Timing of methamphetamine exposure during adolescence differentially influences parvalbumin and perineuronal net immunoreactivity in the medial prefrontal cortex of female, but not male, rats

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Abstract

Adolescence involves significant reorganization within the medial prefrontal cortex (mPFC), including modifications to inhibitory neurotransmission mediated through parvalbumin interneurons (PV) and their surrounding perineuronal nets (PNNs). These developmental changes, which result in increased PV neuron activity in adulthood, may be disrupted by drug use and cause lasting changes in mPFC function and behavior. Methamphetamine (METH), which is a readily available drug used by some adolescents, increases PV neuron activity and could influence the activity-dependent maturational process of these neurons. In the present study, we used male and female Sprague Dawley rats to test the hypothesis that METH exposure influences PV and PNN expression in a sex- and age-specific manner. Rats were injected daily with saline or 3.0 mg/kg METH from early adolescence (EA; 30-38 days old), late adolescence (LA; 40-48 days old), or young adulthood (60-68 days old). One day following exposure, effects of METH on PV cell and PNN expression were assessed using immunofluorescent labeling within the mPFC. METH exposure did not alter male PV neurons or PNNs. Females exposed in early adolescence or adulthood had more PV neurons while those exposed in later adolescence had fewer, suggesting distinct windows of vulnerability to changes induced by METH exposure. In addition, females exposed to METH had more PNNs and more intense PV neuron staining, further suggesting that METH exposure in adolescence uniquely influences development of inhibitory circuits in the female mPFC. This study indicates that the timing of METH exposure, even within adolescence, influences its neural effects in females.

Keywords: PV, adolescent vulnerability, PNNs, METH

1. Introduction

Adolescence is a time when plasticity remains relatively high as prefrontal cortical regions finish development (Casey et al., 2008). This may result in vulnerability to the long-lasting consequences of exposure to drugs. Work in humans indicates that significant drug use during adolescence is associated with an increase likelihood of developing a substance use disorder (Chen et al., 2009; Grant & Dawson, 1997), increased rate of relapse and psychosocial problems (Poudel and Gautam, 2017). Those who begin using methamphetamine (METH) during adolescence may be at an especially higher risk as they have higher rates of relapse than those who use other drugs (Rawson et al. 2005). Furthermore, females may be especially vulnerable to the negative consequences of drug use, exhibiting a "telescoping effect" that is characterized by a faster escalation from initial to problematic drug use (Brecht et al., 2004; Haas & Peters, 2000; Piazza et al., 1998; Towers et al., 2023). Concomitantly, females also initiate METH use at a younger age than males and may have greater behavioral and neurobiological consequences of early life drug exposure (Hammerslag and Gulley, 2016; He et al., 2013; Hser et al., 2005; Mayo et al., 2019).

Work in rats has further characterized differences between adolescent- and adult-onset stimulant use (Westbrook et al., 2020a). Rats that begin long-access self-administration of METH during adolescence escalate their METH intake to a greater extent, suggesting adolescents progress to compulsive drug taking faster than adults (Anker et al., 2012; Westbrook and Gulley, 2020; Westbrook et al., 2020b). When sex differences have been studied, adolescent females have been found to escalate METH intake to a greater (Westbrook et al., 2020b) or equal extent (Westbrook and Gulley, 2020) compared to male adolescents. They also appear to be less sensitive to the acute, stimulant effects of amphetamine, but more sensitive to its ability to induce locomotor sensitization following repeated exposure, when compared to female adults and males (Mathews and McCormick, 2007). Additionally in females, but not males, METH self-administration during adolescence resulted in impaired cognitive flexibility in adulthood (Hankosky et al., 2018a).

The long-term consequences of adolescent METH use may be due to METH influencing ongoing developmental processes within the prefrontal cortex (PFC) as METH has been shown to alter GABAergic transmission in the PFC (Armenta-Resendiz et al., 2022). Inhibitory circuits in the PFC change during adolescence. Fast-spiking interneurons express the calcium binding protein parvalbumin (PV) in an activity dependent manner with increased expression across the juvenile and adolescent periods (Caballero et al, 2014; Solbach et al., 1991; Alcántra et al., 1993; Fung et al., 2010), and induce feedforward inhibition of pyramidal cells (Dilgen et al., 2013; Kvitsiani et al., 2013; Lewis et al., 2022). Concurrently, spontaneous EPSCs recorded in PV neurons increase across adolescence, ultimately resulting in increased IPSCs recorded in pyramidal cells (Caballero et al., 2014; Cass et al., 2014). Indeed, disrupting this maturational process during adolescence by downregulating the PV protein leads to long lasting deficits in inhibitory tone in the mPFC (Caballero et al., 2020).

