4). See Figure 4.1A.

4.4.1.2 First sample-30 minute delayed test

Somewhat surprisingly, there was a significant impairment in the performance of the chronic KET treated group as compared to saline controls, which was evident by day 5-6 of testing and was sustained over the remaining days of KET treatment. RMANOA's indicated a significant effect of treatment ($F(1.7) = 14.92$, $p < 0.01$), a significant effect of trial ($F(6,42) = 6.78$, $p < 0.01$), in addition to a significant trial by treatment interaction ($F(6,42) = 4.77$, $p < 0.01$). Post-hoc analyses indicated significant differences during the day 5-6 block (p's < 0.05) and all subsequent testing blocks during treatment period. This finding contrasts with the absence of any memory deficits in 6 and 12 month old rats performing similar tasks following chronic KET treatment without a specific proactive interference procedure (2^{nd} sample goal) within a day. See Figure 4.2B. It is clearly evident that the age of the rats and/or the degree of training experienced may explain the clear difference between this sample of 2-3 month old rats, and older rats with many months of training on the DMTS-RWM task.

4.4.1.3 Second sample-5 minute delayed test

As expected, KET treated rats also exhibited a significant impairment in memory performance during the specific proactive interference procedure during each day (second sample-5 minute delayed test task). RMANOVA's indicated a significant effect of treatment ($F(1,7) = 12.44$, $p < 0.01$), a significant effect of trial ($F(6,42) = 9.78$, $p < 0.001$), and a significant trial by treatment interaction ($F(6,42) = 7.68$, $p < 0.001$). Post-hoc analyses indicated significant differences during the day 7-8 block (p's < 0.05) and all subsequent testing blocks during the treatment period (see Figure 4.2C).

4.4.2 Effects of chronic KET treatment on PV expression in the neocortex

To determine the effect of chronic KET treatment on PV expression within the

neocortical areas of interest, a one-way ANOVA was conducted. Analyses revealed that there were no significant effects within any cortical region of interest including prefrontal (anterior cingulate; $F(1,15) = 0.06$, $p = 0.81$), retrosplenial ($F(1,15) = 0.29$, $p = 0.60$), or somatosensory ($F(1,15) = 0.003$, $p = 0.96$) cortices (see Figure 4.3 for summary, and Figure 4.4 for representative photomicrographs).

4.4.3 Effects of chronic KET treatment on PV expression in the HPC

A significant effect of chronic KET administration was seen specifically within the CA1 region of the HPC, with no significant effect of treatment in CA3 or DG regions. To explore the effect of chronic KET administration on PV expression within the HPC sub-regions, a one-way ANOVA was conducted for each sub-region (CA1, CA3, DG). Analysis revealed a significant effect of chronic KET administration exclusively within CA1 ($F(1,47) = 10.83$, $p < 0.01$), such that KET-treated rats had significantly less PV expression than saline-treated controls (Figure 4.5). There were no effects of treatment seen in CA3 ($F(1,47) = 1.56$, $p = 0.22$) or DG ($F(1,31) = 0.68$, $p = 0.42$). See Figure 4.5 for summary of data, and Figure 4.6 for representative photomicrographs of the HPC regions of interest. Descriptive data for all regions can be seen in Table 4.1.

4.5 Discussion

The current findings generally provide support to previous work in our lab indicating that PV expression is dynamically modulated by a myriad of influences (e.g. age; see Chapter 2). We provide further nuance to the dynamic nature of PV expression within the data presented herein, suggesting that chronic KET administration impairs cognition with a concomitant decrease in PV expression within the HPC. Previously, we noted

that in adult rats, chronic KET administration impairs cognitive performance, but does not change PV expression within the HPC (see Chapter 3). These null data were seen in adult (6 months) rats; conversely, we find in the present study – which utilized 2-3 month old young rats – that impaired cognition and decreases in PV expression show a clear relationship which is likely age-dependent. The lack of an effect of KET treatment on PV expression within the neocortex was expected based on our previous findings, and is in line with a small number of studies indicating no change in cortical PV expression as a function of NMDA antagonism (e.g. Benneyworth et al., 2011).

Within the present data, it is clear that chronic KET administration not only impaired cognition, but also decreased CA1 PV expression, thereby revealing PV as a possible underlying mechanism in KET-induced cognitive impairment (Gonzalez-Burgos & Lewis, 2012). Importantly, we see this effect in the current set of data which was done in young (approximately 2-3 months of age) rats that were behaviorally trained, while we do not see this effect in adult rats (Chapter 3). While age is likely driving the effect seen in chronically KET treated rats in the present and previous studies conducted in our lab, it is also possible that behavioral training may buffer some of the deleterious effects of KET treatment. The present data does suggest that behavioral training may buffer KET-induced loss of PV expression, minimally within the CA3 and DG regions of the HPC (see Figure 4.5), which is in line with literature describing increases in PV expression following enrichment (e.g., Iuvone et al., 1996; Komitova et al., 2013). However, additional research is warranted to determine the developmental time course of KET susceptibility to HPC PV expression decreases, in addition to a controlled study specifically aiming to determine the interaction between age and behavioral training.

Overall, the present study describes chronic KET mediated deficits in DMTS-RWM cognitive performance when compared to saline controls, with a concomitant PV decrease selectively seen in HPC CA1 of KET treated rats. Though we observed decreases in PV expression in CA1, it remains possible that cognitive impairment may be relatively independent of aberrations in PV expression within the cortex and HPC inhibitory circuitry, as many regions appeared resilient to the KET treatment. As PV expression has been repeatedly linked to the coordination of neural excitability integral to sustaining cognitive processes, we believe it is unlikely that the behavioral impairments in the DMTS-RWM task are independent of KET- induced changes in PV-containing neuron functionality (Carlen et al., 2012; Gonzalez-Burgos et al., 2015). We must also consider the fact that the rats used in the current study were approximately 2 months of age, and previously we have noted that 1 month old rats have significant higher PV expression overall than 6 month old rats. It stands to reason that 2 month old rats would have a similar high density of PV expression compared to adult rats, and therefore a KET induced decrease might be more apparent due to higher baseline levels of expression (see Chapter 2). In order to better characterize the effect of chronic KET administration on PV expression in HPC and neocortex, examination of PV expression following KET treatment at different developmental ages, in addition to behaviorally trained and naïve groups, is warranted.

4.6 Conclusion

In summary, the present work provides compelling evidence for schizophrenic-like cognitive deficits in a DMTS-RWM task in rats following chronic treatment with KET

compared to saline controls. Additionally, we provide data showing a parallel decrease in PV expression within the CA1 region of the HPC. These findings also suggest that cognitive-behavioral training may serve to buffer possible deleterious effects of chronic KET administration, as there were no effects seen in CA3 and DG HPC sub-regions as a function of treatment. This, in turn, ostensibly supports the use of cognitive-behavioral therapies in SCZ cognitive symptom mediation. Based on the current data, and in comparison with data observed in Chapter 3, we propose that young rats (e.g. 1-3 months of age) are more likely to show KET-induced deficits in PV expression than adult rats. This differential effect of age may be due to the developmental trajectory of inhibitory circuit maturation, making a developing circuit more prone to insult than an established one. To better understand these nuances in the dynamic nature of the PV protein, we propose that additional investigations systematically exploring the impact of age in mediating chronic KET-induced changes be carried out.

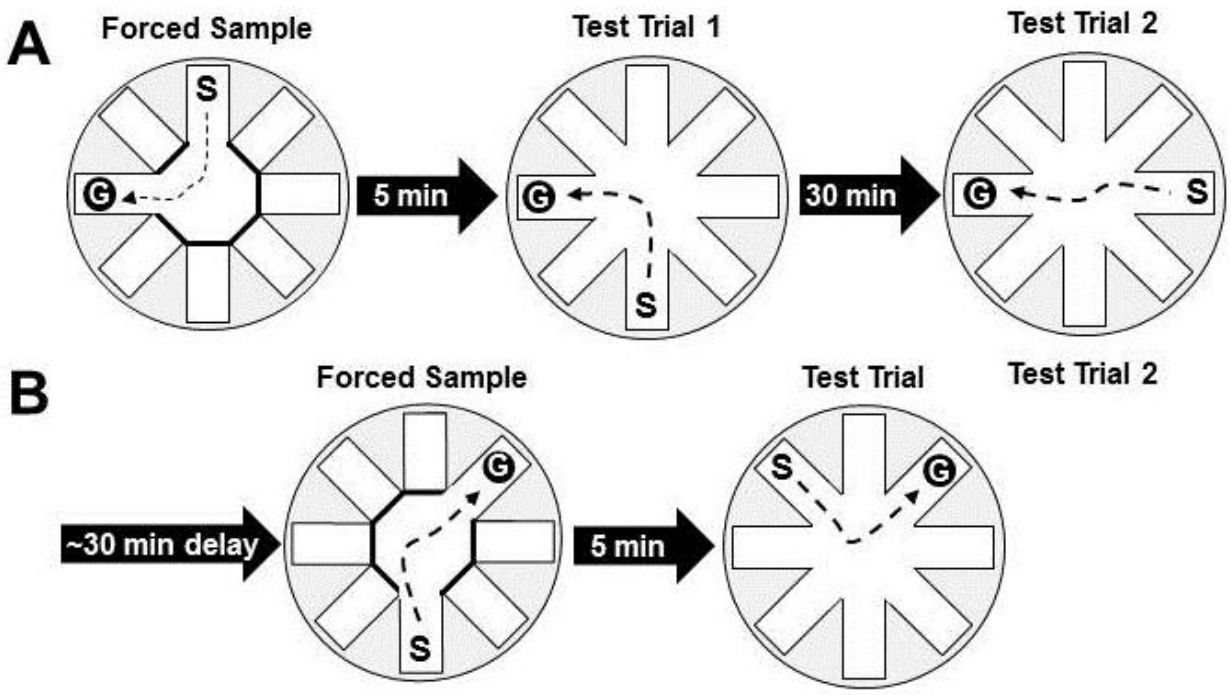

Figure 4.1: Delayed matching-to-sample radial water maze task procedure

For the first month of training, rats were trained in the DMTS-RWM task every day, with an initial forced sample trial to learn the goal location for the day, followed by a 5 minute delayed test, which was then followed by a 30 minute delayed second test; the correct goal for both tests being the goal shown in the forced sample (**A**). Upon beginning chronic KET treatment, rats continued the standard daily trials (**A**), in addition to the introduction of a new, second goal location for the day given in a second forced sample and followed by a 5 minute delayed test (**B**). This second set of trials was separated by an approximately 30 minute delay between the 2nd test trial and the 2nd forced sample. This procedure allowed for the investigation of how chronic KET treatment influences DMTS-RWM cognitive performance, as well as examine whether rats can discriminate between a previous within-day goal location and the most recent location.

