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Prenatal alcohol and cannabis exposure can have opposing and region-specific effects on parvalbumin interneuron numbers in the hippocampus

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Abstract

Background: We have recently shown that alcohol and cannabis can interact prenatally, and in a recent review paper we further identified Parvalbumin-positive (PV) interneurons in the hippocampus as a potential point of convergence for these teratogens.

Methods: A 2 (EtOH, Air) x 2 (THC, Vehicle) design was used to expose pregnant Sprague-Dawley rats to either EtOH or air, in addition to either THC or the inhalant vehicle solution, from gestational days 5-20. Immunohistochemistry was performed to detect parvalbumin-positive interneurons in one male and one female pup from each litter at post-natal day (PND) 70.

Results: Significant between-group, and sub-region specific, effects were found in the dorsal CA1 subfield, as well as the ventral DG. In the dorsal CA1 subfield, there was an increase in the number of PV interneurons in both the EtOH and EtOH + THC groups, but only a possible decrease with THC alone. There were fewer changes in interneuron numbers overall in the DG, however, a sex difference was found, and a decrease in the number of PV interneurons in the THC exposed group was observed in the male ventral DG. There was also an increase in cell layer volume between the EtOH + THC group and control group in the DG, and an increase from the control and THC group to the EtOH group in the CA1 region.

Conclusions: Prenatal alcohol and prenatal THC exposure differentially affect parvalbumin interneuron numbers in the hippocampus, indicating that both individual and combined exposure can impact the balance of excitation and inhibition in a structure critically involved in learning and memory processes.

Keywords: Hippocampus, Dentate Gyrus, Interneuron, Parvalbumin, Alcohol, Cannabis, FASD

1. Introduction

With the legalization of cannabis in many regions of the world, there is increasing interest in the potential for cannabis, particularly its psychoactive component Δ^9-tetrahydrocannabinol (THC), having teratogenic effects (Fish et al., 2019). Approximately 3% of women use alcohol and/or cannabis heavily throughout pregnancy (Goldschmidt et al., 2004; Hayatbakhsh et al., 2012; Young-Wolff et al., 2019), so it is important to understand the effects of each compound alone, as well as any potential synergy between THC and alcohol (Goldschmidt et al., 2004; Young-Wolff et al., 2019). Indeed, our recent studies indicate that the simultaneous consumption of alcohol and THC (A/C) during pregnancy can affect blood alcohol levels (Breit et al., 2020).

Both THC and alcohol (ethanol, EtOH) affect the hippocampus, which plays a central role in learning and memory processes (Christie et al., 2000; Fontaine et al., 2016; Hill et al., 2004; Li et al., 2019). The hippocampal formation is a brain region that consists of two main subdivisions, the dentate gyrus (DG) and the cornu ammonis (CA) (Andersen et al., 2007). The DG is known to play a role in spatial memory and object recognition (Eadie et al., 2012; Jessberger et al., 2009), whereas the CA subfield is implicated in autobiographical and social memory (Bartsch et al., 2011; Okuyama et al., 2016).

We have recently published a systematic review (Reid et al., 2020) that suggested that hippocampal interneurons may be a focal point for the actions of both THC and ethanol in the developing brain. In part, this is because THC activates CB1 receptors, which can change GABA release, and further because repeated THC exposure causes a change in CB1 receptor levels (Sadrian et al., 2013; Vargish et al., 2017). CB1 receptors are $G_{i/o}$ coupled receptors activated by the endogenous cannabinoids anandamide and 2-arachidonoylglycerol under normal conditions (Mackie, 2006). Their activation in excitatory cells inhibits adenylyl cyclase activity, preventing neurotransmitter release at the synapse (Mackie, 2006). For the CB1 receptors on interneurons, this means that cannabinoid binding suppresses GABA release during acute activation. However, repeat exposure to exogenous cannabinoids (e.g., THC) can cause downregulation of these receptors and therefore increase GABA release (Sim-Selley, 2003). Although there is a ~20 fold increase in CB1 receptors on GABAergic cells compared with other cell types, CB1 receptors also exist on astrocytes, microglia, and glutamatergic cells (Cabral, 2005; Kawamura et al., 2006). When glutamatergic and astroglial CB1 receptors are activated in the hippocampus, a signal transduction cascade is activated that causes the removal of AMPA receptors (Han et al., 2012). $GABA_A$ receptors are chloride ion channels found

post-synaptically (Andersen et al., 2007). In most cases, the extracellular chloride concentration is much higher than the intracellular concentration of an excitatory neuron. This means that when GABA is released and the $GABA_A$ channel opens, negatively charged chloride ions travel into the cell along its concentration gradient and cause the cell to become hyperpolarized and unable to propagate excitatory inputs. However, in development, the KCC2 receptor (a potassium-chloride co-transporter), is not yet upregulated (Leonzino et al., 2016), so these inputs act as excitatory inputs because the intracellular chloride concentration is higher than the extracellular concentration. This is an important process for circuit establishment, and also a reason for hyperexcitability with prenatal A/C exposure.

Interneurons play an important role in the hippocampus, controlling excitatory cells through $GABA_A$ receptors, and research suggests that they can also exhibit synaptic plasticity (Christie et al., 2000). While there are several immunohistochemically distinct interneurons in the hippocampus (Pelkey et al., 2017), the parvalbumin (PV) interneurons play a distinct role in hippocampal memory-related processes (see **Figure 1** for PV cell location). They coordinate excitatory networks in memory consolidation (Ognjanovski et al., 2017), are essential for spatial working, but not reference, memory (Murray et al., 2011), and act as a discriminator in social memory (Deng et al., 2019). PV positive interneurons are also integral in producing gamma oscillations and are considered central to the maintenance of the inhibitory/excitatory balance (Reha et al., 2020).