During maturation, PV neurons become enwrapped in aggregations of extracellular matrix proteins called perineuronal nets (PNNs). PNNs are widely expressed throughout the central nervous system, where they influence PV neuronal function, increasing cell excitability and firing rate (Balmer, 2016; Carceller et al., 2020). PNNs have been shown to limit plasticity in the visual cortex, and their degradation restores juvenile ocular dominance plasticity (Lensjø et al., 2017; Mueller et al., 2016; Pizzorusso et al., 2002; Ueno et al., 2018). Furthermore, PNNs have been implicated in reduced plasticity in several regions as they limit and stabilize synaptic connections onto PV neurons (Carstens et al., 2016; Carulli et al., 2020; Thompson et al., 2018; Xue et al., 2014;). PNN expression increases across adolescence in both density and number (Baker et al., 2017; Drzewiecki et al., 2020; Ueno et al., 2017), which may indicate stabilization of function in the mPFC from the juvenile to the adult period.

PNNs are highly plastic structures that are influenced by a myriad of environmental factors including the diurnal rhythm (Harkness et al., 2021), environmental enrichment (Slaker et al., 2016a), high fat diet (Dingess et al., 2018), female puberty (Drzewiecki et al., 2020), and exposure to cocaine (Slaker et al., 2018). Long term changes in PNN expression occur after intermittent ethanol exposure during adolescence (Dannenhoffer et al., 2022). This suggests that while PNNs respond to many stimuli, experiences during adolescence may result in enduring changes. In addition, the developmental trajectory of PNNs in the mPFC could make female PV neurons particularly vulnerable following pubertal onset, where there is a transient decrease in the number of PNNs (Drzewiecki et al., 2020). This could make the post-pubertal period for females one of unique vulnerability to the effects of the environment. PV neurons are also influenced by drugs of abuse, with METH increasing PV neuronal excitability (Armenta-Resendiz et al., 2022) and PV immunoreactivity (Veerasakul et al., 2016). Given that the maturation of PV neurons appears to be activity dependent, METH's impact on their excitability could influence this process.

In the current study, we hypothesized that exposure to METH would influence the maturation of PV neurons and PNNs in a sex- and age-specific manner. The preponderance of evidence suggests that the female pubertal period results in large changes within the PFC, including modifications to inhibition, PNNs, and synapses (Piekarski et al., 2017; Drzewiecki et al, 2020; Drzewiecki et al., 2016). In addition, female puberty also influences the effects of amphetamine on drug-associated behaviors (Kang et al., 2016), suggesting female puberty alters how amphetamine influences the underlying neural circuitry. Because of this, we hypothesized that METH dosing during the peri-pubertal period would influence maturation of PV neurons and PNNs. To test this, we quantified PV and PNN immunoreactivity in the mPFC of male and female rats following METH exposure during early adolescence (female peri-pubertal period), late adolescence (male peri-pubertal period), or adulthood.

2. Methods

2.1 Animals and Housing

Sprague-Dawley rats (obtained from Envigo) were bred in the Psychology vivarium and 12 litters, across four breeding cohorts, were used. The day of birth was marked as postnatal day (P) 0. At weaning (P22), male and female pups were assigned to an exposure group counterbalanced across litter. Rats were double or triple housed in a temperature-controlled room with a reverse 12:12 light/dark cycle (lights on at 0900). Food and water were available *ad libitum*. Rats were weighed daily at 0800 starting at P25 to acclimate them to handling and to minimize injection stress. Daily checks for puberty started at P30 and ended when puberty was reached. For females, vaginal opening was used since it marks when levels of luteinizing hormone and ovarian hormones increase denoting female rat puberty (Castellano et al., 2011). In males, separation of the prepuce from the glans penis was used as an indicator, given this process is dependent on the high levels of androgens released at male puberty (Korenbrot et al., 1977). All animal procedures were performed in accordance with guidelines of the National Institutes of Health and the university's animal care committee regulations.

2.2 Non-Contingent METH Exposure

Rats were given once daily injections of 3.0 mg/kg methamphetamine HCl (Research Triangle Institute; distributed by The National Institute on Drug Abuse) or saline via an i.p. injection for 9 days in either early adolescence (EA), P30-38, late adolescence (LA), P40-48, or adulthood, P60-68 **(Figure 1)**. Injections took place within the first hour of the dark cycle (0900-1000).

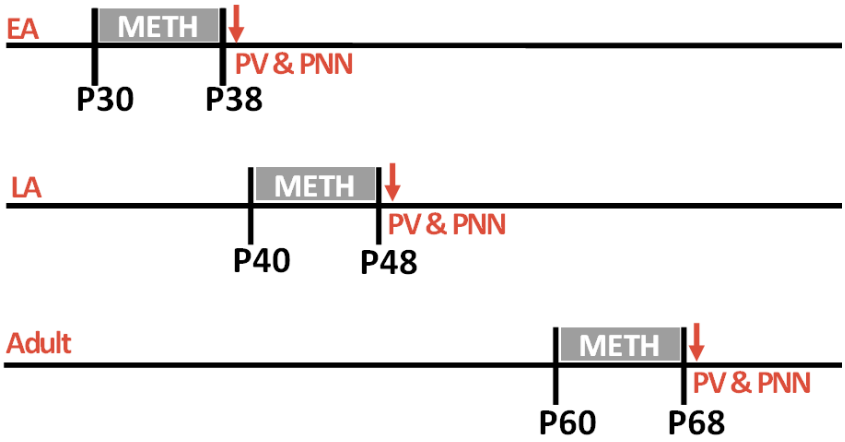

Figure 1: Study timeline. METH exposure occurred for 9 days during either EA (early adolescence), LA (late adolescence), or adulthood. Tissue was collected one day following final METH exposure for immunofluorescent PV neuron and PNN labeling. (P) = postnatal day.