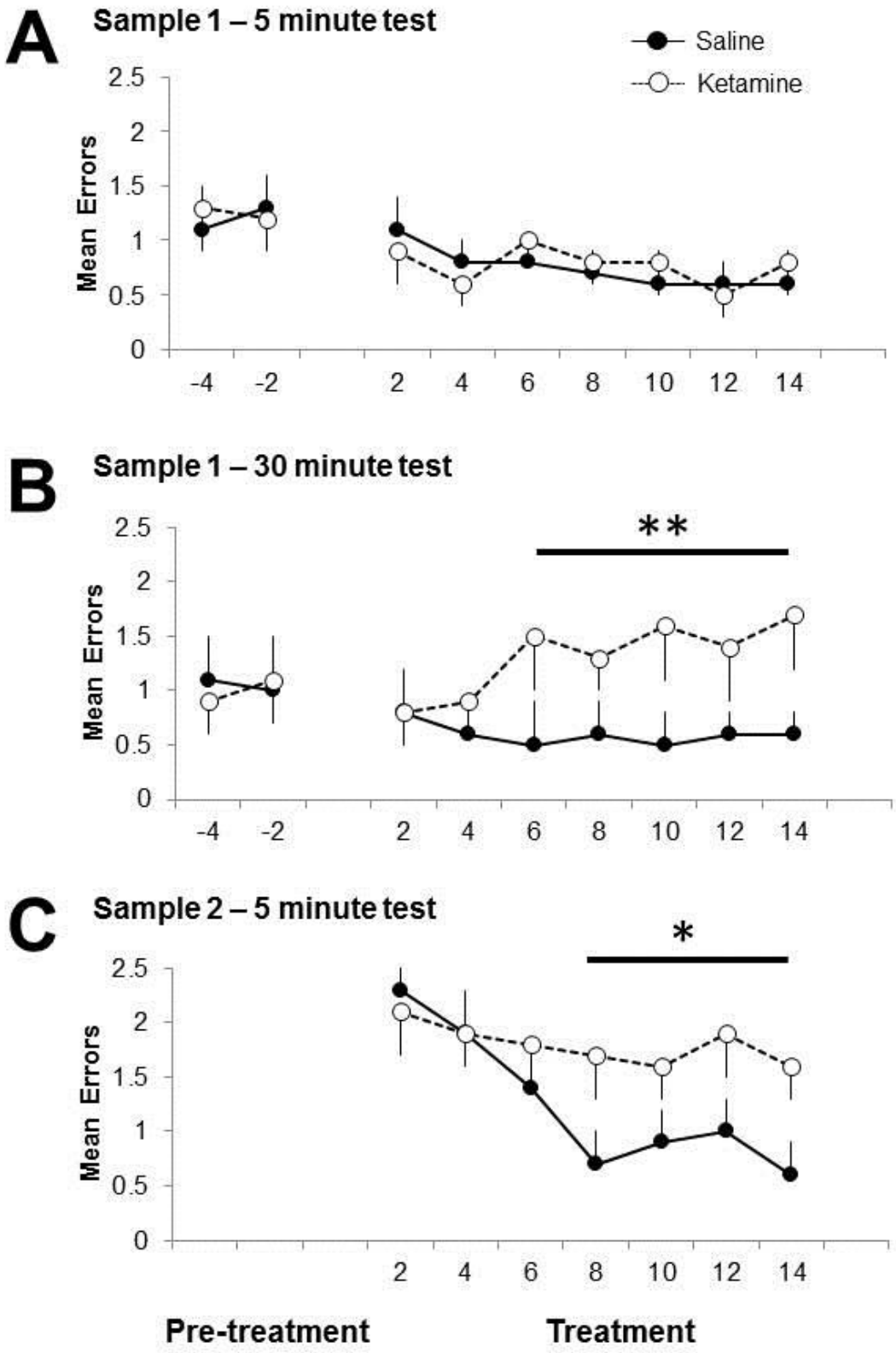
A
Sample 1 – 5 minute test
Saline
Ketamine
Mean Errors
2.5
2
1.5
1
0.5
0
-4
-2
2
4
6
8
10
12
14
B
Sample 1 – 30 minute test
**
Mean Errors
C
Sample 2 – 5 minute test
*
Mean Errors
Pre-treatment
Treatment

Figure 4.2: Chronic KET impairs DMTS-RWM performance

Rats were assessed for cognition on the DMTS-RWM task (as described in Figure 4.1), and performance was analyzed based on the mean number of errors made before locating the most recent goal location. Chronic KET administration did not impair task performance during the initial 5 minute delayed test (**A**), but significantly impaired performance beginning on Day 6 during the first 30 minute delayed test (**B**). During the trials initiated at the start of KET treatment, a significant difference in performance emerged starting at Day 8, which continued to the final day of testing (Day 14), such that the control rats learned to go to the most recent goal location, while KET treated rats made significantly more errors than controls (**C**). These findings indicate a role for KET enhanced proactive interference on performance.
*$p < 0.05$
**$p < 0.01$

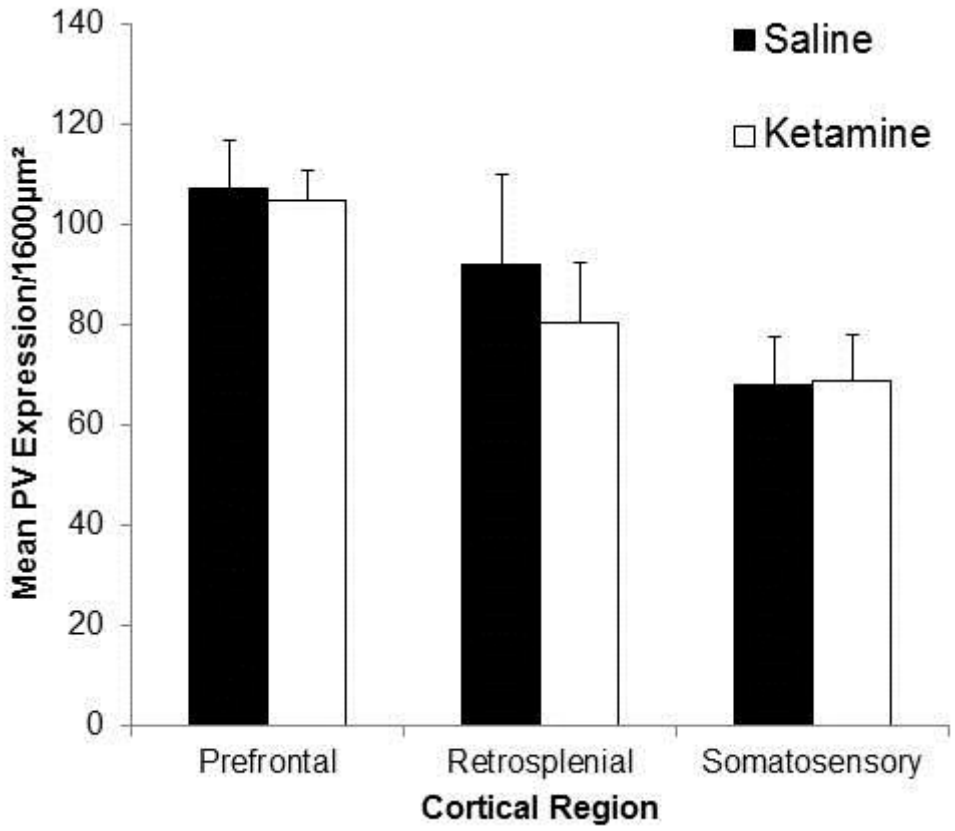

Figure 4.3: No effect of chronic KET administration on cortical PV expression in behaviorally trained rats

Despite showing impairment in cognitive processes, rats that were treated with chronic KET did not show changes in average PV expression within the prefrontal (anterior cingulate) cortex, retrosplenial cortex, or the somatosensory barrel fields compared to saline-treated controls.

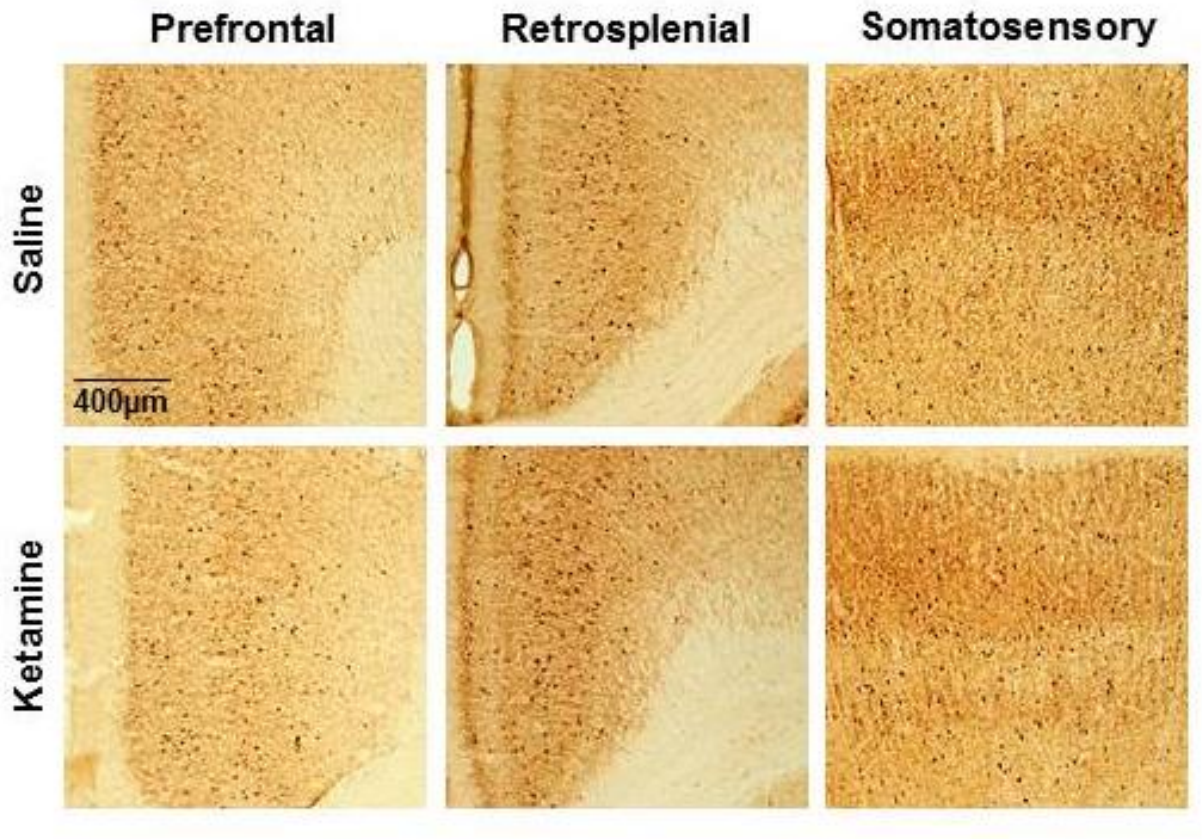

Figure 4.4: Representative photomicrographs of PV expression in neocortical regions of interest

There were no significant effects of chronic KET treatment on PV expression within any neocortical regions of interest observed (prefrontal, retrosplenial, or somatosensory).

