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Highlights for 'Effects of chronic exposure to haloperidol, olanzapine or lithium on SV2A and NLGN synaptic puncta in the rat frontal cortex':

- SV2A-binding radiotracers assess synaptic density in human psychiatric disorder
- Separating illness from drug exposure effects on SV2A binding remains challenging
- We exposed rats to clinically relevant doses of haloperidol, olanzapine and lithium
- SV2A and Neuroligin puncta in the frontal cortex were quantified after 28 days
- SV2A and synaptic puncta were unaffected but lithium increased Neuroligin puncta

Effects of chronic exposure to haloperidol, olanzapine or lithium on SV2A and NLGN synaptic puncta in the rat frontal cortex

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Abstract

Positron emission tomography studies using the synaptic vesicle glycoprotein 2A (SV2A) radioligand [^{11}C]-UCB-J provide *in vivo* evidence for synaptic dysfunction and/or loss in the cingulate and frontal cortex of patients with schizophrenia. In exploring potential confounding effects of antipsychotic medication, we previously demonstrated that chronic (28-day) exposure to clinically relevant doses of haloperidol does not affect [^{3}H]-UCB-J radioligand binding in the cingulate and frontal cortex of male rats. Furthermore, neither chronic haloperidol nor olanzapine exposure had any effect on SV2A protein levels in these brain regions. These data do not exclude the possibility, however, that more subtle changes in SV2A may occur at pre-synaptic terminals, or the post-synaptic density, following chronic antipsychotic drug exposure. Moreover, relatively little is known about the potential effects of psychotropic drugs other than antipsychotics on SV2A. To address these questions directly, we herein used immunostaining and confocal microscopy to explore the effect of chronic (28-day) exposure to clinically relevant doses of haloperidol, olanzapine or the mood stabilizer lithium on presynaptic SV2A, postsynaptic Neuroligin (NLGN) puncta and their overlap as a measure of total synaptic density in the rat prefrontal and anterior cingulate cortex. We found that, under the conditions tested here, exposure to antipsychotics had no effect on SV2A, NLGN, or overall synaptic puncta count. In contrast, chronic lithium exposure significantly increased NLGN puncta density relative to vehicle, with no effect on either SV2A or total synaptic puncta. Future studies are required to understand the functional consequences of these changes.

Key words: antipsychotics, lithium, synaptic density, SV2A, schizophrenia, bipolar disorder

1. Introduction

Schizophrenia (SCZ) and Bipolar Disorder (BD) are psychiatric disorders that affect approximately 0.5% and 1% of the global population, respectively (WHO). For each of these disorders there is accumulating evidence that synaptic dysfunction and/or loss are hallmarks of disease pathophysiology. Neuroimaging and *post-mortem* studies of individuals with SCZ or BD show reduced brain volume and spine density, respectively, particularly in the prefrontal and anterior cingulate cortex and hippocampus relative to healthy controls[1–4]. These changes coincide with reduced protein and/or mRNA levels of various pre- and postsynaptic markers[5–12]. Whereas the majority of these data were derived from *post-mortem* human material, the recent development of the UCB-J PET tracers, which specifically interact with the presynaptic Synaptic Vesicle glycoprotein 2A (SV2A), has enabled visualisation of pre-synaptic terminal density in the living human brain[13–15]. SV2A is thought to play a role in neurotransmitter release and vesicle recycling[16–19], and, due its localisation at both inhibitory and excitatory synapses throughout the brain, is considered to be a proxy for overall synaptic terminal density. Consistent with the aforementioned *post-mortem* evidence, studies using [^{11}C]-UCB-J PET imaging provide evidence for a reduction in SV2A binding in the prefrontal and anterior cingulate cortex of chronic SCZ patients[20]. Whilst there is also evidence for reduced SV2A ligand binding in major depressive disorder[21], data for BD is as yet lacking.

In our recent study, which demonstrated decreased SV2A-binding in the frontal cortex of chronic SCZ patients, we also provided evidence that total SV2A protein levels and [^{3}H]-UCB-J radioligand specific binding in the rat frontal cortex were unaffected by chronic exposure to two different antipsychotic drugs (APD), Haloperidol (HAL) and Olanzapine (OLZ)[20]. Another study likewise reported no statistically significant changes in synaptosomal SV2A protein levels in the auditory cortex of monkey brains after >1 year of treatment with the same drugs[12]. These data suggest that the reduced SV2A ligand binding observed in patients is related to SCZ pathophysiology rather than an effect of antipsychotic treatment on overall SV2A levels. This does not exclude the possibility, however, that more subtle changes may occur at SV2A-containing synaptic terminals following antipsychotic drug exposure, which may not be captured by studying overall protein levels. Moreover, aside from antipsychotics, to date no studies have reported on the effects of other psychotropic drugs on SV2A, such as the mood stabilizer lithium (Li) used in the treatment of BD. We previously demonstrated contrasting effects of the antipsychotic drugs HAL and OLZ compared to Li on rat