In this paper, we assess the effect of prenatal alcohol exposure, prenatal THC exposure, and the combination of the two on PV interneuron numbers in the CA1 and DG subfields of the hippocampal formation. PV interneurons are fast-spiking, integral to the processes of feedforward, feedback, and lateral inhibition and are abundant in the stratum pyramidales (SP) layer of CA1 and the granule cell layer (GCL) of the DG (Espinoza et al., 2018). Loss of these interneurons has been associated with schizophrenia, Lewy Body Dementia, and chronic stress (Bernstein et al., 2011; Czeh et al., 2005; Konradi et al., 2011). Further, these cells are thought to influence adult neurogenesis and play a role in spatial working memory, novel object exploration, and novel object location recognition (Fuchs et al., 2007; Song et al., 2013). This work seeks to investigate population changes in PV interneurons in adult rats that were prenatally exposed to prenatal ethanol vapor, THC vapor, or a combination of both.

2. Materials and Methods

Animals and Vapor Administration Protocol: Female Sprague-Dawley rats (n=24) were assigned to receive 1 of 4 experimental conditions (EtOH or Air), and (THC or Vehicle (VEH; propylene glycol, Sigma-Aldrich; see **Figure 2**). Thus, there were 4 total conditions: EtOH + THC (n=6), EtOH + VEH (EtOH; n=6), Air + THC (THC; n=5) and Air + VEH (VEH; n=6). Pregnancy was confirmed in all dams by the presence of a seminal plug, and this was designated as gestational day (GD) 0.

Pregnant rats were exposed to their assigned drug exposure condition once daily from GD 5 to GD 20 in a vapor inhalation chamber (La Jolla Alcohol Research Incorporated, La Jolla, CA). Pregnant rats were exposed to 95% EtOH (68 mL/hour; Sigma-Aldrich) or Air for 3 consecutive hours at an airflow of 2 L/min. Then, subjects were either exposed to THC (100 mg/mL; NIDA Drug Supply Program) or the VEH (propylene glycol; Sigma-Aldrich) at an airflow rats of 2 L/min; THC and VEH were administered in single 6-second puffs every 5 min for a 30-min period (7 puffs total). An additional 10 minutes of airflow was administered before opening the chambers to remove any residual vaporized drug. Dams gave birth on GD 22, and litters were culled on postnatal day (PND) 2.

One male and one female pup from each dam were used in this study. All animals used in this study had previously been tested in an open field (PND 31-34); measuring activity levels in the open field is not expected to influence hippocampal interneurons (Breit et al., 2019).

Immunohistochemistry: Adult offspring (PND 60-70) were deeply anesthetized with a lethal ketamine (67 mg/mL) and xylazine (6.7 mg/mL) dose (0.001 mL/g) and transcardially perfused with cold saline followed by 4% paraformaldehyde (PFA) in a phosphate buffer. Brains were extracted and post-fixed in 4% PFA for 24 hours. Brains were sectioned using a Leica vibratome into 50 µM slices. Immunohistochemistry was performed using a 1 in 6 series of tissue. A 5-minute incubation in 1% sodium borohydride in PBS was used for antigen retrieval. MilliporeSigma mouse anti-PV antibody (MAB1572) was used at 1:1000 in 3% normal goat serum (NGS) and 0.5 % Triton X-100 in PBS (0.5% PBST) for 48 hours at 4 °C with shaking. Biotinylated goat anti-mouse secondary antibody (ThermoFisher # 31804) was used at 1:500 in 3% NGS and 0.5% PBST for 2 hours at room temperature with shaking. Development for light microscopy was performed with Vectastain ABC

Elite avidin-biotin peroxidase complex and 3,3'-Diaminobenzidine (Vector laboratories Burlingame, CA) . ABC and DAB were prepared as per supplier instructions. For fluorescent microscopy, Alexa 488 conjugated goat anti-mouse secondary (ThermoFisher # A-11001) was used at a 1:500 concentration in 3% NGS and 0.5% PBST. Cresyl Violet staining was performed on a 1 in 12 section of tissue from the same set of brains as described previously (Meconi et al., 2018).

PV Cell Counts and density analysis: A profile count was performed throughout the hippocampus (1 in 6 series) at 40x using an Olympus brightfield CX21 microscope (Olympus Corporation, Center Valley, PA, USA). To ensure that sex-specific differences in hippocampal volume did not affect our results, we obtained approval from the university to enter the laboratory during the pandemic and obtained images of these sections for area analysis using an upright Olympus BX51 microscope equipped with Stereoinvestigator (MBF Bioscience, Williston, VT, USA).

Due to Covid-19 restrictions on lab access, the area analysis was performed outside of the lab using FIJI-Image J (Version 1.52p, National Institutes of Health, USA) (see figure **S1** for areas measured and **S2** for representative DAB stained images). Reconstruction of PV cell density was performed in two steps. First, each cell count was divided by the measured area for the region being counted (in mm^2). Following this the cell densities were averaged for each brain in the dorsal CA1, ventral CA1, dorsal DG, and ventral DG. A calculation of per region was done by multiplying the average density by 2400 μM; the normalized length for an adult rat hippocampus (Palkovits, 1983).

Cell density analysis with Cresyl Violet: To add context to any observed changes in PV cell density, the number of overall cells was also assessed using Cresyl Violet staining. For Cresyl Violet analysis, images of the granule and pyramidal cell layers (in the DG and CA1 respectively) were taken using StereoInvestigator (MBF Bioscience, Williston, VT, USA) and an Olympus BX51TF microscope. Images were analysed in FIJI-ImageJ (Version 1.52p, National Institutes of Health, USA (**Figure S3**)) to allow analysis to be completed from home computers. Stained sections were assessed by counting the cells in 3 2000 μm^2 boxes per animal in both the dorsal and ventral regions. For each cell layer, a region of interest from the top, middle, and bottom of the cell layer was selected for this analysis, to ensure that any potential changes in density throughout a cell layer were captured. Within these regions of interest, sampling frames were randomly selected for actual cell counts.