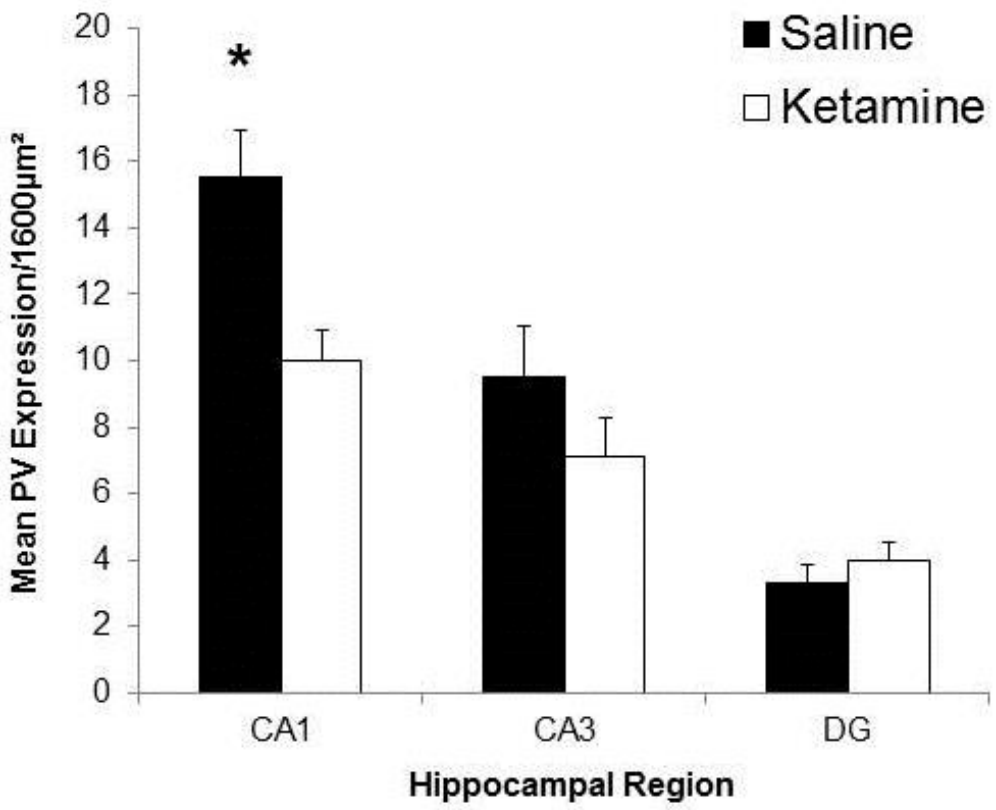

Figure 4.5: Chronic KET administration decreases CA1 PV expression

Chronic KET administration significantly decreased the number of PV expressing neurons in the CA1 sub-region of the HPC. However, this effect was selective for CA1, as CA3 and DG sub-regions did not show an effect of KET treatment.
*$p < 0.01$

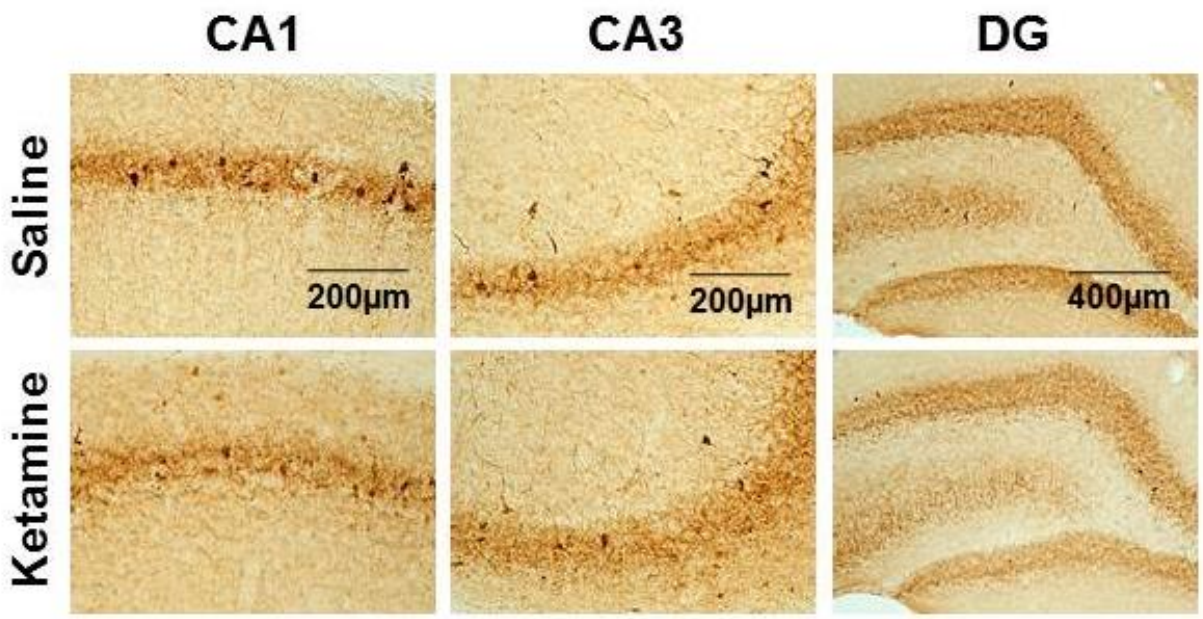

Figure 4.6: Representative photomicrographs of PV expression within the HPC

Chronic KET administration significantly decreased average PV expression exclusively within the CA1 sub-region of the HPC. There was no effect of KET treatment on PV expression within CA3 or DG sub-regions.

Table 4.1: Descriptive Data

		Saline	Ketamine
Prefrontal (Cingulate; n = 8)		107.4 ± 9.2	104.8 ± 5.9
Retrosplenial (n = 8)		92.0 ± 18.1	80.4 ± 12.1
Somatosensory (n = 8)		68.1 ± 9.5	68.8 ± 9.0
Hippocampus			
	CA1 (*n* = 24)	15.5 ± 1.4	10.0 ± 0.9*
	CA3 (*n* = 24)	9.5 ± 1.5	7.1 ± 1.2
	DG (*n* = 16)	3.3 ± 0.6	4.0 ± 0.6

Descriptive data in the above table is expressed as M ± S.E.M. and represents the average number of PV expressing cells per 1600µm² for each region (n's indicated above)*.*

An asterisk (*****) following the M±SEM indicates that the PV expression is significantly different from saline control at the p < 0.01 level

CHAPTER 5

Age and time of sacrifice mediate expression of the calcium-binding protein parvalbumin in a model of NMDA hypofunction

5.1 Abstract

A marked decrease in the expression of the calcium-binding protein, parvalbumin (PV), has been a consistently replicated finding in postmortem tissue of patients with schizophrenia. This reduction is selective to PV, and is not seen in other calcium-binding proteins (e.g. calretinin), and alterations are exclusively seen within the prefrontal cortex and hippocampus of patients. Rodent models of NMDA-receptor hypofunction utilizing non-competitive NMDA antagonist drugs such as ketamine (KET) to induce schizophrenic-like cognitive impairments show a comparable decrease in PV immunoreactivity, which is often considered a marker of underlying neuropathology. However, previous studies looking at the effects of chronic KET administration on cognitive impairment and concomitant changes in PV expression are contradictory, with findings suggesting decreased, increased, or even no change in PV expression. Upon examination of the procedures used across studies it is clear that there many inconsistencies, particularly with regard to the age of animals used, as well as the timeline of tissue collection. In order to better understand the possibility of methodological differences impacting PV expression, the present study specifically investigated the impact of age and time of sacrifice/brain tissue collection in behaviorally-naïve male Sprague-Dawley rats following chronic KET treatment (14 consecutive days of 30 mg/kg IP) compared to controls. Our findings suggest a dynamic interaction between age and time of sacrifice on prefrontal cortical and hippocampal PV expression based on treatment. Additionally, we provide compelling evidence for age-related differences in PV response to chronic KET treatment, such that 1 month old rats exhibit decreased PV, while

naïve adult rats show increased PV following KET treatment. Based on our data, we propose that PV expression is a highly dynamic marker, and that changes in expression following NMDA antagonist treatment should be considered in the context of the age of the animals used.

5.2 Introduction

Pervasive cognitive deficits are a hallmark of schizophrenia (SCZ) and are predictive of long term outcome and functionality in patient populations (Elvevag & Goldberg, 2000; Keefe & Harvey, 2012).Postmortem findings in patients have revealed a selective decrease in the expression of the calcium-binding protein (CBP), parvalbumin (PV), within the prefrontal cortex (Beasley & Reynolds, 1997) and hippocampus (HPC; Zhang & Reynolds, 2002). PV is localized to GABAergic inhibitory neurons targeting the perisomatic region of postsynaptic pyramidal cells, and plays a key role in the coordination of neural synchrony within frontal and limbic circuitry important for maintaining effective cognitive and memory processes (Gonzalez-Burgos et al., 2015).

Rodent models of schizophreniform cognitive impairments have been integral to advancing our understanding of the pathophysiology underlying these deficits and provide insight into their putative neural mechanisms. NMDA hypofunction induced by NMDA antagonist pharmacological agents (e.g. ketamine, MK-801, PCP) is a widely accepted and used model in large part to its ability to replicate the cognitive-behavioral impairments characteristic of SCZ (Meltzer et al., 2013). Additionally, administration of NMDA antagonist drugs have been shown to replicate neuropathological features of the disorder; specifically, many groups have observed a decrease in PV expression prefrontal and HPC brain regions following acute and/or chronic administration of these drugs (Adell et al., 2012). Despite general agreement that decreased PV expression following pharmacologically induced NMDA hypofunction is a valid model of schizophrenic neuropathology, there exists a growing number of studies presenting

conflicting findings, calling into question the aptness of utilizing PV as a marker of pathology.

Within the literature discrepancies regarding the outcomes of NMDA antagonism on neuropathology, particularly PV expression, are apparent with some groups indicating significant decreases in PV expression following treatment (e.g. Kittelberger et al., 2012), and some reporting no change (e.g. Benneyworth et al., 2011). Interestingly, upon examination of the methods employed across various studies, it clear that differences in methodology exist, including: 1) whether or not behavioral assays were used to assess concomitant cognitive impairment; 2) the age of the animals used in the studies; and 3) the timeline of tissue collection following treatment and/or behavioral assays. Previously, we have provided evidence suggesting that behavioral training increases the expression of PV expression across brain regions (Corriveau, Master's Thesis, 2013; in agreement with Gomes da Silva et al., 2010; Urakawa et al., 2013), and therefore the act of behavioral experience may serve to mask possible NMDA antagonist-induced decreases in PV(see Chapters 3 and 4).