2.3 Tissue Collection

Between 24 and 28 h following the last injection, rats were deeply anesthetized using a minimum dose of 50mg/kg sodium pentobarbitol i.p. until loss of the foot withdrawal reflex, followed by a cardiac perfusion using 0.1M phosphate buffered saline and 4% paraformaldehyde as a fixative. Brains were post-fixed in 4% paraformaldehyde for 24 hours and then submerged

in a 30% sucrose solution for 72 hours. The entire mPFC was then coronally sectioned using a freezing microtome and stored at -20 C in a cryoprotectant solution of 30% glycerol, 30% ethylene glycol, 30% deionized water, and 10% 0.2M phosphate buffered saline. Tissue was coded with random IDs to ensure experimenters were blind to age, sex, and treatment for all tissue processing and analysis.

2.4 Volume Quantification

Every fourth section of mPFC was mounted and Nissl stained to obtain mPFC volume. First, sections were mounted in order on charged microscope slides and allowed to dry completely. Next, tissue was placed in increasing concentrations of ethanol followed by 0.2M phosphate buffered saline to re-hydrate the tissue. Slides were then placed in 1% periodic acid followed by Methylene Blue/Azure II, rinsed with 0.2M PBS and dehydrated using increasing concentrations of ethanol, placed in Citrisolv and coverslipped using Permount.

Volume was assessed using StereoInvestigator under a 2.5x objective. The area of the mPFC was traced on each section of tissue. The anterior boundary of the mPFC was denoted when the white matter of the forceps minor was evident. The most posterior section traced was the section before the appearance of the genu of the corpus callosum. The dorsal boundary of the mPFC was marked by thinning of layer 1 along with cell orientation. The ventral boundary of the mPFC was denoted by loss of visible cortical layers. From these parcellated boundaries, the area of each sampled slice was calculated, multiplied by the average section thickness, multiplied by 4, and then all volumes of sampled tissue were summed to obtain the entire mPFC volume for each animal.

2.5 Immunofluorescent Labeling and Imaging

For each subject, three sections containing mPFC (bregma +3.0 - +3.4) were stained for PV and PNNs. First, sections were washed in tris buffered saline (TBS) in triplicate followed by a 1-h incubation in a blocking solution containing 1% hydrogen peroxide, 20% normal goat serum, and 1% bovine serum albumin. Sections were then incubated for 48 h in the primary antibodies: Monoclonal mouse anti-parvalbumin (1:5000; P3088; Sigma-Aldrich) and Wisteria floribunda lectin (1:500; L-1516; Sigma-Aldrich). Next, sections were washed in triplicate using TTG, which is a mixture of tris buffered saline, 0.3% Triton-X, and 2% normal goat serum. Sections were next incubated in fluorescent secondary antibodies: AlexaFluor Plus 488 goat anti-mouse IgG (H + L) (1:500; A32723; ThermoFisher Scientific) and Texas Red Streptavidin conjugate (1:1000; S872; ThermoFisher Scientific). After a 2-h incubation, sections were rinsed in duplicate with TTG and in duplicate with TBS, mounted onto a charged microscope slide and coverslipped using EverBrite™ Mounting Medium with DAPI (Biotium, #23002). Negative controls were also included in each stain run to ensure minimal auto-fluorescence was maintained. Slides were stored protected from light at 4 °C until imaging within 5 days.