***Inhibitory cell ratio calculation*:** To determine if the ratio of interneurons to principal cells was changing in a given hippocampal region, we divide the density of PV neurons (in the pyramidal layer of the CA1 or the granule layer of the DG) by the density of Cresyl Violet stained cells in the same region. We did this for a total sample area of 6000 μm^2 in each cell layer. The resulting number is reported as a "inhibitory cell ratio". We further subdivided the hippocampus into dorsal (bregma -1.88 to -4.16) and ventral (bregma -4.52 to -6.04) subregions to help determine if there were regional differences in PV cell density using this inhibitory cell ratio. All statistics using the general linear model were done using IBM SPSS statistics software and estimation statistics and figures were made using R (Stull, 1994; R Computing, 2018). A 95% confidence interval was used as the statistical threshold in this study, and all post-hoc tests were performed using a Tukey test.

Representative fluorescent images were taken using and Olympus BX61WI confocal laser scanning microscope and Olympus FluoView FV10-ASW 1.7c software (Olympus Corporation, Center Valley, PA, USA).

3. Results

Data was analysed with a multi-way ANOVA with Tukey post-hoc tests, and supported with estimation statistics.

An initial multivariate analysis of variance (ANOVA) showed a statistically significant effect of dorsal versus ventral location and a significant effect of treatment in the CA1 and DG for parvalbumin positive cell density ($p = 0.008$, $p = 0.003$, see **Supplementary Tables 1 and 2**). Power analysis performed with G* Power software based on cell counts showed a large effect size and suggest the number of animals and number of slices per animal was appropriate.

In the dorsal CA1, there was a trend towards increase in the inhibitory ratio of PV cells in the EtOH group ($p = 0.081$) compared with the THC alone group, and a significant increase in the EtOH + THC groups compared with the THC group ($p = 0.021$) (**Figures 3 and 4**). There was no significant difference between the Air + Vehicle group and the THC exposed group in the Tukey post hoc test, however, estimation statistics suggests that there may be a decrease in the number of cells (**Figure 4**). This is also reflected in cell count data (reconstructed without area measurement) (**Figure S4**). In the ventral CA1, in contrast to the dorsal CA1, no significant effects were found in the density of PV

interneurons (**Figures 3 and 4**). Interestingly, there was a significant increase in the cell layer volume between the Air + Vehicle and THC groups when compared with the Ethanol alone group (**Figure S5**). Further, the cell count data alone (**Figure** S4), reflected the trend of dorsal CA1 with the exception that the decrease in cell number from control to THC reached the threshold of significance. When the number of Cresyl Violet stained cells was sampled in three 2000 μm^2 boxes of the cell layer, no differences were found (**Figure S6**).

The initial three-way ANOVA in the dentate gyrus showed a potential interaction between sex and dorsal or ventral location relative to PV neuron density ($p = 0.054$), so for this analysis, results were split by both sex and dorsal or ventral location (Cohen's D = -0.738). Tukey post-hoc analysis showed no changes were observed in the dorsal DG in any groups (**Figures 5 and 6**). In the ventral DG, there was a trend towards a decrease in the number of PV cells in the THC exposed group ($p = 0.057$). The likelihood of this trend being real is supported by estimation statistics with a Cohen's D value of -1.49. There was a significant increase in granule cell layer volume between the EtOH + THC group and control group in the dentate gyrus ($p = 0.004$, **Figure S5**).

We also investigated the relative change between the density of PV interneurons in the dorsal and ventral regions relative to each exposure group (**Figure S7**). There was a trend towards a decrease between the THC alone and Ethanol alone groups in the male dentate gyrus ($p = 0.092$).

4. Discussion

To our knowledge, this is the first paper to study PV interneurons using this model. Although we are unable to compare directly with previous research, the present findings are in line with those by Lu *et al.* who used a similar model with the more general interneuron marker glutamic acid decarboxylase 67 (Lu et al., 2018). Between estimation and ANOVA statistics, it is likely that there is a decrease in PV interneuron density in the dorsal CA1 region, suggesting that in our model, there is a main effect of THC that is not seen in the dual exposure (**Figures 3 and 4**). Given that there is an increase in the cell layer volume in the EtOH group but no change in cell numbers, there could be an increase in the overall number of cells in the ethanol group not seen in the EtOH + THC group.

Unlike the CA1 region, there were probable sex differences in the DG. This suggests that neurogenesis is differently affected in males and females exposed to these teratogens. The only significant change seen in this group in the density of PV interneurons was a decrease in the THC exposed group compared with all other groups in the male ventral region (**Figures 5 and 6**). However, the fact that there is also an increase in the cell layer volume in the EtOH + THC group compared with the control group, and no change in the overall cell density, suggests that there are more cells in the EtOH and THC group.

Considering that prenatal alcohol exposure is known to cause $GABA_A$ receptor hyperexcitability, these results are perhaps surprising, as one might expect that PV interneurons may be downregulated in response to the increased efficacy of GABA (Olney et al., 2002). However, one must also consider that the net effect of FASD is network hyperexcitability, which is seen clinically as a comorbidity with epilepsy in more extreme cases (Bell et al., 2010). Further, the greatly differing effects of prenatal versus postnatal exposure must be considered. Prenatal exposure not only effects circuit development, but also occurs at a time before the GABA switch, a process where KCC2 chloride receptors are upregulated (Leonzino et al., 2016). Before this upregulation, the intracellular concentration of chloride is so high that when the existing chloride channels open, the ions exit the cell along its concentration gradient, causing interneurons to have an excitatory effect *in utero* (Leonzino et al., 2016). Lastly, interneurons in the hippocampus all migrate from either the caudal or medial ganglionic eminences during development and the cannabinoid system has been implicated in this (Vargish et al., 2017). Therefore, it is possible that an upregulation of the number of neurons is a response to prenatal ethanol exposure that is inhibited by THC− leading to worse behavioral outcomes (Fish et al., 2019). Further research is required to parse out what changes occur due to the initial teratogenic insult and what effects, if any, result from the body's attempt to mitigate the injury.