Findings indicating developmental changes in PV expression from young to adult rodents provide a compelling argument for considering age as a factor, since there appears to be a decrease in PV expression within the HPC in adult compared to young animals (see Chapter 2; Corriveau, Master's Thesis, 2013; in agreement with De Jong et al., 1996). Time of tissue collection is also likely to be a significant mediator of PV immunoreactivity, as emerging evidence suggests that PV phenotype begins to recover as a function of time since treatment cessation (Behrens et al., 2008). In order to better understand the normative relationship between PV and these factors, the present study

sought to investigate: 1) changes in PV expression from 1 to 6 month old behaviorally naïve rats; 2) the impact of chronic KET administration on PV expression as a function of age; and 3) the role of tissue collection time line post KET cessation on PV expression. Here, we provide compelling evidence suggesting a differential effect of chronic KET administration based on age, such that 1 month old rats show significant decreases in PV expression, whereas 6 month old rats show a pattern of increased PV expression. Furthermore, we provide evidence suggesting a role of tissue collection timeline on PV immunoreactivity. Our work suggests that PV expression is highly dynamic and can be mediated by a myriad of factors, and therefore may not be a reliable marker of pathology.

5.3 Methods

5.3.1 Subjects and drug administration

Thirty male Sprague Dawley rats sourced from Charles River Laboratories (Wilmington, MA) were used for the present study and were aged 1 month (approximately PD30; *n* =15) and 6 months (*n* = 15). Prior to pharmacological manipulations, all rats were individually housed in polycarbonate caging with access to food and water *ad libitum*. Rats were housed in a temperature and humidity controlled vivarium with a 12 hour light/dark cycle (lights on at 0800h) and were exposed to normal husbandry procedures, but were otherwise behaviorally naïve. Subsets of rats from each age group (1- and 6-months) were assigned to one of 3 distinct treatment groups consisting of: 1) chronic ketamine regimen with tissue collection immediately following treatment cessation (30 mg/kg IP; *n* = 5 per age group); 2) chronic ketamine regimen with tissue collection delayed 10 days post treatment cessation (30 mg/kg IP; *n* = 5 per age group); or 3)

comparable IP injection treatment of physiological saline (*n* = 5 per age group). All housing and experimental procedures were performed in accordance with, and approved by, the University of Connecticut Institutional Animal Care and Use Committee.

5.3.2 Immunohistochemistry

Rats were anesthetized with Euthasol (pentobarbital sodium solution; .5 – 1mL via IP injections) and transcardially perfused with ice-cold 0.9% physiological saline solution immediately followed by ice-cold 3.7% paraformaldehyde solution. Following perfusions, brains were removed and individually stored in scintillation vials filled with 3.7% paraformaldehyde solution for 1 week. Prior to sectioning, brains were transferred into a 30% sucrose solution for cryoprotection. All brains were sliced into 60µm serial coronal sections on a cryostat and stored in phosphate-buffered saline (PBS)-containing well-plates for storage prior to immunohustochemical procedures.

One series of tissue sections from each brain was selected to examine PV immunoreactivity, with sections encompassing tissue approximately +2.5mm through +0.25mm from Bregma to include anterior cingulate cortex, and -2.5mm through -6mm relative to Bregma to include the septotemporal extent of the HPC (Paxinos & Watson, 1997). Free floating sections were initially blocked in 5% normal goat serum (NGS; Jackson Immuno), 0.1% triton-X, and PBS solution for 1 hour, followed by 3 five-minute rinses in PBS. Sections were then transferred into primary antibody solution (1:16000 anti-rabbit parvalbumin polyclonal antibody (ABCAM), 0.1% triton-X, and PBS) for a 1 hour incubation period at room temperature. Sections were then rinsed in PBS 3 times at five minutes each, and transferred to secondary solution (horseradish peroxidase

(HRP) labeled polymer anti-rabbit; DAKO) for a 1 hour incubation at room temperature. Following a final set of 3 five-minute rinses in PBS, sections were transferred into a diaminobenzidine (DAB) chromogen solution (DAKO) for ten minutes to develop the stain. Sections were then mounted and dried on glass slides, then cover-slipped for microscopy using DPX.

5.3.3 Quantification of PV expression

Slides containing tissue processed for PV immunoreactivity were analyzed using a Nikon Eclipse E600 (Melville, NY) upright microscope equipped with an Insight SPOT digital camera (Diagnostic Instruments, Inc.). Photomicrographs of regions of interest including: prefrontal cortex (anterior cingulate), retrosplenial cortex, somatosensory barrel fields, and HPC (including the CA1, CA3, and DG sub-divisions; as sampled in Nomura et al., 1997), were catalogued and stored digitally for analysis by a researcher blind to treatment and age (refer to Figure 2.1 for sampling examples). To ensure precision in the quantification of PV-expressing cells within each region of interest, photomicrographs were taken at either 20x magnification (CA1, CA3) or 10x magnification (cortical areas, DG). Refer to Figure 2.2 for photomicrograph sampling locations for each analyzed region.

Digital photographs were analyzed using ImageJ software (NIH), and the number of PV-immunoreactive cells was quantified using macros written to automate particle counting within each region of interest (refer to Figure 2.3 for an example of color-thresholding analysis performed). The sizes of the analyzed regions were as follows: HPC CA1/CA3, 800μm^2; HPC DG and cortical areas, 1600μm^2. The average number of PV expressing cells for HPC CA1 and CA3 were normalized to represent the average

number of PV-positive cells per 1600μm² for cross regional comparisons and consistency across data sets.

5.3.4 Statistical Analysis of PV expression

In order characterize the differences in PV immunoreactivity based on treatment and time of sacrifice, the average number of PV expressing cells was calculated for each rat as measure using ImageJ, and group means were computed for each treatment group within each age group. This was done across brain regions for each rat, and for each region the average number of PV cells was computed from 3-6 photomicrographs per region (2-3 photomicrographs from each of the left and right hemispheres). Data was collapsed across hemispheres for all brain regions as there were no significant hemispheric differences in expression. Within the HPC sub-regions (CA1, CA3, DG) averages for septotemporal regions (septal, MST, temporal) were considered individual samples and contributed to overall averages for respective regions. Differences in PV immunoreactivity were assessed using separate one-way ANOVA analyses for 1 and 6 month old animals to determine whether there were changes within each group as a function of treatment and/or time of sacrifice. An additional one-way ANO VA was conducted between age groups to determine baseline differences in PV expression in saline-treated controls as a function of age. Significant effects were followed up with post-hoc analyses where appropriate.

5.4 Results

5.4.1 Age-related effects

Overall, there was a significant effect of developmental age, which was especially

apparent in the control rats, such that 1 month old rats had significantly higher levels of PV expression within the HPC and prefrontal (cingulate) cortex regions than 6 month old rats. Furthermore, 1 month old control rats had significantly higher levels of PV across all regions of the HPC compared to 1 month old KET treated rats. Conversely, KET treated 6 month old rats showed a general trend toward an increase in PV expression compared to 6 month controls. Additionally, KET administration appears to equalize PV expression when comparing 1 and 6 month old KET treated rats. Thus, our observations consistently evidence age-related decreases in PV expression most prominent in the HPC, and an interaction between age and KET treatment on PV expression across multiple brain regions.

5.4.1.1 Neocortex Results

Analyses revealed a significant decrease in PV expression in 6 month old compared to 1 month old saline controls exclusively within the prefrontal (cingulate) cortex ($F(1,9) = 8.81$, $p < 0.02$; Figure 5.1). No effect of age was seen in the retrosplenial cortex ($F(1,9) = 1.83$, $p = 0.21$), or in the somatosensory barrel fields ($F(1,9) = 2.29$, $p = 0.17$). Additionally, there was no effect of age observed between 1 and 6 month old rats in the KET group that was immediately sacrificed, or the KET group that was sacrificed 10 days post drug cessation (all p's > 0.05). For representative photomicrographs see Figure 5.2.

5.4.1.2 Hippocampus Results

Within the HPC, there was an overall decrease in PV expression as a function of age, such that 6 month old rats expressed significantly less PV than 1 month old rats in the control condition. This age-related decrease was seen in CA1 ($F(1,29) = 9.45$, $p < 0.01$),

CA3 (F(1,29) = 8.63, p < 0.01), and DG (F(1,19) = 12.62, p < 0.01). Age-related alterations in PV expression were only observed in controls, as there were no significant differences seen as a function of age in either of the KET groups (immediate or delayed sacrifice; all p's > 0.05). See Figure 5.3 for summary of finding in the HPC, Figure 5.4 for representative photomicrographs, and Table 5.1 for descriptive statistics.

5.4.2 KET administration and time of sacrifice in 1 month old rats

5.4.2.1 Neocortex Results

KET administration did not significantly impact the number of PV expressing cells within the neocortical regions of interest, nor did time of sacrifice (immediate or delayed post drug-cessation) in 1 month old rats (Figure 5.5A). Analyses revealed no significant main effect of treatment in the prefrontal (anterior cingulate) cortex (F(2,14) = 2.33, p = 0.14), retrosplenial cortex (F(2,14) = 1.15, p = 0.35), or somatosensory barrel fields (F(2,14) = 0.87, p = 0.44). Representative photomicrographs can be seen in Figure 5.2.

5.4.2.2 Hippocampus Results

There was a significant decrease in PV expression as a function of KET treatment, and this decrease was seen in both immediate and delayed tissue collection groups and was significant at all levels of the HPC: CA1 (F(2,44) = 3.97, p < 0.05), CA3 (F(2,44) = 5.52, p < 0.01), and DG (F(2,29) = 5.68, p < 0.01). Post-hoc LSD analysis of 1 month old CA1 PV expression as a function of treatment revealed that saline controls had significantly higher expression than immediate and delayed KET groups (p's < 0.05), but that KET groups did not differ from one another (p = 0.80). Within CA3 and DG, PV expression in control rats was significantly higher than in immediate and delayed KET groups (p's < 0.01), with KET groups not differing from each other (p = 0.96 and 0.94, respectively). Data summary can be seen in Figure 5.6, and

representative photomicrographs can be found in Figure 5.4.

5.4.3 KET administration and time of sacrifice in 6 month old rats

5.4.3.1 Neocortex Results

PV expression in 6 month old rats was not significantly altered by treatment within the neocortical regions of interest (Figure 5.5B).There were no significant main effects for treatment in prefrontal (anterior cingulate) cortex ($F(2,14) = 1.35$, $p = 0.30$), retrosplenial cortex ($F(2,14) = 0.89$, $p = 0.44$), or somatosensory barrel fields ($F(2,14) = 0.57$, $p = 0.58$).