cortical grey matter volume, showing an increase upon Li treatment but reduced cortical volume upon exposure to HAL and OLZ[22,23]. To improve our understanding of how these different psychotropic medications affect SV2A-containing synaptic terminals, we investigated the effects of chronic (28-day) exposure to clinically relevant doses of HAL, OLZ, and Li on SV2A puncta in the rat frontal cortex, specifically the prefrontal cortex (PFC) and anterior cingulate cortex (ACC) subregions. These brain regions are most commonly associated with synaptic dysfunction in SCZ, BD and MDD, as well as a reduction in SV2A tracer binding[20,21]. Importantly, as SV2A localises to pre-synaptic terminals, analysis of SV2A puncta alone does not fully address the question of whether exposure to either antipsychotic drugs or Li affects overall synaptic density or not. Therefore, we additionally investigated drug exposure effects on a global marker of both inhibitory and excitatory postsynaptic puncta, Neuroligin (NLGN). This combination enables quantification of SV2A/NLGN puncta overlap as a proxy for overall synaptic density. We hypothesised that puncta count would be unaffected by chronic exposure to either HAL or OLZ, concurrent with our earlier data[20], however we anticipated that chronic exposure to Li, which has been suggested to have neurotrophic and neuroprotective effects[24,25], would lead to an increase in synaptic puncta. Our research provides one of the first investigations into the effect of these different psychotropic medications on SV2A puncta.

2. Materials and Methods

2.1 Animals

Animal experiments were carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986) and European Union (EU) Directive 2010/63/EU, with the approval of the local Animal Welfare and Ethical Review Body (AWERB) panel at King's College London (KCL). Male Sprague-Dawley rats (Charles River UK Ltd, Margate, UK), initial body weight 220-270 gram (6-10 weeks of age) were housed four per cage in conventional plastic cages (38 x 59 x 24 cm, Tecniplast, UK) containing sawdust, paper sizzle nest and cardboard tunnels. Animals were maintained under a 12-hour light/dark cycle (07.00 lights on) with food and water available *ad libitum*. Room temperature and humidity were maintained at 21 ± 2°C and 55 ± 5%, respectively. Animals were habituated for a minimum of 7 days before experimental procedures.

2.2 Experimental Design (animals)

With the exception of Li-treated animals, data reported in this study is derived from the same animals as those used in our previous study[20]. In brief, two separate batches of animals received continuous administration of psychotropic medication using osmotic minipumps (Alzet Model 2ML4; Alzet, Cupertino, CA, USA) for 28 days (equivalent to approximately 2.5 human years based on 11.8 rat days as equivalent to 1 human year[26]). Specifically, cohort 1 comprised animals of initial body weight 240-270 gram (9-10 weeks of age), treated with a common vehicle (β-hydroxypropylcyclodextrin, 20% wt/vol, acidified by ascorbic acid to pH 6; n=11), 0.5 mg/kg/day haloperidol (HAL; n=11), or 2 mmol/L equiv/kg/day lithium chloride (Li; n=10). Cohort 2 comprised animals of initial body weight 220-240 gram (6-7 weeks old) treated with vehicle (n=4), or 7.5 mg/kg/day olanzapine (OLZ; n=12) (all chemicals from Sigma-Aldrich, Gillingham, UK). The slightly younger age of cohort 2 was related to the limited solubility of olanzapine, as we were unable to dissolve Olanzapine at the concentration required for the weight of animals in cohort 1. Blood plasma levels achieved using these doses and delivery by osmotic pumps have previously been shown to be consistent with clinically comparable dopamine D2 receptor (D2R) occupancy and plasma levels[22,23,27].