THC tended to cause either a trend towards a decrease or a significant decrease in the number of PV cells. While no completely parallel literature was identified, Vargish *et al.* found a decrease in the number of cholecystokinin-positive positive interneurons in the DG, however only in those derived from the caudal ganglionic eminence (Vargish et al., 2017). It is important to note that while cholecystokinin-positive and PV interneurons are found in the hippocampus, most cannabinoid receptor 1 (CB1) receptors are found on cholecystokinin-positive positive interneurons and not PV

positive interneurons (de Salas-Quiroga et al., 2020), although there is evidence of this outside the hippocampus (Huang and Kirkwood, 2020). This is relevant because THC acts predominantly on CB1 receptors thus it is expected that THC would affect PV and CCK interneurons differently. CB1 receptors are $G_{i/o}$ coupled; CB1 receptor activation results in a signal cascade that inhibits the release of neurotransmitter vesicles at the synapse (Scheyer et al., 2019). For these reasons, future work should assess cholecystokinin-positive interneurons. Work by Tortoriello *et al.* found an increase in the number of CB1 receptors the CA1 of adult rats exposed to THC prenatally (Tortoriello et al., 2014). While more research is needed to strengthen these results, it possible that the decreased number of cholecystokinin-positive interneurons each express relatively more CB1 receptors. This is relevant to PV interneurons as interactions between cholecystokinin-positive interneurons and PV interneurons may be a good line of enquiry for future research to determine why this drop in PV interneurons is observed. Loss of PV interneurons could be directly from prenatal THC exposure, or could be selective degeneration as the brain's attempt to curb excessive inhibition. Fish *et al.* recently published a paper suggesting that prenatal cannabinoids interact with alcohol through a CB1-smoothened (Smo) pathway. Smo is a G-protein coupled receptor (GPCR) that acts to lower adenylyl cyclase activity (Fish et al., 2019). Specifically, they found that prenatal ethanol exposure inhibits sonic hedgehog (Shh) signalling, thereby increasing intracellular oxysterol, which then activates the smoothened protein (Fish et al., 2019). CB1 is found in heteromeric subunits with Smo, therefore overall adenylyl cyclase activity is decreased by a CB1 mechanism during prenatal THC exposure, and Smo via prenatal alcohol exposure, thus GABA is further released due to a loss of inhibition of neurotransmitter vesicle fusion (Fish et al., 2019).

After our initial results we were curious if differing numbers of inhibitory neurons could reflect a differing requirement for inhibition. So, to contextualize our results we analyzed the area measurements collected alone as well as a sampled density of Cresyl Violet stained cells. Interestingly, we found no change in density in any region, but an increase in cell layer volume in the dentate gyrus of EtOH + THC exposed animals and in the CA1 pyramidal layer of EtOH exposed animals. This suggests that in these cases that the numbers of total cells are increasing in this layer, which may speak to changes in neurogenesis, in the case of the dentate gyrus. But, considering that the changes are also seen in CA1, future research should take early timepoints into account. As a

caveat, it is worth noting that the magnitude of these changes is not resolvable with our analysis, and could partially be attributed to the changes in interneuron number. Further work is needed to elucidate the immediate and latent effects of combined prenatal alcohol and cannabis.

PV interneurons have been implicated in lateral inhibition, network synchrony, and neurogenesis (Deng et al., 2019; Espinoza et al., 2018; Song et al., 2013). A decrease in the density of PV interneurons in adulthood could therefore decrease the efficacy of lateral inhibition, decrease network synchrony, and decrease neurogenesis while an increase in the density of PV interneurons could lead to too much lateral inhibition, over-inhibition of basal network repression, and a loss of cell survival. Regarding neurogenesis, contacts from PV interneurons on adult born granule cells (abGCs) in the DG are thought to be necessary for cell survival. It would therefore be interesting to investigate the correlation between changes in PV interneuron density and neurogenesis (Song et al., 2013). Further, it is possible that the greater or lesser density of cells have altered amounts of synaptic connections in order to overcome an abundance or loss of inhibition so electrophysiological and ultrastructural studies would further elucidate the consequences of the changes in PV interneuron density seen in prenatal THC, ethanol, and combined exposure.

5. Conclusions

This study assessed the effects of prenatal alcohol, THC, and their combination on the number of PV cells in the rat hippocampus. While no sex differences were found, differences in the dorsal and ventral dentate gyrus, and the dorsal and ventral CA1 were observed. These dorsal-ventral differences should be considered in future work, as they reflect differences between neocortical versus amygdalar inputs. Significant effects were found in the dorsal and ventral CA1, as well as the ventral DG. In the dorsal CA1, there was an increase in the number of PV interneurons in both the EtOH and EtOH + THC groups, and no change in the THC group. In the ventral CA1, there was a decrease in the number of PV interneurons in the THC group, an increase in the EtOH group, and no change from air-exposed in the EtOH + THC group. In the ventral dentate gyrus there was a decrease in the number of PV interneurons in the THC exposed group but not in either of the other two groups. More work is

required to better understand the underlying mechanisms of the current results, and further work should address other interneuron types in the hippocampus.

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Figure 1. Schematic of Parvalbumin positive cell axonal and dendritic position in the hippocampus. (A) Schematic showing the relative position of PV positive cells in the CA1 and DG of the hippocampus. Note how the majority of connections are located in the CA1 and DG cell layers. *S.O.: stratum oriens; S.P.: stratum pyramidales; S.R.: stratum radiatum; S.L.M.: stratum lacunosum moleculare; M.L.: molecular layer; G.L.: granule layer; H.: hilus.*