5.4.3.2 Hippocampus Results

Generally, there was no effect of treatment in 6 month old rats within the HPC sub-regions, with the exception of DG. However, there appears to be a trend toward an increase in overall PV expression in 6 month old rats treated with KET (Figure 5.6). Analyses revealed no significant effect of treatment in CA1 ($F(2,44) = 1.77$, $p = 0.18$) or CA3 ($F(2,44) = 1.41$, $p = 0.26$) of 6 month old rats, regardless of tissue collection timeline. However, the general increase in PV expression as a function of KET treatment reached significance in DG ($F(2,29) = 4.61$, $p < 0.05$), such that both the immediate and delayed groups had significantly more PV than controls (p's < 0.05 and 0.01, respectively).

5.5 Discussion

Overall, the present study provides compelling evidence supporting a selective age-related decrease in PV expression within the prefrontal (cingulate) cortex. This cortical finding is in addition to the robust age-related decrease in PV expression within the HPC, which was non-specific and seen in all 3 sub-regions observed (CA1, CA3, DG).

The present work also presents a novel dissociation between the effects of chronic KET administration based on the age of the animal being treated. However, our findings suggest that time of sacrifice does not have a significant impact on PV expression in either age group, though the data appears to suggest a possible period of recovery when rats are sacrificed at a delayed time point post drug cessation. Here, we discuss the main findings observed in this work, including: 1) age is a significant factor in normative baseline PV expression; and 2) KET administration differentially modulates PV expression based on age.

5.5.1 Age significantly impacts normative PV expression

In the present work we provide strong evidence for a developmental decrease in PV expression, which is very clear when comparing 1 and 6 month old control groups. These findings are in strong agreement with work presented in this dissertation (see Chapter 2), as well as findings previously presented from our lab (Corriveau, Master's Thesis, 2013). Within the literature, there have been few reports describing age-related changes in PV expression, but of those available, the findings suggest a clear role of age in regulating PV expression (e.g., Lolova & Davidoff, 1992; Miettinen et al., 1993; Vela et al., 2003; Lee et al., 2013). The present findings provide clarity to this body of work, as the rats utilized in the current study were behaviorally naïve, whereas many studies employ behavioral assays on their subjects which likely changes baseline PV expression due to experience/enrichment (Iuvone et al., 1996; Komitova et al., 2013). These robust alterations in PV expression as a function of age should be thoroughly considered, as differences in baseline expression can interfere with experimental manipulations, leading to lack of validity as well as difficultly in interpreting and generalizing results.

5.5.2 Chronic KET treatment differentially impacts PV expression based on age

Acute and/or chronic KET administration is often linked to decreases in PV expression comparable to those seen in schizophrenic postmortem brain tissue. However, the fact that some studies find decreases in PV expression (e.g. Kittelberger et al., 2012), while others find no change (e.g. Benneyworth et al., 2011) following KET treatment begs the questions as to whether there may be additional factors impacting the reactivity of PV to such treatments. The present findings provide clear evidence indicating that age plays a key role in post-KET treatment PV expression.

Our work indicates a differential role of KET treatment that is specific to the age of the animals receiving the treatment. Specifically, we see clear evidence for a decrease in PV expression within 1 month old chronically KET treated rats compared to controls, which is in agreement with previous findings (e.g. Kittelberger et al., 2012). Conversely, we describe an opposite effect in 6 month old rats, such that those treated with KET show a general trend toward an increase in PV expression compared to controls, which reached significance in the DG. The significant elevation in PV expression within the DG seen in the current study has not been well documented in the literature, but is in agreement with a report by Sabbagh and colleagues (2013) reporting an increase in HPC PV expression in adult rats following chronic KET administration similar to that employed herein. A possible explanation for KET-induced increases in PV expression in 6 month old rats exclusively within the HPC may be due to KET's ability to promote neurogenesis within the HPC (Keilhoff et al., 2004); a phenomenon which may also explain increases in PV seen in our lab as a result of cognitive-behavioral enrichment (Corriveau, Master's Thesis, 2013).

5.6 Conclusion

The present work elucidates upon the importance of age on PV expression within the prefrontal cortex and HPC in rodents, and highlights the need for controlling for this variable when examining KET-oriented models of SCZ dysfunction. Additionally, we provide clear evidence indicating that KET-induced changes in PV expression may be exclusively seen in young rats, and that the timeline of administration is likely crucial to understanding the relationship between PV and KET-induced cognitive impairments. To our knowledge, this is the first time a differential effect of KET as a function of developmental age has been systematically described, and provides important nuance to the literature describing KET-induced changes in PV expression. Additional investigation into the impact of chronic KET administration on concomitant cognitive-behavioral impairment across differing age groups is warranted in order to better understand the role of PV in regulating cognitive processes, and whether these processes may be sensitive to aging.

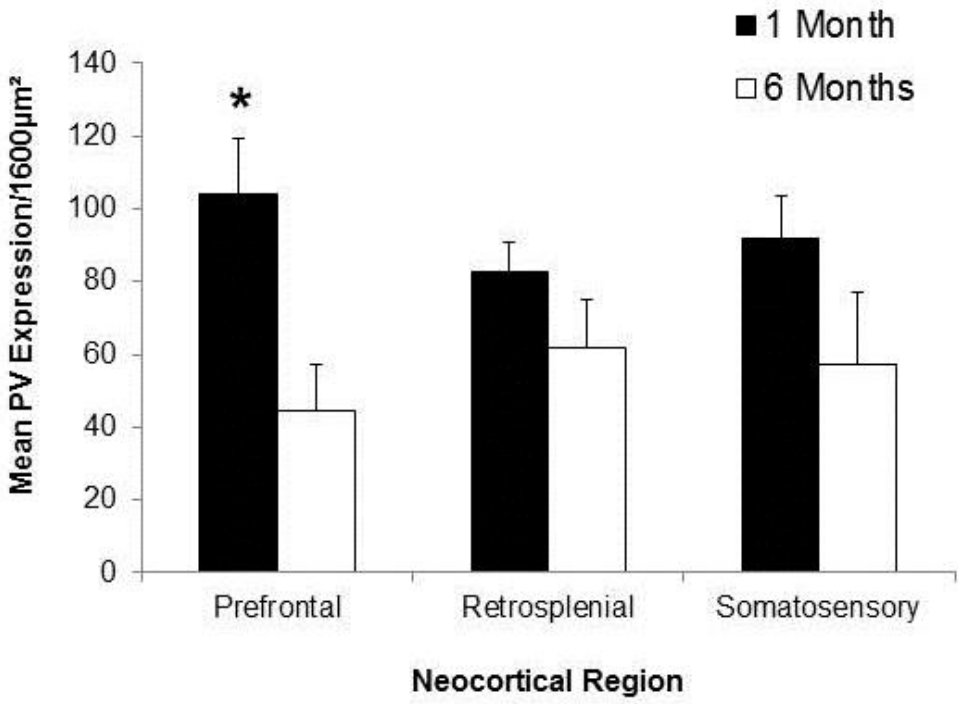

Figure 5.1: PV expression is selectively decreased in the prefrontal cortex in 6 month old controls

PV expression within the prefrontal (cingulate) cortex of 6 month old behaviorally naïve saline-controls was significantly decreased compared to 1 month old behaviorally naïve saline-controls. This decrease was selective for the prefrontal cortex, and was not seen in other neocortical regions observed.
***p* < 0.05

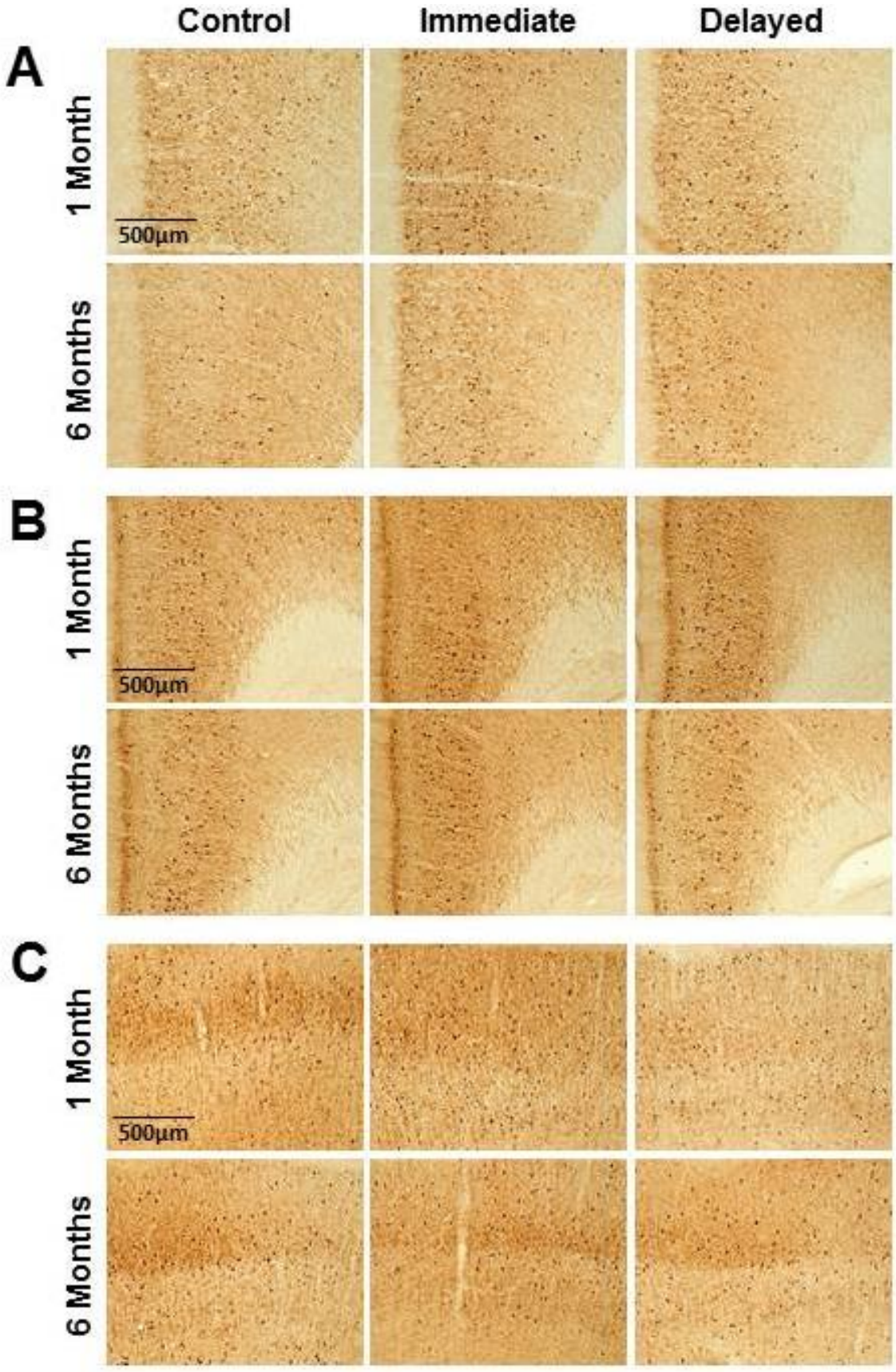
Control
Immediate
Delayed
A
1 Month
500µm
6 Months
B
1 Month
500µm
6 Months
C
1 Month
500µm
6 Months

Figure 5.2: Representative photomicrographs of neocortical regions of interest

Within the neocortical regions of interest, there was a significant effect of age in the prefrontal (cingulate) cortex (**A**), with no effect of age in the retrosplenial (**B**) or somatosensory (**C**) cortices. In the prefrontal cortex, 1 month old controls had significantly higher PV expression than 6 month old controls. There was no effect of treatment in any of the regions for either age group. Photomicrographs were taken at 10x magnification.