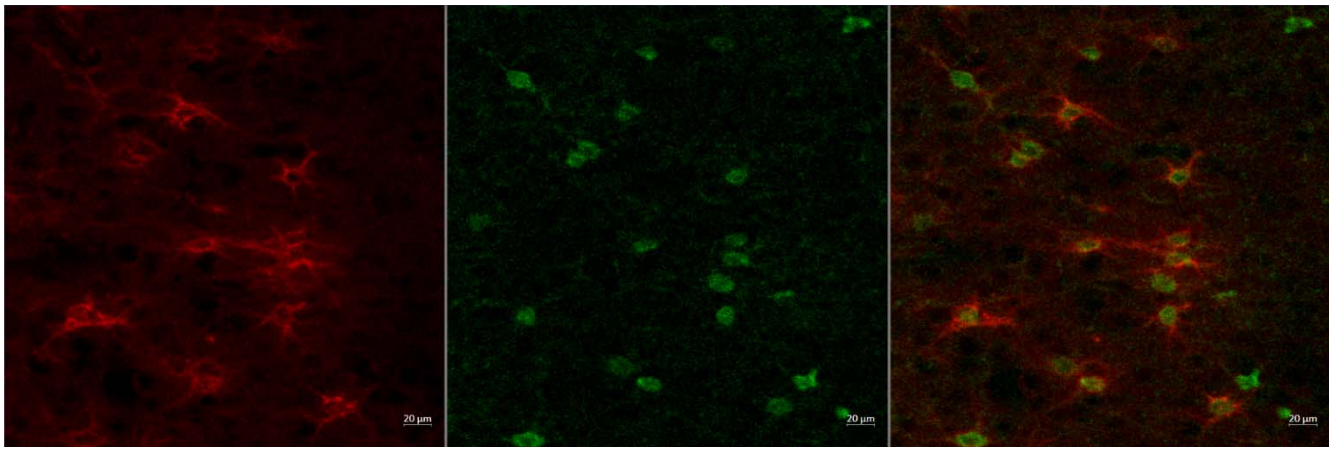

Figure 2: Immunofluorescent labeling of PNNs using wisteria floribunda lectin (left), PV using Mouse Monoclonal Anti-parvalbumin (middle), and their colocalization (right). Imaged with 10x objective. Scale bar = 20µm

Sections containing the mPFC were imaged using an LSM700 confocal microscope at 10x magnification **(Figure 2)**. 16-22 Z-stacked images were taken of each mPFC, with each photo being 320.9 µm x 320.9 µm x 17.66 µm. A total of 2,402 images were analyzed.

Images were analyzed in ImageJ using a protocol adapted from Drzewiecki et al. (2021a). Briefly, z-stacks were first separated by fluorescent channel, so that PNN and PV intensity could be measured separately. Next, the z-stacks for each channel were projected into a single photo. Then PV neurons and PNNs were manually selected as regions of interest (ROIs). To calculate the intensity of both PV neurons and PNNs, corrections for background fluorescent intensity were made using methods adapted from Slaker et al. (2016b). First, the image was processed using the Rolling Ball Radius function. Next, three regions in the background were randomly selected to measure average intensity and standard deviation of the background. The background stain intensity was set as the highest average intensity measure plus two SDs above this mean. Pixels within ROIs below the background stain threshold were not used to calculate the mean intensity of PV neurons or PNNs using the "NaN" function. The stain intensity of each cell in the mPFC was averaged resulting in mean stain intensity for each animal. The number of cells counted in all the pictures was divided by the total volume of the pictures counted to give neuron density. Neuron density was then multiplied by the volume of the mPFC to obtain neuron number.

2.6 Statistical Analysis

All statistical tests were run using Rstudio, version 1.2.1335 (https://www.rstudio.com). Mixed linear effects models were run using the "lmerTest" package (version 3.1.0) for the number of PV neurons and PNNs, as well as for their stain intensity, using two-way ANOVAs for age and treatment with litter as a random factor. Males and females were analyzed separately due to differences in the age of pubertal onset. Significant effects of age were analyzed using

Tukey's post hoc test with a pairwise comparison in the "emmeans" package. Significant interactions between age and treatment were assessed by running one-way ANOVAs in each age group, looking for main effects of treatment with litter as a random factor. Age of pubertal onset was analyzed within the groups that were exposed to METH during puberty (EA females and LA males) using one-way ANOVAs with the main effect of treatment, and with litter included as a random factor.

Data from 121 animals was included in this study. 63 males were used in 6 groups: 10 EA saline, 11 EA METH, 8 LA saline, 11 LA METH, 11 adult saline, and 12 adult meth. There were 58 females in 6 groups: 10 EA saline, 11 EA METH, 10 LA saline, 7 LA METH, 10 adult saline, 10 adult METH.

3. Results

3.1 Pubertal Onset

Age of pubertal onset was analyzed in groups treated with saline or METH during puberty, early adolescent females and late adolescent males, using one-way ANOVAs split by sex. Peri-pubertal METH treatment did not influence the age of pubertal onset in either males or females **(Table 1)**.

	Saline (Mean ± SD)	**METH (Mean ± SD)**
Females	35.80 ± 2.20	35.70 ± 2.71
Males	42.00 ± 0.93	42.00 ± 1.48

Table 1: The average age of pubertal onset in females treated during the peri-pubertal period- early adolescence, and males treated during their peri-pubertal period- late adolescence. No effects of METH were observed in either males or females.

3.2 PV Neuron Number and Stain Intensity

The number of PV neurons showed a significant interaction between METH exposure and age in female animals ($F_{2,52} = 2.14$, $p = 0.02$). Tukey's post hoc testing revealed a significant effect of METH in all female age groups, with EA ($p = 0.02$) and adult ($p = 0.01$) females having more PV neurons in the mPFC while LA females ($p = 0.04$) had fewer PV neurons **(Figure 3A)**. There was also a significant main effect of age in females ($F_{2,52} = 4.85$, $p = 0.012$), where adult females had more PV neurons than EA ($p = 0.016$). No main effects of age or treatment, or interactions were observed in males **(Figure 3B)**.