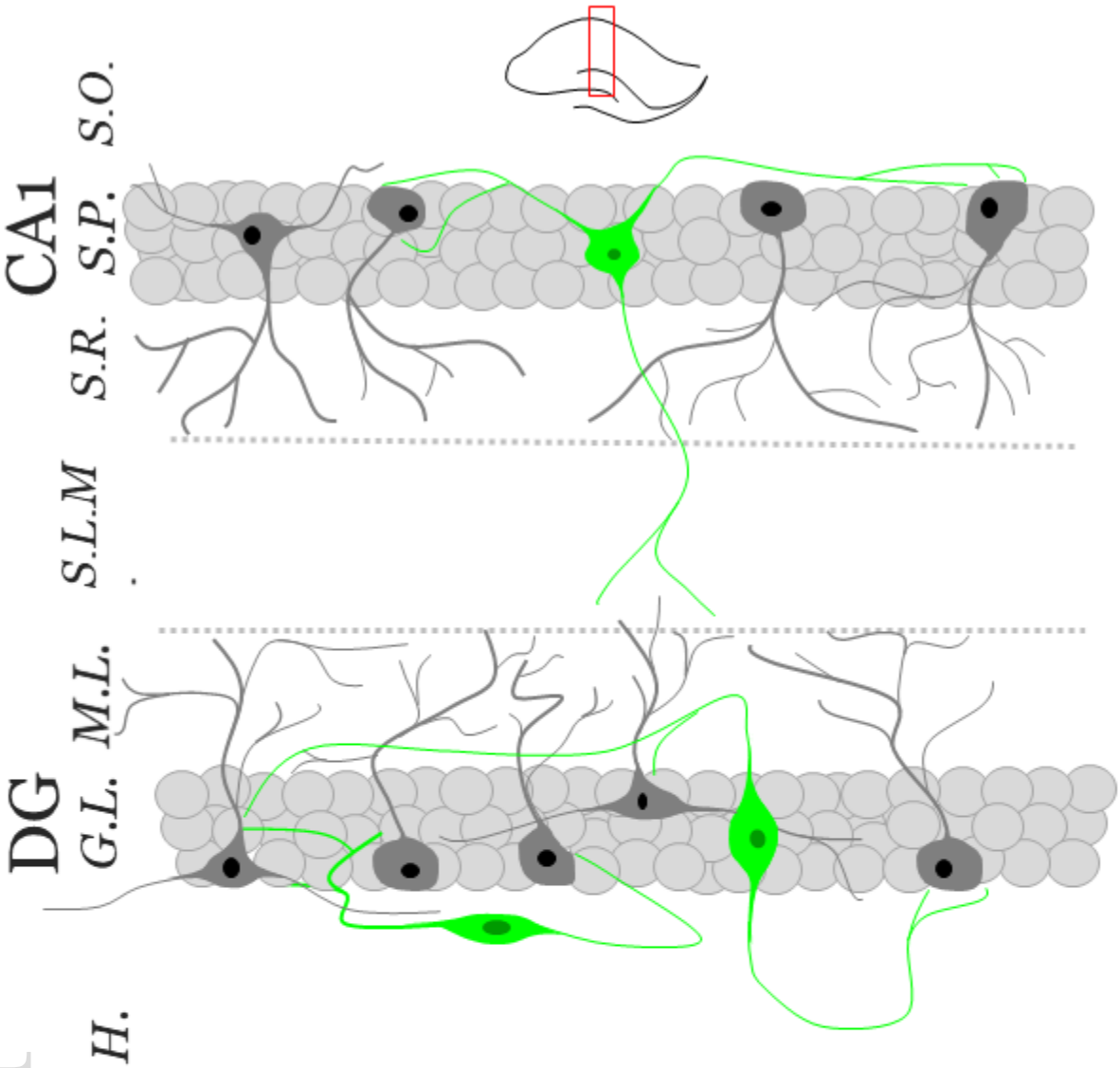
Figure 2: Pregnant rats were exposed to 95% EtOH or Air for 3 consecutive hours at an airflow of 10 L/min. Afterwards, all rats were either exposed to THC or propylene glycol vehicle at an airflow rats of 2mL/min. Blood samples were taken on GDs 5, 10, 15, and 20. Between PND60-70, the adult offspring were deeply anesthetized with lethal ketamine (67 mg/mL) and xylazine (6.7 mg/mL) and underwent transcardiac perfusion. Brains were collected and post-fixed in 4% paraformaldehyde for 24 hours. Brains were sliced at 50 μm for histology.

Figure 3: Representative confocal images of cells positive for parvalbumin (green) in the CA1 region of the hippocampus in P70 Sprague-Dawley rats, counterstained with Hoechst (blue). The images show representative examples of of stained sections from (A) the air alone exposure group; (B) the THC exposure group; (C) the EtOH exposure group; and (D) the EtOH + THC exposure group. Scale bar = 50 microns.

Figure 4: Representative confocal images of PV cells in the DG of P70 Sprague-Dawley rats. Calculated numbers of parvalbumin-positive cells in rats exposed to air, THC, EtOH, and EtOH + THC from GD5-20 in the dorsal and ventral CA1 hippocampal region. Animals assessed at P70. Data collected as profile counts of a DAB stained 1 in 6 series of tissue. Cohen's D of effect size shown where each experimental group is compared with control.

Figure 5: Representative confocal images of PV cells in the DG of P70 Sprague-Dawley rats, counterstained with Hoechst. A. Air exposure group. B. THC exposure group. C. EtOH exposure group. D. EtOH + THC exposure group.

Figure 6: Calculated numbers of parvalbumin-positive cells in rats exposed to air, THC, EtOH, and EtOH + THC from GD5-20 in the dorsal and ventral dentate gyrus. Animals assessed at P70. Data collected as profile counts of a DAB stained 1 in 6 series of tissue. Cohen's D of effect size shown where each experimental group is compared with control.