Minipumps filled with drug or vehicle solutions were inserted subcutaneously on the back flank under isoflurane anaesthesia (5% induction, 1.5% maintenance delivered in an 80/20% medical air/oxygen mix). Weight was monitored daily in the week following surgery, and twice a week in the following weeks. Dyskinetic behaviour, *i.e*. vacuous chewing movements (VCMs) were measured at 2 and 4 weeks *post* surgery as follows. Room temperature was maintained at 21 $\pm$ 2 °C and ambient light settings were used (125-300 lux). Prior to behavioural assessment, animals were individually removed from their home cage and placed on a raised platform, out of eyesight from their home cage. After habituation for 1 minute, VCMs were manually scored during 2 minutes, blinded to treatment group, after which animals were returned to their home cage. Movements counted as VCM were typical 'licking' movements (sticking out of the tongue), and jaw tremors that lasted at least 2 seconds. VCMs are stereotypical motor behaviours that commonly develop in animals treated with antipsychotics[28,29] and are considered a proxy measure for tardive dyskinesia (TD) observed in patients undergoing chronic antipsychotic treatment. Animals undergoing Li treatment were given access to 0.9% saline instead of tap water to minimise hyponatremic properties of Li[30].

2.3 Post-mortem tissue handling and blood plasma analysis

After 28 days of administration, animals were terminally anaesthetised by injection of sodium pentobarbital (60 mg/kg, intraperitoneal) and culled by cardiac perfusion using heparinised (12.5 U/ml) ice-cold PBS. In order to prevent masking of synaptic target proteins and obtain a better quality of immunostaining[31,32], animals were not perfusion-fixed. Following PBS perfusion, brains were extracted, hemisected and post-fixed overnight in 4% PFA at 4°C. Brain hemispheres were then washed once in PBS, incubated in PBS-buffered 30% sucrose solution for 48h at 4°C, and then snap-frozen on dry ice and stored at -70°C until further processing for immunostaining.

At termination a blood sample was collected for estimation of drug levels. HAL and OLZ levels were commercially measured using tandem mass spectrometry (Cyprotex, Macclesfield, UK); Li levels were measured on an Thermo Scientific X-Series 2 ICP-MS (Durham University, Durham, UK) optimised for sensitivity and minimal oxide interferences, using an external calibration curve of 10, 25, 50 and 100 ppb

standards (Romil, Cambridge, UK). Good agreement was seen between both Li 6 and 7 isotopes indicating that spectral interferences were minimal.

2.4 Fluorescence immunostaining

The left hemisphere of each animal was serially sectioned (30 µm-thick coronal sections, interval 1/12, 360 µm spacing between sections within a series) on a cryostat at -18°C and stored in tissue cryoprotection solution (25% glycerol, 30% ethylene glycol, 45% 1x PBS pH 7.4, 0.05% azide) at -20°C until further processing. Free-floating sections from each brain in each group were washed for 10 min in phosphate buffer (PB; 0.1M) and 2x10 min in PBS. For antigen retrieval sections were incubated for 10-15 mins in 10 mM sodium citrate (pH 6.2) at RT, followed by a 15 min incubation in pre-heated 10 mM sodium citrate (pH 6.2) in a water-bath at 78°C. Sections were then allowed to cool down to RT in the same solution while gently shaking for 30 min. Sections were then washed twice (2x5 min) in PBS supplemented with 0.05% Triton-X100 and incubated for 3-4h in blocking solution (10% Normal Goat Serum, 1.5% BSA, 0.3% Triton-X100 in PBS), followed by overnight incubation at 4°C with primary antibody diluted in blocking solution (1:1000 Rabbit-α-SV2A, Abcam ab32942, Cambridge, UK; specificity of this antibody has been verified using mouse SV2A knockout tissue and SV2A blocking peptides[33]; and 1:300 Mouse-α-Neuroligin1/2/3/4, Synaptic Systems 129211, Göttingen, Germany; this antibody was shown to localise exclusively to the postsynaptic density in mouse brain sections[34]). Sections were then washed in PBS (3x10 min) and incubated for 2h in secondary antibody diluted in blocking solution (1:1000 Goat-α-mouse AlexaFluor488, abcam ab150113; 1:1000 Goat-α-rabbit AlexaFluor555, abcam ab150090) and washed again in PBS (4x10 min). Finally, sections were mounted on Superfrost Plus slides (ThermoFisher Scientific, UK), and air-dried at RT for 1h before coverslipping with mounting medium containing DAPI (Vectashield; Vector Laboratories, Peterborough, UK).