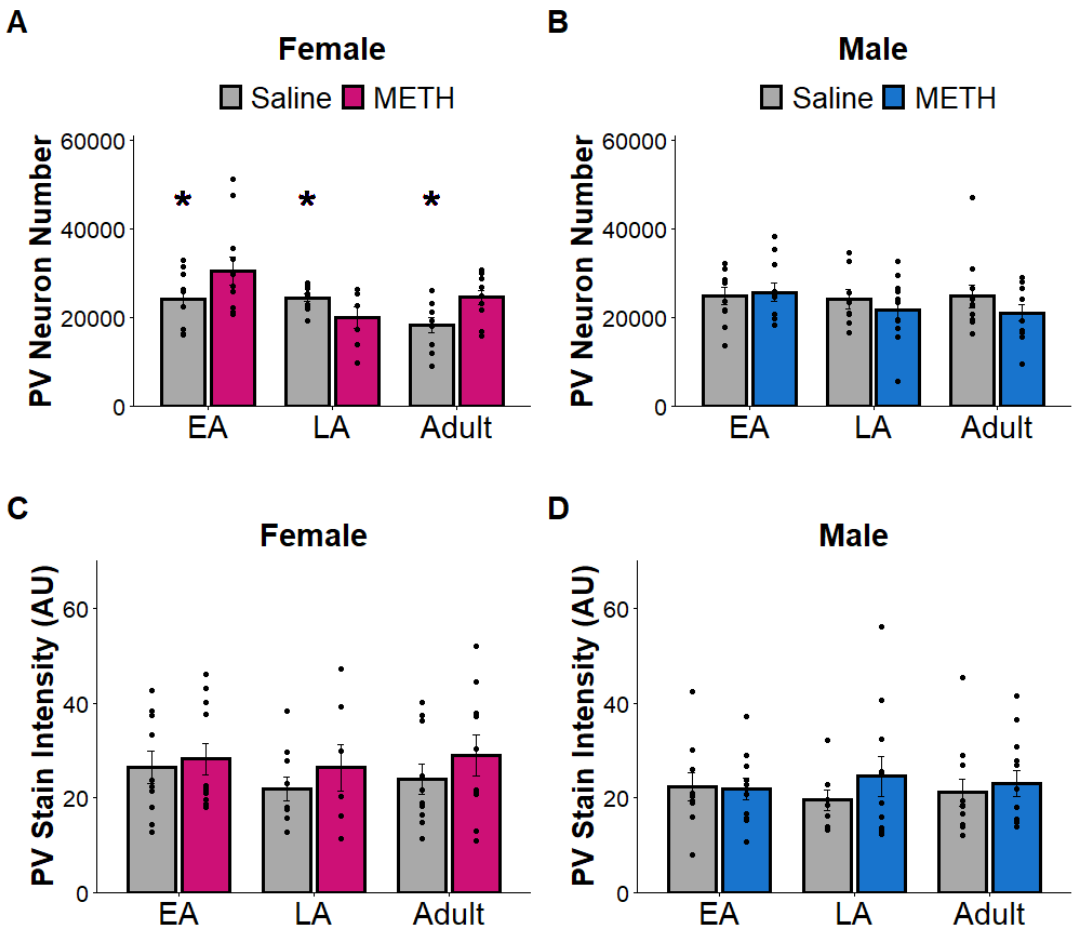

*Figure 3: The number of PV neurons in females (A) and males (B). Significant main effects of age occurred between EA and adult females. A significant interaction between age and treatment was also observed. The fluorescent stain intensity of PV neurons of females (C) and males (D). Significant main effect of METH treatment seen in females. * $p < 0.05$, vs. METH by age*

There was a significant main effect of METH treatment on parvalbumin intensity in females ($F_{1, 52} = 5.70$, $p = 0.021$), such that METH treated females had more intensely labeled PV neurons **(Figure 3C)**. No effects of age on PV stain intensity were observed in either males or females. Male PV stain intensity also had no significant effects from METH treatment **(Figure 3D)**.

3.2 PNN Number and Stain Intensity

Significant main effects of METH treatment on PNN number were observed in females **(Figure 4A),** with increasing PNN number following METH treatment ($F_{1,52} = 8.09$, $p = 0.0067$). No effects of age or interactions with age and METH treatment on PNN number were observed in females. No effects of age or METH treatment were seen in male subjects **(Figure 4B)**.

METH treatment did not significantly influence fluorescent stain intensity of PNNs in either males or females. In females, age significantly influenced PNN stain intensity ($F_{2,52}$ = 6.83, p = 0.003), with the PNN label being more intense in adult females than EA (p = 0.004) or LA (p = 0.024) females **(Figure 4C)**. Age significantly influenced PNN stain intensity in males as well ($F_{2,57}$ = 8.93, p = 0.0005) **(Figure 4D)**, with adult males having more intense PNNs than EA males (p = 0.0003).