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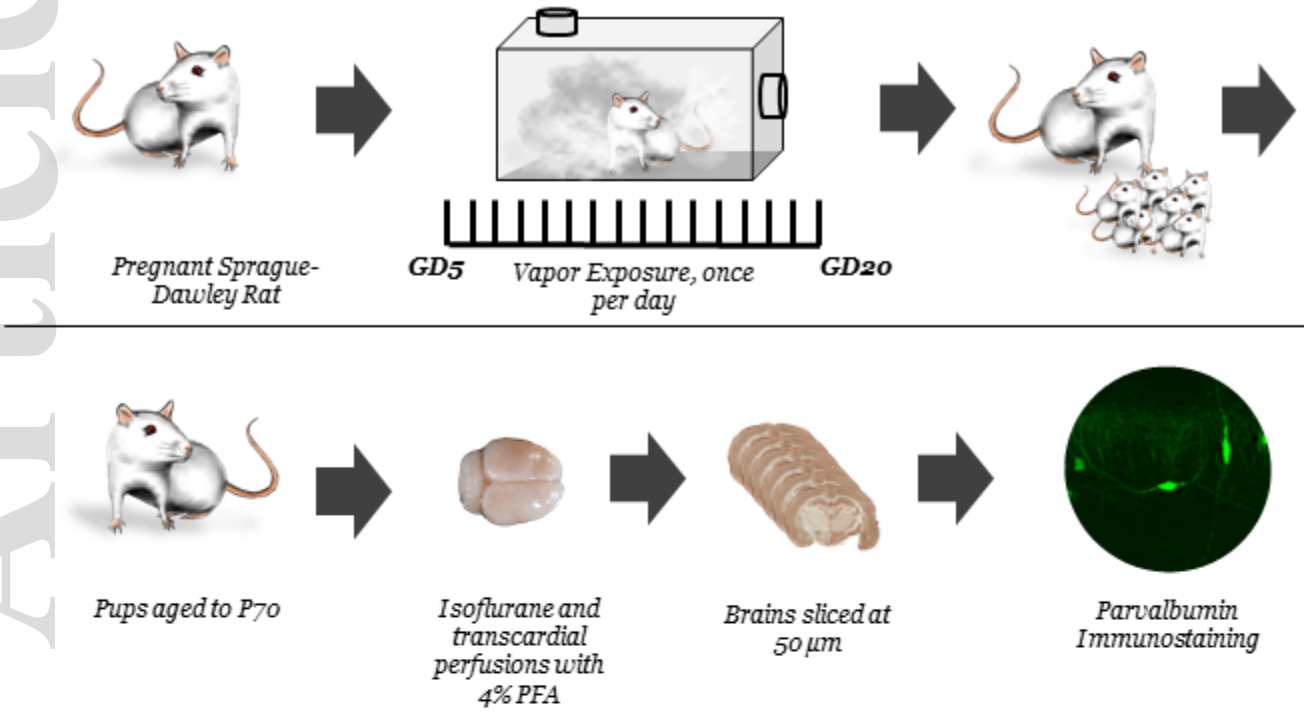

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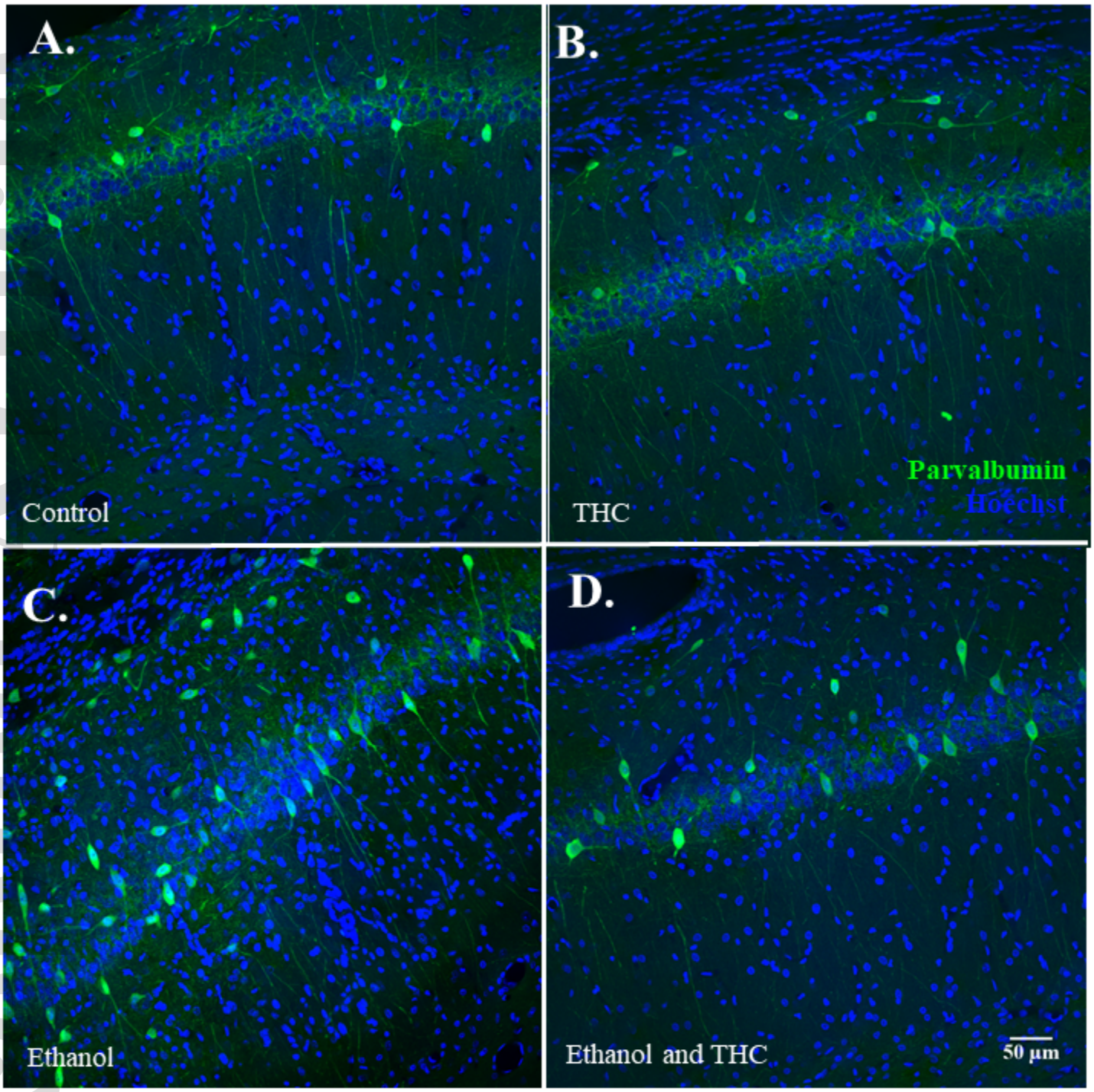