2.5 Confocal image acquisition and analysis

Overview images of fluorescent staining in brain sections (Figure 1A) were acquired in automated batch mode on a VS120-L100-W slide scanner (Olympus, UK), using a 4x lens objective and automated tiling. Confocal images for quantification of synaptic puncta were acquired at the Wohl Cellular Imaging Centre using an Inverted Spinning Disk confocal microscope (Nikon, Japan) and 60x oil immersion lens objective (NA 1.4). Exposure time was kept constant for the entire dataset. Images were 102.65 x 102.65 µm in size (512 x 512 pixels), acquired as a stack spanning 6-10 µm, at an interval of 0.3 µm. Image stacks were acquired from 4-5 consecutive sections containing the pre-specified brain regions, either the PFC, (Bregma +4.2 to +2.5mm, 6 stacks per section of which 3 in layer II/III and 3 in layer V) or the ACC (Bregma +2.3 to +0.0 mm, 4 stacks per section of which 2 in layer II/III and 2 in layer V). Synaptic puncta were analysed in ImageJ (*https://imagej.net/Welcome*), using a previously published macro[35] that we adapted for our images and analysis requirements. In brief, the macro performs the following operations: 3 consecutive optical sections (equating 0.9 µm total thickness) within each image stack were manually selected based on quality of staining and contrast in the image and were then combined by maximum intensity projection. After background correction using a rolling ball with radius of 5 µm, the image was thresholded automatically using the "Moments Dark" algorithm. The thresholded image for each channel was used as a mask to measure puncta count, size and total intensity within the puncta in the image created by maximum intensity projection. To avoid including noise in the puncta count, and in accordance with a previously published protocol for quantification of synaptic puncta[36], puncta smaller than 0.12 μm^2 (i.e. 3 pixels) were excluded. Maximum puncta size was determined empirically by measuring ~5 larger perisomatic puncta that were considered synaptic from at least 3 images per marker; using this method, puncta larger than 5 μm^2 (SV2A) or larger than 2.4 μm^2 (NLGN) were excluded. The number of overlapping puncta was determined after dilating puncta in the thresholded images of each channel by 1 pixel on all sides and overlaying both dilated masks; this ensured inclusion of synapses of which pre- and postsynaptic signal were located on adjacent pixels. Overlapping puncta smaller than 0.12 μm^2 (i.e. 3 pixels) were excluded.

196 *2.6 Statistical analysis*

197 Statistical analyses were performed using Prism version 8 (GraphPad Software, La Jolla, California,
198 USA). Samples sizes were based on prior studies[20,22,23]. Two animals (one VEH-treated in cohort 1 and one
199 OLZ-treated) were excluded from puncta analysis on the basis of sub-optimal saline perfusion of the brain,
200 which negatively impacted on staining quality. Data were tested for statistical outliers based on ROUT test
201 (0.5%) but no outliers were identified. Data were tested for normal distribution using the Shapiro-Wilk test.
202 Scores for VCMs and immunostaining data were analysed by two-way ANOVA, assessing treatment, time,
203 and treatment x time interaction (VCM), or treatment, region, and treatment x region interaction
204 (immunostaining), with post-hoc Bonferroni's multiple comparison test where appropriate. Linear
205 correlation analysis was computed based on Pearson's correlation coefficient (2-tailed). Researchers were
206 blinded to the treatment groups during sample preparation, imaging and image analysis.

207

3. Results

3.1 Behavioural analysis and blood plasma levels

Following osmotic pump implantation, rats were monitored for weight loss or gain. All animals gained weight comparably with no significant differences between drug treatments and their respective control groups (Supplementary Figure S1A,B).

Administration of HAL, OLZ or Li by osmotic pump achieved plasma levels (mean ± SD) of 3.03 ± 0.94 ng/mL for HAL; 15.5 ± 5.54 ng/mL for OLZ and 0.3 ± 0.04 mmol/L for Li, respectively. The levels of HAL and OLZ are consistent with clinically comparable dopamine D2 receptor (D2R) occupancy and plasma levels[22,23,27]. Plasma levels of Li fell just below the therapeutic range and earlier observed plasma levels[23]

Vacuous chewing movements (VCMs) were measured at 2 and 4 weeks after the start of drug exposure. Rats exposed to HAL or OLZ, but not Li, showed a statistically significant increase in VCMs as compared to their respective control groups (Supplementary Figure S1C,D; C, main effect of treatment: $F(2,29)=17.18$, $p<0.001$, $\eta_p^2=0.55$; D, main effect of treatment: $F(1,15)=11.33$, $p<0.01$, $\eta_p^2=0.60$). There was no effect of time or treatment x time interaction on VCM count. There was also no correlation between HAL or OLZ plasma level and the number of VCMs (Supplementary Figure S1E,F), consistent with our prior work[22].