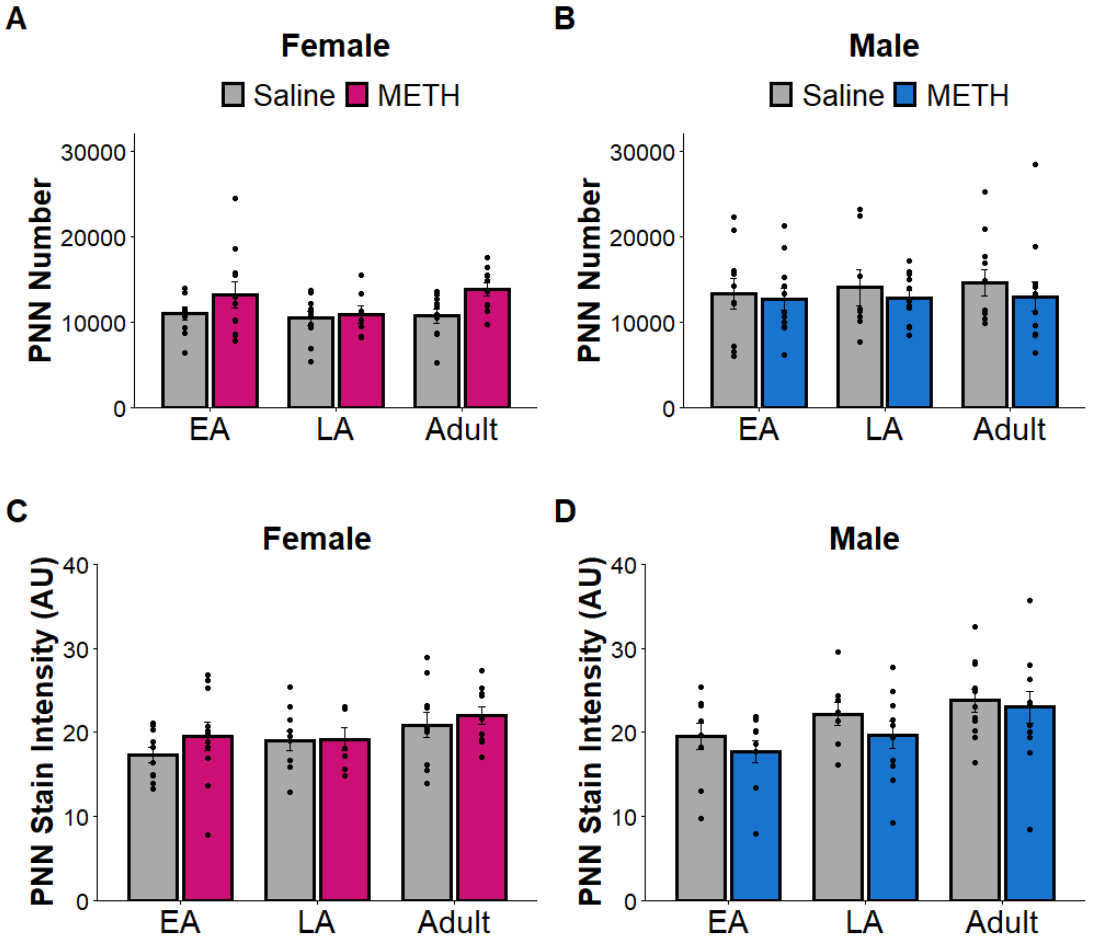

Figure 4: The number of PNNs in the mPFC of females (A) and males (B). Significant main effect of METH treatment on female PNN number. There were no effects of METH treatment on fluorescent stain intensity of PNNs in the mPFC of females (C) and males (D). Significant main effects of age were observed in both males and females.

3.3 PV and PNN Colocalization

Colocalization of PV neurons and PNNs was also analyzed **(Figure 5)**. No significant effects of METH treatment were observed in either males or females. Significant effects of age were found in females ($F_{2,52}$ = 5.66, p = 0.007), with the adult animals having a higher proportion of PV neurons surrounded by a PNN than EA (p = 0.007). In males, the proportion of

PNNs surrounding PV neurons was significantly influenced by age ($F_{2,57} = 3.34$, $p = 0.04$), decreasing between EA and LA ($p = 0.04$).