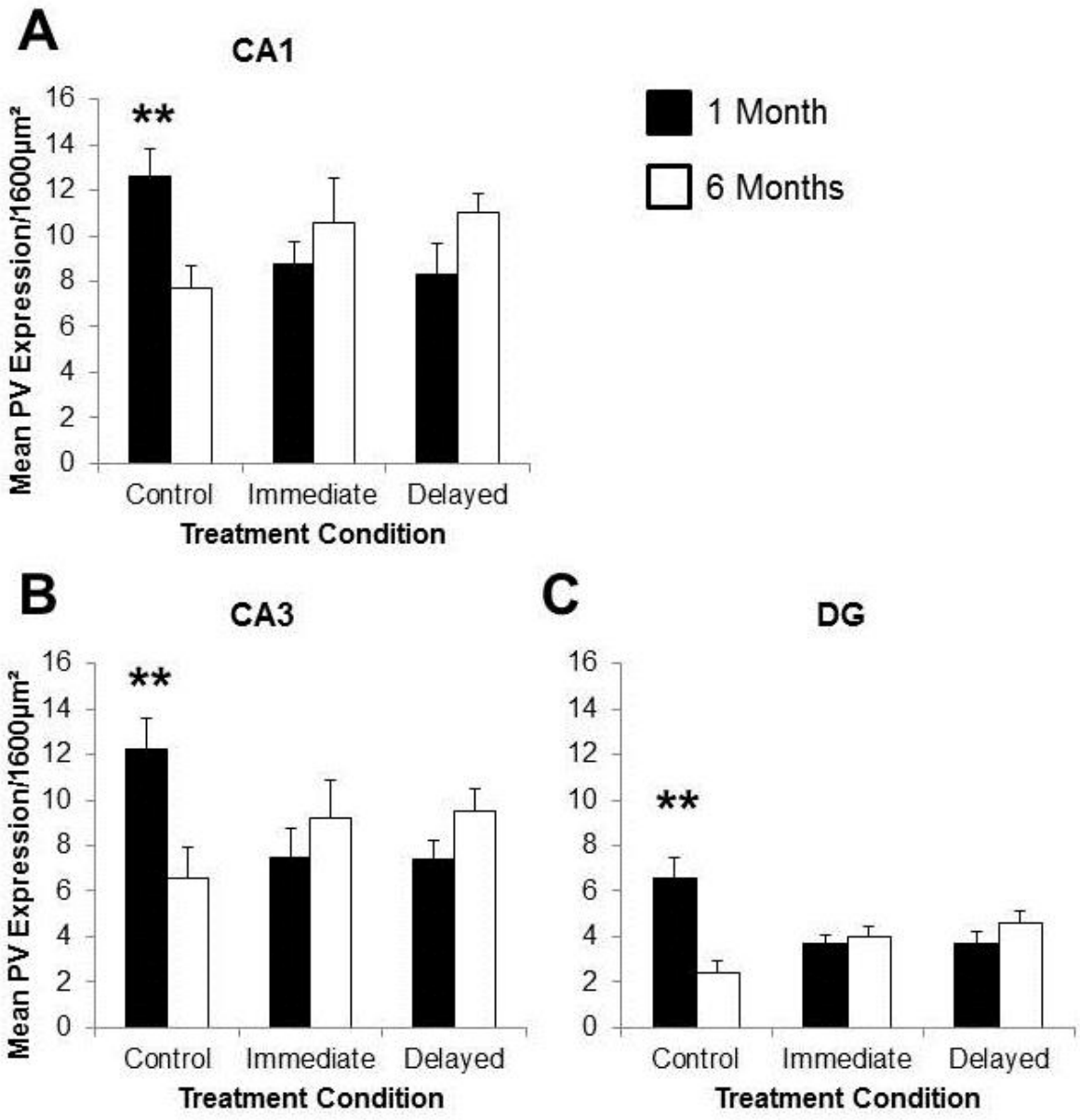

Figure 5.3: Age-dependent decline in PV expression in control rats

In the HPC, there is a significant age-dependent decrease in PV expression from 1 month to 6 month old control rats. This decline in PV expression as a function of age is significant across all HPC sub-regions: CA1 (A), CA3 (B), and DG (C). There was no effect of age on PV expression in rats chronically treated with KET at either the immediate or delayed sacrifice time points.
***p* < 0.05
****p* < 0.01

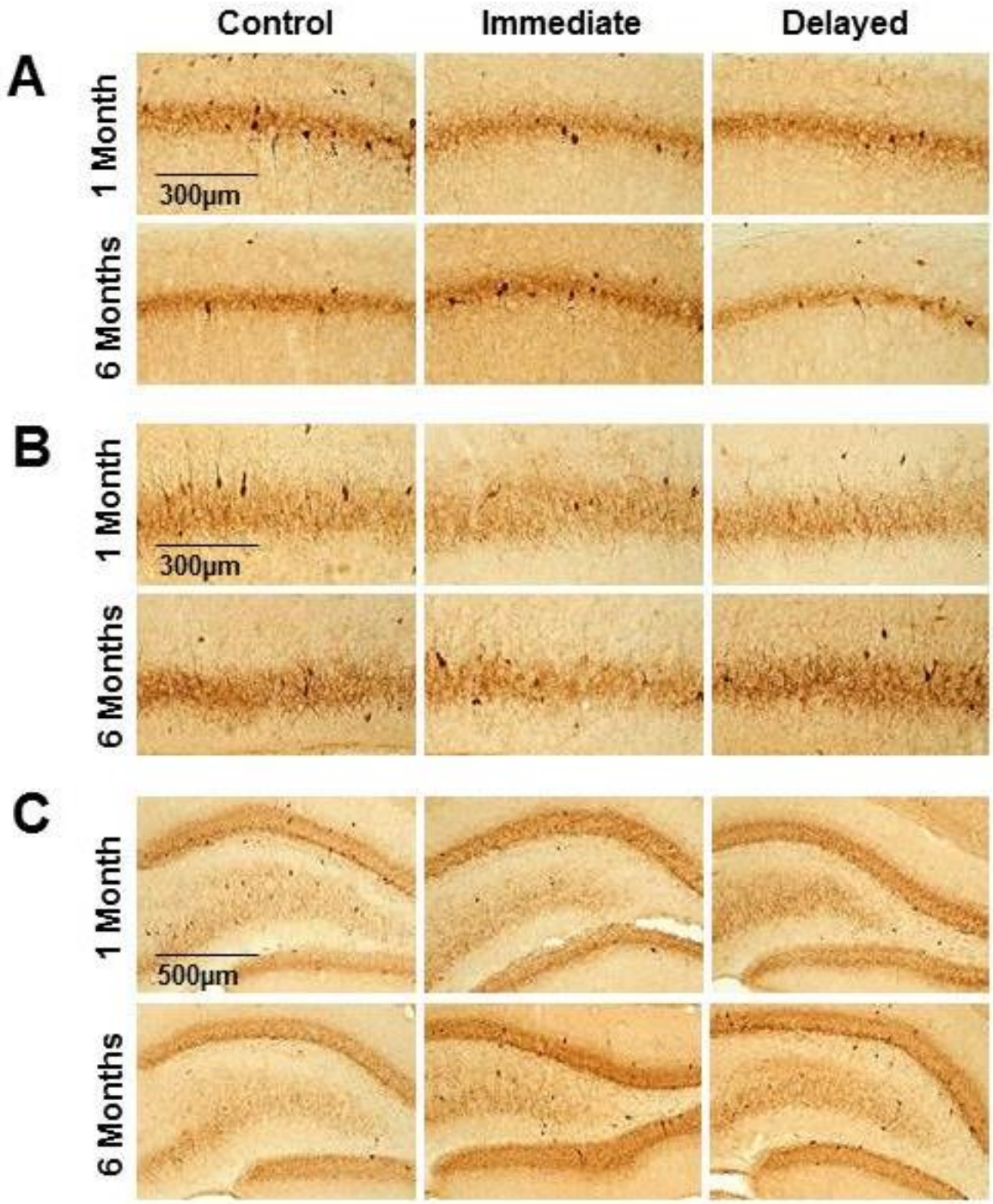
Control
Immediate
Delayed
A
1 Month
300μm
6 Months
B
1 Month
300μm
6 Months
C
1 Month
500μm
6 Months

Figure 5.4 Representative photomicrographs of HPC regions of interest

1 month old rats had significantly higher PV expression than 6 month old rats in CA1 (**A**), CA3 (**B**), and DG (**C**) in the control condition. There were no significant effects of age in any of the KET treated groups (immediate or delayed time of sacrifice post cessation). There was a significant decrease in PV expression in 1 month old rats in KET (immediate and delayed) treated rats compared to controls. In 6 month old rats treated with KET (immediate and delayed) there was a significant increase in PV expression compared to controls. Photomicrographs of CA1 (**A**) and CA3 (**B**) were taken at 20x magnification, while DG (**C**) photomicrographs were taken at 10x magnification.