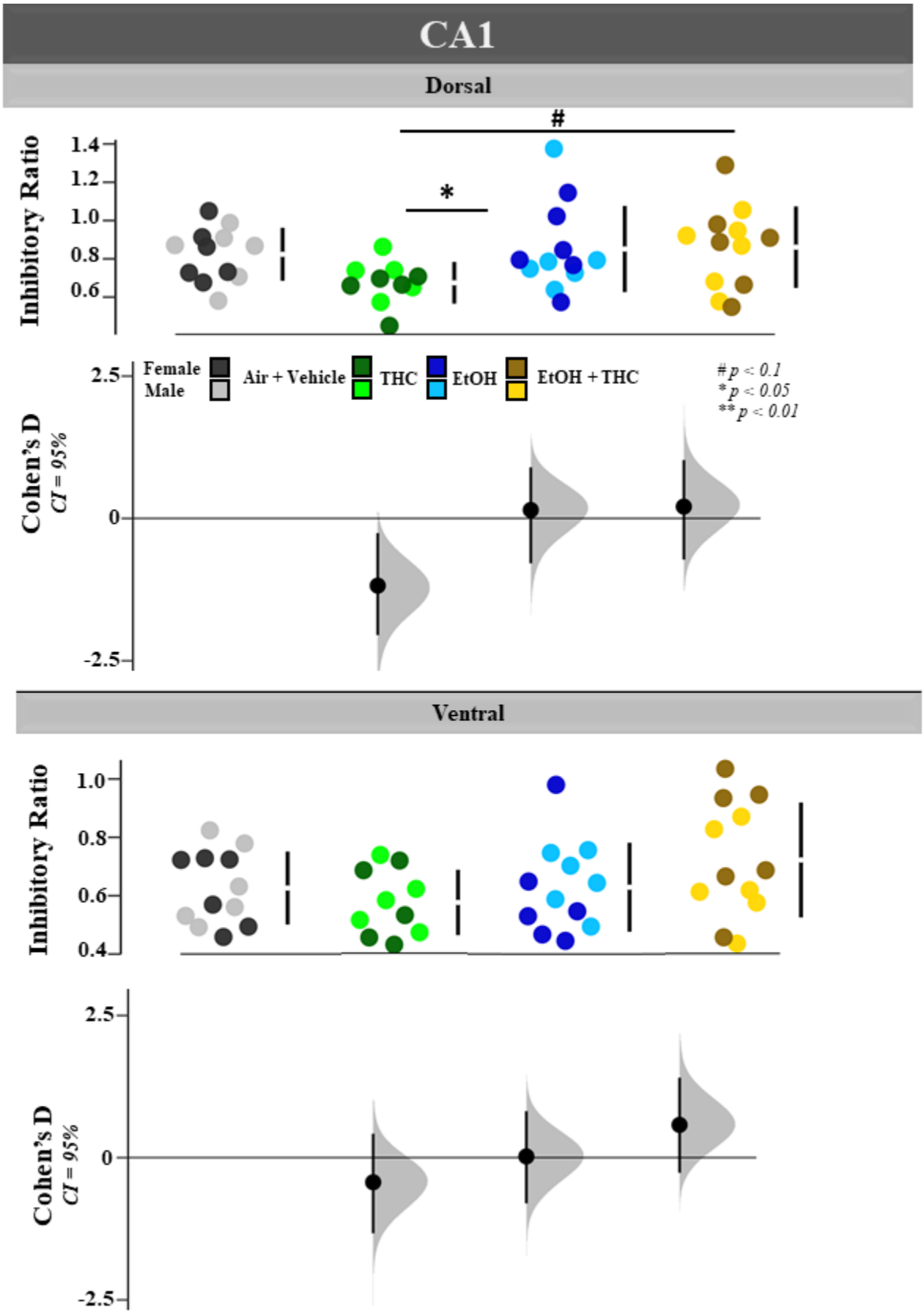

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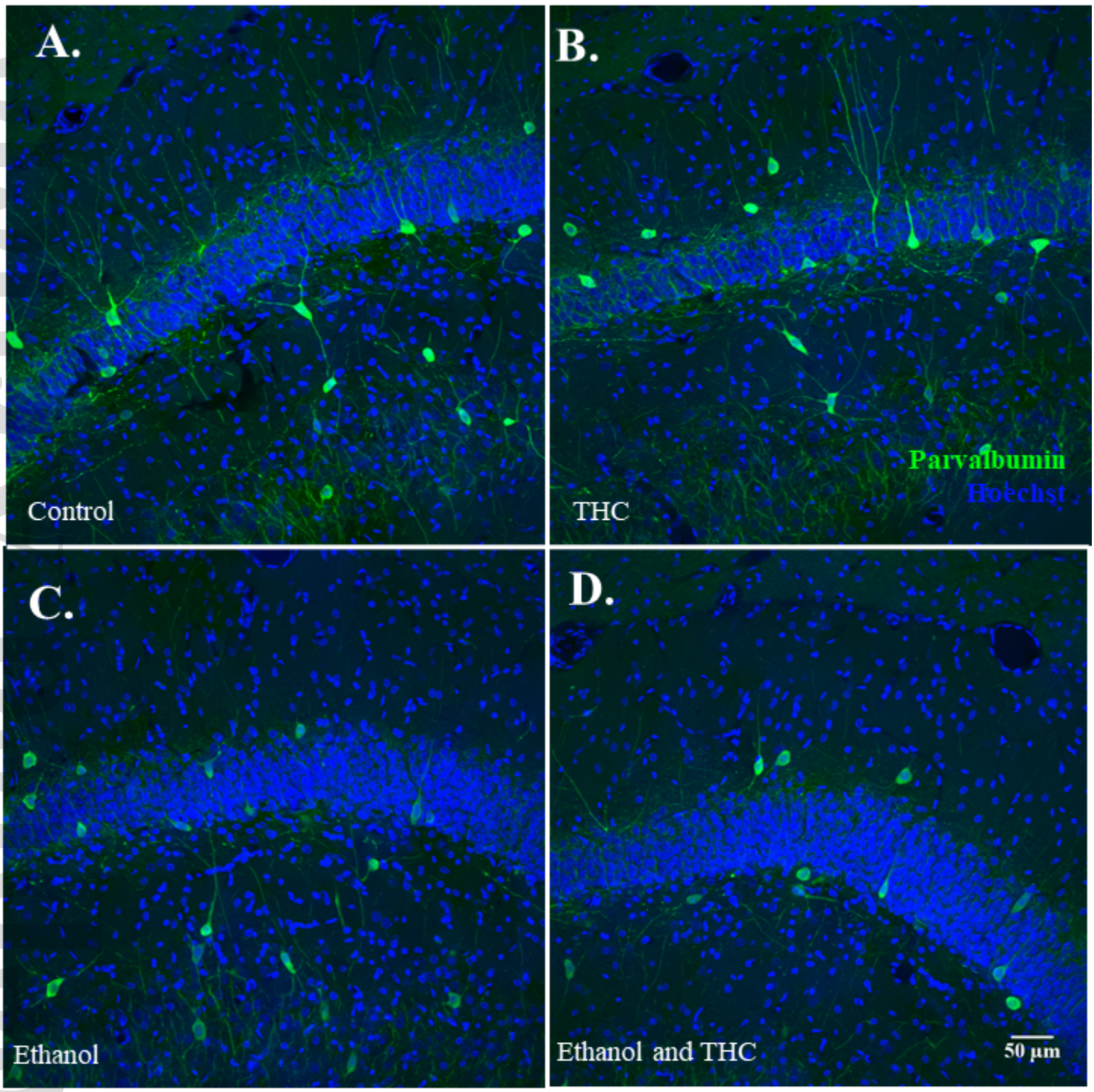