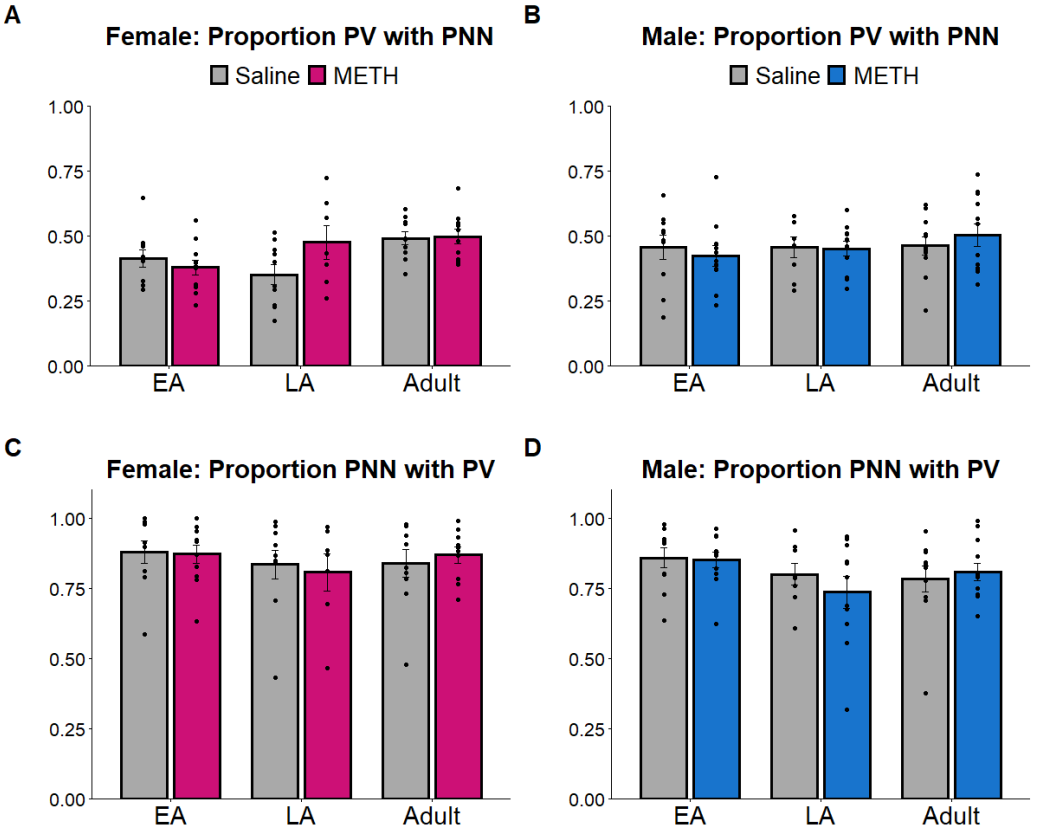

Figure 5: Colocalization of PV neurons and PNNs. Proportion of PV neurons with a PNN in females (A) and males (B) and proportion of PNNs that surround neurons positive for PV in females (C) and males (D). No main effects of METH treatment observed in males or females. Proportion of PV neurons with PNNs increases with age in females while PNNs surrounding PV neurons decreases with age in males.

4. Discussion

METH exposure over nine days affected both PV neurons and PNNs in the mPFC during adolescence and adulthood but only in female, not male, rats. Interestingly, the effects of exposure were nonlinear such that drug exposure in early adolescence has different effects from exposure in late adolescence. It is also notable that effects of exposure in adult females were more similar to those in early, rather than late, adolescents.

In females, METH exposure increased the number of PV expressing neurons during both early adolescence and in adulthood, and METH exposure increased the intensity of PV expression in all groups. The late adolescent females were the exception to this pattern with a significant decrease in the number of PV neurons following METH. Likewise, the number of PNNs significantly increased across groups following METH exposure with a minimal increase

in the late adolescent group. Thus, the pattern of changes in both PV expression and PNNs suggests a similar influence on METH exposure in early adolescence and adulthood with late adolescence being an exception.

The different effects of METH exposure in early versus late adolescence indicate that adolescence is not a single entity but rather a series of cellular changes that interact with environmental events such as METH exposure, so that timing is important. The role of timing has also been noted following repeated non-contingent amphetamine exposure, where both males and females treated from P27-45 experienced reduced D1-receptor levels in the mPFC while those exposed from P37-55 did not (Kang et al., 2016). Another example of differential timing comes from work on pre-pulse inhibition (PPI), a task that relies on the mPFC (Li et al., 2009; Schneider & Koch, 2005). PPI is deficient in females that experienced daily restraint stress during early, but not late, adolescence (Drzewiecki et al., 2021b). Interestingly, PPI was also deficient in males when the stress was applied peri-pubertally which coincides with late adolescence in the present study, and males were not affected even later, but post-pubertal, in adolescence. Thus, PPI was deleteriously affected when stress occurred peri-pubertally in both sexes but not after puberty. This suggests that early and late adolescence can occur at different ages in each sex.