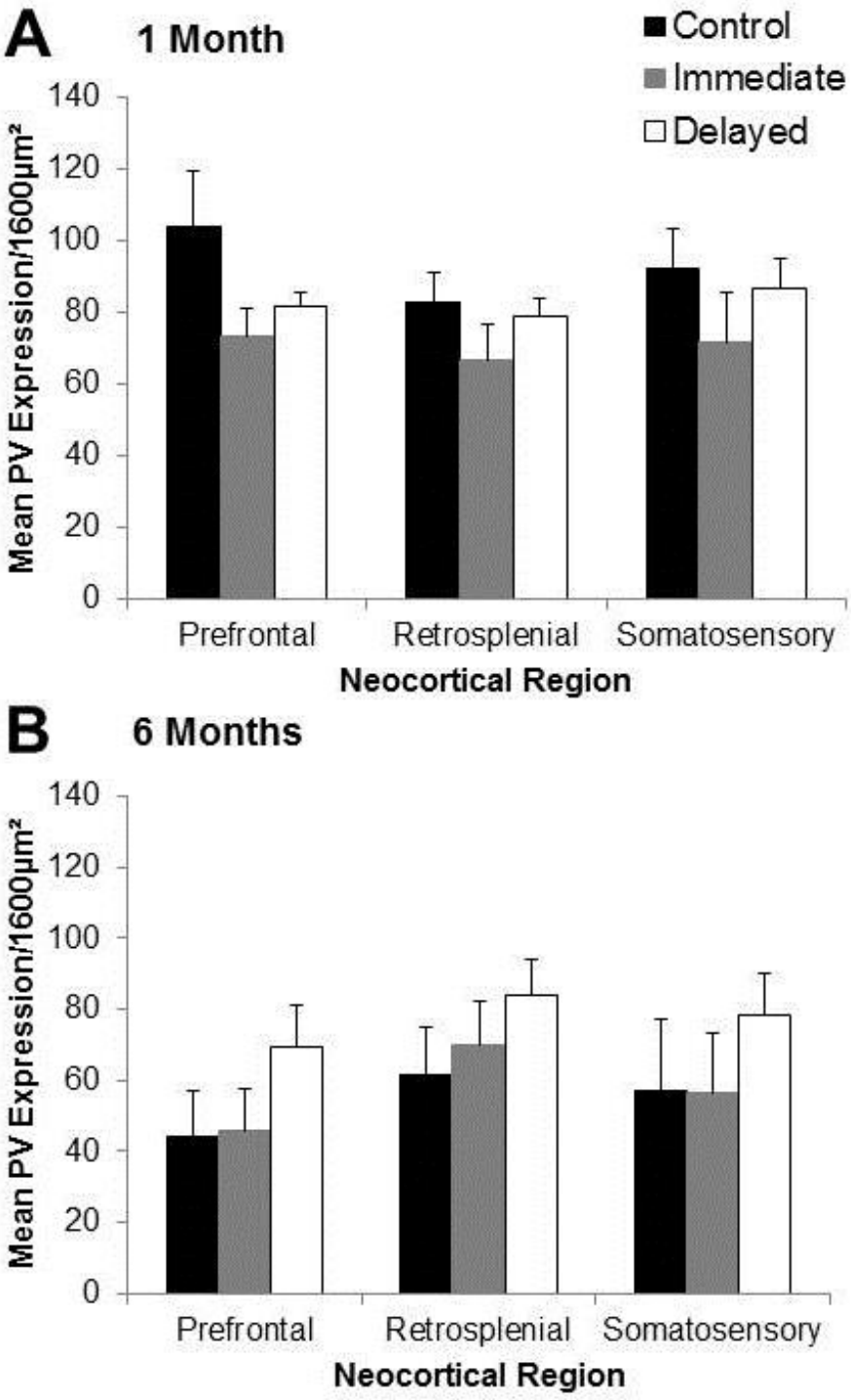

Figure 5.5: Neocortical PV expression was not significantly altered by KET treatment or time of sacrifice

No significant changes in PV expression were observed in neocortical regions of interest in 1 (**A**) or 6 (**B**) month old rats, regardless of KET treatment or time of tissue collection.

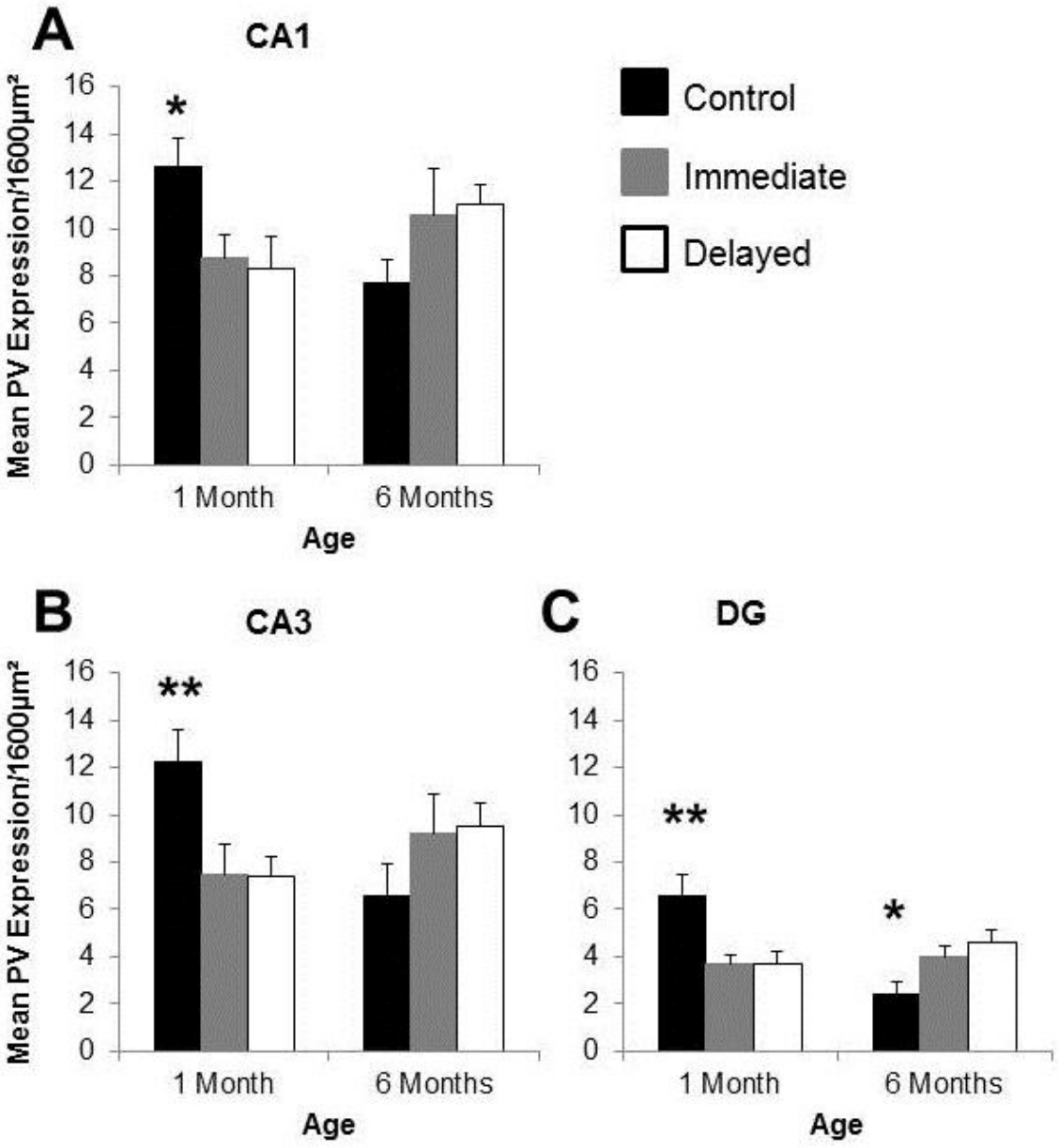

Figure 5.6: PV expression is mediated by age and treatment condition

1 month old rats showed a significant reduction in PV expression as a function of treatment, such that KET treated rats in both the immediate and delayed groups was significantly lower than saline controls in CA1 (**A**), CA3 (**B**) and DG (**C**). There was no significant change in PV expression in these regions based on time of sacrifice. In 6 month old rats, there was no effect of treatment condition in CA1 (**A**) or CA3 (**B**). However, in DG (**C**) there was a significant increase in PV expression as a result of KET treatment, and this increase was stable regardless of tissue collection timeline.

***$p < 0.05$**

****$p < 0.01$**

Table 5.1: Descriptive Data

	1 Month			6 Months		
	Control	**Immediate**	**Delayed**	**Control**	**Immediate**	**Delayed**
Neocortex (*n*'s = 5)						
Prefrontal (cingulate)	103.9 ± 15.5	73.5 ± 7.8	81.7 ± 3.9	**44.5 ± 12.6**	45.8 ± 11.7	69.4 ± 12.0
Retrosplenial	82.9 ± 8.1	66.6 ± 10.0	79.1 ± 4.7	61.6 ± 13.5	70.2 ± 12.2	84.0 ± 10.0
Somatosensory	92.1 ± 11.4	71.4 ± 14.1	86.7 ± 6.6	57.0 ± 20.2	56.4 ± 16.9	78.5 ± 11.6
Hippocampus						
CA1 (*n*'s = 15)	12.6 ± 1.2	8.7 ± 1.0*	8.3 ± 1.3*	**7.7 ± 1.0**	10.6 ± 1.9	11.0 ± 0.9
CA3 (*n*'s = 15)	12.2 ± 1.4	7.5 ± 1.2*	7.1 ± 1.1*	**6.6 ± 1.3**	9.2 ± 1.7	9.5 ± 0.9
DG (*n*'s = 10)	7.1 ± 1.1	3.9 ± 0.4*	4.0 ± 1.9*	**2.6 ± 0.7**	4.3 ± 0.5*	5.0 ± 1.7*

Descriptive data in the above table is expressed as M ± S.E.M. and represents the average number of PV expressing cells per 1600µm² for each region of interest (*n*'s indicated in table).

*An asterisk (*****) following the M ± S.E.M. indicates that the PV expression is significantly different from saline controls within the age group. Bold **M ± S.E.M.** within the 6 month old data indicates that the average PV expression for that region is significantly decreased compared to 1 month olds rats.*

CHAPTER 6

Discussion

6.1 Summary of findings

Overall, the current work sheds light on the need for considering age in rodent models of psychiatric disorders to account for the dynamic expression of neuronal markers – such as parvalbumin (PV) – throughout the lifespan. This is evidenced through multiple results indicating significant decreases in PV expression in adult rats compared to high baseline levels of PV expression in adolescent rats. Additionally, the present findings indicate that age and behavioral training interact with chronic ketamine (KET) administration. Specifically, we describe: 1) that chronic KET administration decreases PV expression in 1 month old rats, while it appears to increase PV expression in 6 month old rats; and 2) chronic KET administration significantly impairs cognitive performance in the DMTS-RWM task in both young and adult rats, but only young rats show concomitant decreases in PV expression. We also report herein that the effects of age and behavioral experience appear to be negated when rats are given a chronic KET treatment, . Together, the present work systematically describes the dynamic nature of PV expression across the lifespan, and provides compelling evidence for: 1) a significant age-dependent decline in PV expression from adolescent to adult rats; 2) differential impact of chronic KET administration on PV expression dependent on age; and 3) a role for cognitive-behavioral training/experience in the modulation of PV expression across multiple brain regions.

6.2 Age-related effects on parvalbumin expression

The present findings from Chapter 1 of this dissertation provide evidence for age (1, 6, 12 months), regional (CA1, CA3, DG) and septotemporal location (septal, MST, temporal) dependent variation in PV expression within the HPC. Conversely, we do not observe significant age-dependent variation in PV expression within neocortical and striatal regions. These findings are discussed with reference to their regional and age-dependent differences below. The present work provides the first systematic characterization of PV expression within the HPC in key sub-regions and across the entire septotemporal axis as a function of age; a topic poorly characterized within the literature.