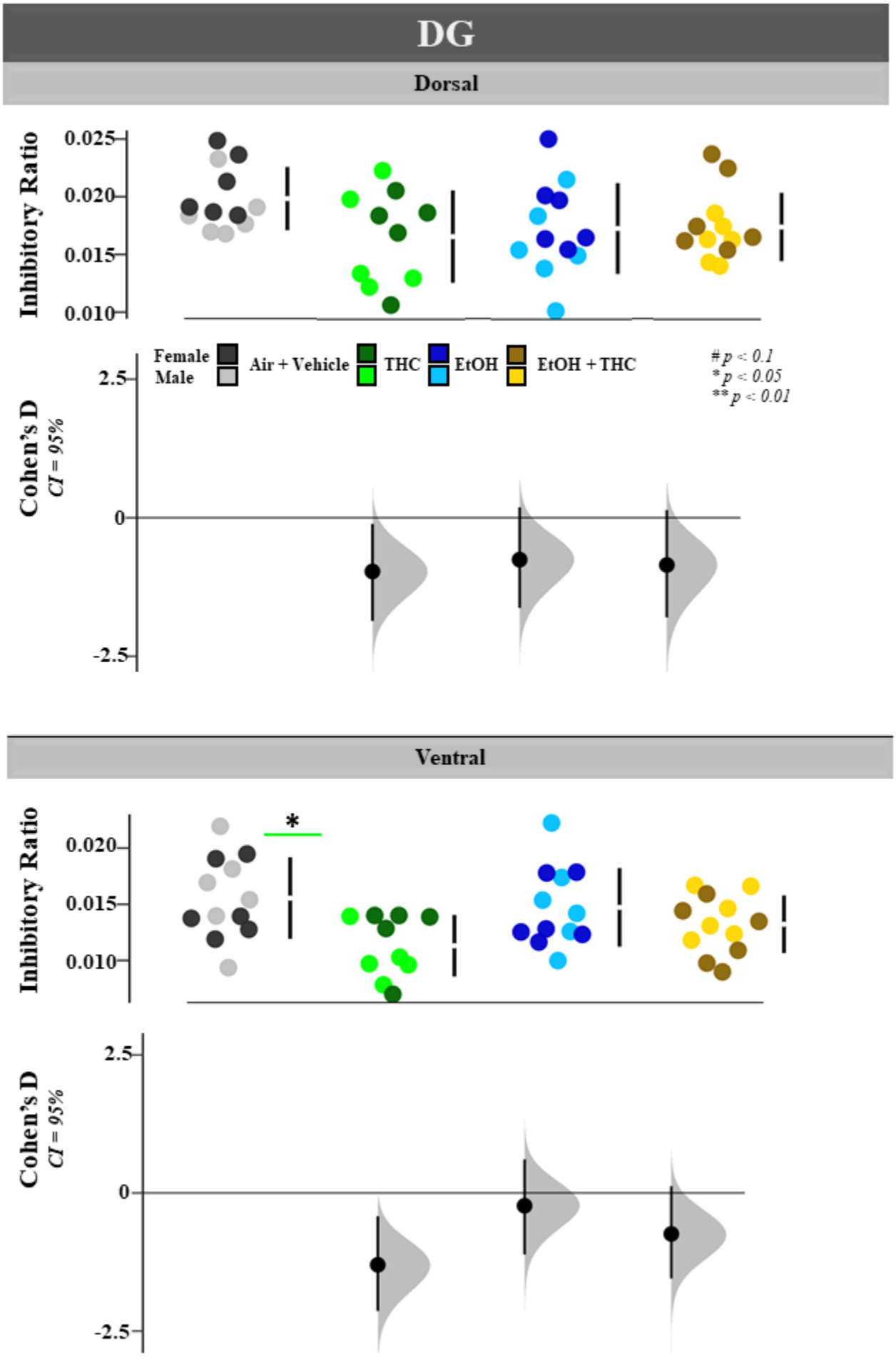

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