In females, exposure to METH during the early adolescent period had contrasting effects on PV neuron number from exposure in later adolescence. This could be due to the onset of puberty. Following female puberty, GABAergic signaling increases in the mouse cingulate cortex. This increase is blocked by a pre-pubertal ovariectomy, but not by post-pubertal ovariectomy, suggesting reorganization of inhibitory neurotransmission by pubertal onset (Piekarski et al., 2017). This increase in GABAergic signaling occurs near the time PNNs being remodeled by female puberty in the mPFC (Drzewiecki et al., 2020). Interestingly, *Esr2* (RNA encoding ERβ) shows a unique developmental pattern in the mPFC, where it too drops in expression following female puberty. This was not seen in the motor cortex and striatum, suggesting this is not a global reduction in *Esr2* and may be specific to the mPFC (Drzewiecki et al., 2021a). In the mPFC, ERβ colocalizes extensively with PV neurons (Blurton-Jones & Tuszynski, 2002) and has been shown to mediate estrogen's influence on inhibition across the estrous cycle (Clemens et al., 2019). These changes in estrogen signaling may render PV neurons vulnerable during the time immediately following female puberty, which could be compounded by METH administration further increasing PV neuron activity.

We found that females exposed to METH in adulthood had more PV neurons than adults exposed to saline. This result is somewhat puzzling given that neurogenesis in the adult cerebral cortex is negligible and does not involve PV expressing neurons (Cameron & Dayer, 2008); however, increased PV neuron density in the anterior cingulate cortex one day after repeated non-contingent amphetamine exposure has been noted in males (Mohila & Onn, 2005). Between the juvenile and adolescent periods PV expression increases, followed by decreased expression into adulthood (Du et al., 2018; Gildawie et al., 2020). During this time, it is unclear if PV neurons are being pruned or if they are simply reducing PV expression such that they no longer meet stain intensity thresholds to be counted as PV neurons. If the latter is true, neurons could

increase their PV expression in response to METH exposure, a notion supported by increased PV stain intensity in females exposed to METH. However, if the developmental changes in PV expression are the consequence of PV cell apoptosis, it is possible that exposure to METH decreased pruning of interneurons fated to express PV.

In the present study, females exposed to METH had more PNNs; however, this increase seems to be in proportion to the changes in PV neuron number in both the early adolescent and adult exposed groups. While females exposed in late adolescence had fewer PV neurons, they did not have fewer PNNs leading to the speculation that PV neurons without PNNs might have been pruned or have reduced PV expression to negligible levels following METH exposure at this age. PNNs have many functions, one of which is giving protection from oxidative stress (Cabungcal et al., 2013). PV neurons are prone to oxidative stress due to their activity level (Kann, 2016) and METH has been shown to induce oxidative damage (Bu et al., 2013; Tata & Yamamoto, 2007). At a speculative level, it is possible that given PNN number temporarily decreases in the mPFC after female puberty (Drzewiecki et al., 2020), PV neurons could be particularly vulnerable to oxidative stress during this time resulting in cell death.

The males did not have any significant effects of METH exposure on PNNs or PV neurons in the mPFC in the current study. While we had hypothesized that the development of PV neurons and PNNs in females would be more influenced by METH, the lack of effects in males is notable. To date, only studies assessing PV neuron density (PV neurons/area or volume), not total number per se, have assessed the effects of METH treatment in adult males and mixed results have been reported (Veerasakul et al., 2016; Robinson et al., 2022). Electrophysiologically, METH administration has been shown to increase inhibitory tone in the mPFC of adult male rats (Armenta-Resendiz et al., 2022). Additionally, repeated amphetamine exposure from P27-45 in male rats resulted in a reduced effect of amphetamine on IPSCs in adulthood (Paul et al., 2016). Non-contingent cocaine exposure from P35-40, but not P75-80, has also been shown to attenuate GABAergic signaling in the mPFC when measured in adulthood while also altering PV immunoreactivity in males (Cass et al., 2013). This suggests psychostimulants can have an enduring effect on inhibitory neurotransmission within the prefrontal cortex. Unfortunately, most studies assessing the influence of stimulants on inhibition only include male groups so that the impact on inhibition in the female mPFC is unknown. Changes in inhibition in males must involve cellular mechanisms other than those examined in the present study.

Future studies will need to address whether the effects within 24 hours of METH exposure continue into later stages of life. This is especially important for the increased number of PV neurons seen following exposure during early adolescence since compensation could occur during drug-free late adolescence. This also needs to be accompanied by a study relating the effects of non-contingent exposure at these ages to later self-administration. Lastly, the effects of other doses of METH need to be examined to examine the extent to which the present results generalize.

The present study underscores the importance of using both sexes in psychostimulant research as sex differences likely exist at all levels. Sex differences in self-administration

behavior (Lynch, 2006; Hankosky et al., 2018b; Westbrook et. al, 2020) and long-term cognitive deficits following METH exposure (Hankosky et al., 2018a) have already been noted. The data presented further supports the notion that METH exposure results in unique changes within the female mPFC. The present study also highlights the importance of not viewing adolescence as a single period, but rather an ongoing set of cellular processes that can interact differentially with drug exposure, making the exact timing of exposure particularly important when studying adolescents.

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