Age as a robust determinant of PV expression is perhaps one of the most compelling and interesting findings described in this dissertation, in addition to being a factor that appears to interact with various other factors to dynamically modulate PV. In addition to the significant age-dependent decline in PV expression outlined in Chapter 2, in Chapter 5 1 and 6 month old saline-control groups were compared, revealing a marked (approximately 50%) decrease in HPC PV expression in 6 month compared to1 month old rats. This stark decrease in PV expression from 1 to 6 months, in conjunction with the age-related decreases presented in Chapter 2, provide compelling evidence for natural alterations in PV expression across development. The consistency of this finding across multiple studies in our lab suggests that this age-dependent decline is robust and brain-region specific, and should therefore be considered when developing and interpreting models of putative neural pathology.

6.2.1 Hippocampal parvalbumin expression

The HPC structure is integral for cognitive processing and memory formation within the

brain (Milner et al., 1998), and can be divided along its septotemporal axis into three distinct regions (Amaral & Witter, 1989). Septal, MST, and temporal regions of the HPC are thought to be differentially associated with distinct roles in information processing, with septal regions receiving spatial and sensory information, while temporal regions are associated with emotionally salient content (Bannerman et al., 2004). Due to the differences in the type of information processed, as well as overall differences in functionality, it follows that anatomical markers might also vary as a function of septotemporal location (Gusev et al., 2005). The distribution of PV expression across the long axis of the HPC are not well documented in the literature, and only a small number of studies (the present work included) detail changes in expression across the entirety of the HPC. Nomura and colleagues (1997) characterized PV expression across the septotemporal axis of young rats, and found no difference in PV expression as a function of septotemporal location. This finding is replicated within the current work, as we describe a lack of septotemporal variation across all HPC sub-regions in 1 month old animals (see Chapter 2). However, to our knowledge, there has been no systematic evaluation of PV expression across the septotemporal axis in adult and/or aged rats. To this end, the present work carried out in Chapter 2 of this dissertation provides clarity to the literature, detailing that 6 and 12 month old behaviorally naïve rats show a selective decrease in PV expression across the CA1 long axis, such that septal regions of CA1 have an elevated number of PV expressing cells compared to MST and temporal regions, with stable septotemporal distribution within CA3 and DG (refer to Figure 2.7).

In terms of age-dependent changes in PV expression within the HPC, we see a general decrease in PV expression in 6 and 12 month old rats compared to 1 month old

rats, with expression remaining stable between these later time points. This finding suggests that PV expression is initially high in young rats, and then decreases in adulthood; therefore PV likely changes as a function of neural development. These findings are consistent with preliminary findings in our lab as previously described (Corriveau, Master's Thesis, 2013).Furthermore, the age-related decline seen in the present work is in agreement with age-dependent changes in PV expression observed by other groups (e.g., Shetty & Turner, 1998; Lee et al., 2008). Providing additional support for age being a key player in PV expression, Chapter 5 presents evidence of a decrease in PV expression in 6 month compared to 1 month old control rats. Generally, we also describe a lack of additional decline in expression from 6 to 12 months of age, indicating that PV expression appears to remain constant during adulthood. We propose that this decrease may be due, at least in part, to pruning during the pubertal period, and may be cell-type specific (Fish et al., 2014). However, further examination into this phenomenon is necessary to pinpoint underlying mechanisms of protein expression alterations due to aging.

6.2.2 Cortical parvalbumin expression

Generally, we found no significant effect of age on PV expression within any of the neocortical or striatal regions of interest we examined across the present studies within the following regions of interest: caudate, anterior cingulate, somatosensory barrel fields, retrosplenial cortex, and ventral auditory cortex. However, we did observe one instance where there was a significant decrease in PV expression in 6 month old rats compared to 1 month old rats within the prefrontal (anterior cingulate) cortex (see Chapter 5). Due to the fact that this decrease was only observed in a single study, it is unlikely to be a stable age-related finding. This is especially true in light of the work

conducted in Chapter 2, where there was a trend toward significance for 6 and 12 month old rats to have elevated PV expression compared to 1 month old rats. Likely, we do not see significant changes in many of these cortical regions due to the early maturation of inhibitory circuitries, compared to the slower maturation of circuits in areas like the HPC (Bergmann et al., 1991) and the prefrontal cortex (Lewis et al., 2004; Crews et al., 2007), and therefore the treatment windows employed in the current work may not encompass critical windows of circuit development.

6.4 Effects of chronic ketamine administration on behavior and neuroanatomy

6.4.1 Effect of chronic ketamine on radial water maze performance

Chronic KET administration had a clear impact on cognitive performance in the DMTS-RWM task, which is evidenced in findings presented in Chapters 3 and 4 of this dissertation (see Figures 3.2 and 4.2, respectively). In both studies, chronic KET administration significantly impaired performance, such that KET treated rats committed significantly more errors when attempting to find the goal location than saline controls. In Chapter 3 KET treated rats also displayed a lasting effect of treatment up to 3 days post drug-cessation, indicating that the impact of chronic KET on cognition may be due, at least in part, to underlying changes in neural pathology (Morgan et al., 2004). Impairment on performance within this task suggests that chronic KET administration interferes with cognitive processes integral to spatial memory and temporal dissociation of goal representations (Chrobak et al., 2008), therefore creating increased instances of proactive interference similar to that seen in SCZ patients (Barch & Smith, 2008).

Interestingly, we describe a differential effect of chronic KET treatment with regard to age and behavioral experience as evidenced in Chapters 3 and 4. Specifically,

in Chapter 3, we describe a significant KET-induced impairment in cognitive performance in 6 month old adult rats, with no parallel changes in average PV expression compared to saline controls. In Chapter 4, we also describe a significant KET-induced impairment in cognitive performance in the DMTS-RWM task in 2-3 month old adolescent rats, in addition to a marked concomitant decrease in PV expression in KET compared to saline treated rats. These findings, when considered together, provide compelling evidence for an age-dependent interaction with chronic KET treatment on PV expression, indicating that young rats (2-3 months) appear to be more vulnerable to KET-induced cognitive impairment, in addition to more sensitive to the deleterious impact of KET treatment on PV expression, specifically within HPC CA1.

6.4.2 Effect of chronic ketamine on parvalbumin expression

6.4.2.1 Hippocampal parvalbumin expression

In the present set of studies, the impact of chronic KET administration varied as a function of age and, to a certain extent, behavioral experience on PV expression within the HPC formation. In Chapter 3, we describe no significant effect of chronic KET administration within any of the HPC sub-regions in adult rats. However, this is likely due to the advanced age (6 months) of the rats, as younger (2-3 month old) KET treated rats showed a significant decline in PV expression within the HPC. This indicates that PV is likely more vulnerable in young rats, thereby making chronic KET treatment more impactful on both cognition and neuroanatomy.

In agreement with the findings from Chapters 3 and 4, Chapter 5 details findings indicating that 6 month old rats receiving chronic KET administration did not generally show changes in PV expression within the HPC, with the exception of the DG, where there was a significant increase in PV expression following KET treatment. In contrast,

we provide compelling evidence for a robust and significant KET-induced decrease in PV expression within 1 month old behaviorally naïve rats. The reduction in PV expression is nearly 30% in KET treated groups across all regions of the HPC in 1 month old rats, and this reduction stays consistent regardless of time of sacrifice, indicating a long-term effect of chronic KET treatment on underlying neural pathology. The fact that we see such a robust reduction in PV expression in 1 month old rats, but not 6 month old rats, following chronic KET administration within the HPC is novel, and suggests that KET may be interacting with PV expressing neurons in different ways based on age and age-related changes in PV-localized NMDA receptors (Cull-Candy et al., 2001). This differential effect speaks to the overwhelming need to control for developmental age when utilizing NMDA hypofunction preparations, as age is a critical factor in normative PV expression.

6.4.2.2 Cortical parvalbumin expression

Generally, within the neocortical regions of interest (prefrontal/cingulate cortex, retrosplenial cortex, and somatosensory barrel cortex), there were no significant effects of KET administration. In Chapters 3 and 4, there are no significant effects of chronic KET treatment on PV expression within the neocortex. Additionally, in Chapter 5, there was no effect of KET administration on cortical PV expression in 1 or 6 month old rats. This indicates that cortical regions appear to be resilient to the deleterious effects of NMDA hypofunction induced by chronic KET treatment, regardless of age and experience. The lack of a KET-driven effect on PV expression in the neocortical regions is likely due an advanced and early maturation in these areas compared to HPC regions, where we see increased susceptibility (Yu et al., 2006).

6.5 Future directions

While the present set of studies provides clarity to the literature, additional research is warranted to further investigate and elucidate upon the findings presented herein. With regard to the significant age-dependent reduction in PV expression across development, it would be useful to further characterize the developmental shift in PV expression across the entire lifespan of the rat. Such a study would provide much needed characterization of the dynamic role of age in overall PV expression, and would allow for researchers to pinpoint, more precisely, the time course during which PV begins to decline toward adult levels from initial elevated baseline adolescent expression.

Further characterization of the impact of enrichment is also an avenue for future research, as the present, and previous (e.g. Corriveau, Master's Thesis, 2013), work provides evidence for a mediating effect of behavioral training on PV expression. While the present studies suggest an upregulation in PV expression based on behavioral training consistent with previous work (Corriveau, Master's Thesis, 2013), it is unclear whether similar training, or minimally early-life enrichment, in very young (<1 month) would elicit a similar result. Such a study could provide evidence for behavioral interventions early on in life to buffer the deleterious effects of insults – whether they be in the form of NMDA hypofunction or otherwise – later in life. Overall, these proposed studies would allow for systematic and thorough investigation into key factors we have shown to be integral in the dynamic regulation of PV expression within the rat brain.

6.6 Final conclusions

Taken together, the current body of work provides a comprehensive characterization of the dynamic impact of age, behavioral experience, and chronic KET administration on PV expression within the rat brain. Our findings provide a solid foundation for future work detailing a time-line of PV expression across multiple developmental time points, in addition to providing a framework for understanding the role of age and chronic NMDA hypofunction on PV immunoreactivity. Our findings provide timely and compelling evidence for a significant role of age in PV expression within the HPC that should be carefully considered when modeling SCZ pathology, as this factor markedly modulates PV. Importantly, the current findings contained in the present dissertation, and those we have proposed, allow for enhanced understanding and interpretation of future studies aiming to understand the dynamic relationship between PV expression and SCZ cognitive dysfunction. Overall, the present work in this dissertation present clear evidence that PV expression is mediated by a myriad of influences, and thus may not be an accurate marker of pathology due to the dynamic nature of its neuronal expression, as well as its unclear relationship to cognitive dysfunction.

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