3.2 Effect of Haloperidol and Lithium treatment on SV2A and NLGN puncta

Using low magnification (4x) fluorescent imaging, we observed that both SV2A and NLGN immunostaining were present throughout grey but mostly absent in white matter (Fig.1A). At higher magnification (60x) and using confocal imaging, we observed that SV2A immunostaining displayed clear punctate structures with high contrast in the pyramidal cell layers. Presynaptic SV2A structures were most prominent at perisomatic regions, but absent from the nucleus and soma (Fig.1B,C). NLGN staining revealed smaller puncta that were also present in the soma, but not in the nucleus (Fig.1B,C). We determined the number of pre- and postsynaptic puncta individually, as well as their overlap as a proxy of overall synaptic density. Furthermore, we determined the total area of puncta as a measure for total synaptic terminal area,

and the intensity of staining within puncta as a relative measure of synaptic SV2A and NLGN protein levels (Fig.2).

Analysis of SV2A puncta number showed a main effect of region, but no significant effects of treatment or region x treatment interaction. Likewise, there were no effects of treatment or region x treatment interaction for the total area of SV2A puncta or total intensity inside puncta (Fig.2A, Table 1). Thus, our data suggests that chronic exposure to either HAL or Li does not affect SV2A-containing presynaptic terminals.

Analysis of NLGN puncta number showed a significant effect of both region and treatment, but no region x treatment interaction (Fig.2B, Table 1). *Post-hoc* analysis revealed that the treatment effect was driven by a significant increase in NLGN puncta number in both PFC and ACC upon exposure to Li as compared to VEH-treated animals. Total NLGN puncta area also showed a significant increase upon Li treatment (Fig.2B, Table 1), although for the ACC this did not survive correction for multiple comparisons (actual p=0.08). There was no correlation between Li plasma level and NLGN puncta number or area (data not shown). Total NLGN staining intensity showed no significant effect of treatment (Fig.2B, Table 1). Thus, our data suggests that chronic Li, but not HAL exposure, induces an increase in postsynaptic puncta number and size.

Finally, we analysed the number of overlapping SV2A/NLGN puncta, as these would be representative of structural synapses. There was an overall effect of region on overlapping puncta count (Fig.2C, Table 1), suggesting an overall difference in synaptic density between PFC and ACC, but there were no statistically significant effects of either treatment or a treatment x region interaction.

3.3 Effect of Olanzapine treatment on SV2A and NLGN puncta.

To test whether the results obtained with HAL exposure generalises to other antipsychotics, we analysed SV2A, NLGN and synaptic puncta in a separate cohort of animals treated for 28 days with vehicle or 7.5 mg/kg/day Olanzapine (OLZ; Supplementary Figure S2A,B).

We found no effect of OLZ treatment on SV2A or NLGN puncta number, or overlapping synapse count, with medium effect size (Supplementary Figure S2C, Table 2). Likewise, we found no effect on

puncta area or staining intensity of either marker (Supplementary Figure S2D-E, Table 2). Thus, we show that OLZ, like HAL, does not affect presynaptic SV2A or postsynaptic NLGN puncta, nor their overlap.

4. Discussion

SV2A is a synaptic protein of great interest due to the development of the UCB-J tracers, which have enabled *in vivo* imaging of presynaptic terminal density in the living human brain[13,14,20,21,37–41]. Specifically, recent studies provided evidence of reduced SV2A tracer binding in the prefrontal (PFC) and anterior cingulate cortex (ACC) in schizophrenia[20]. One potential confounding factor in human PET studies is whether chronic exposure to psychotropic medication has an impact on SV2A levels or the specific binding of the SV2A radioligand. Whereas we previously demonstrated that chronic (28-day) APD exposure in rats did not affect overall SV2A protein levels or [^{3}H]-UCB-J binding in the PFC and ACC[20], suggesting that the decrease observed in patients is primarily disease-related, more research is required to understand whether psychotropic mediation exerts more subtle effects on SV2A and synaptic density. Therefore, we investigated the effect of chronic exposure (28 days) to two different antipsychotics, Haloperidol (HAL) and Olanzapine (OLZ), and the mood stabiliser lithium (Li) on presynaptic SV2A and postsynaptic NLGN puncta in the rat prefrontal (PFC) and anterior cingulate cortex (ACC). In agreement with our hypothesis, and complementing our previous data[20], we find that neither OLZ or HAL alter SV2A or NLGN puncta density, puncta area, or immunostaining intensity, or overall synaptic density as assessed by SV2A/NLGN puncta overlap. Furthermore, we provide evidence for a contrasting effect of chronic Li exposure, which selectively enhances postsynaptic NLGN puncta count and area in both the PFC and ACC, without affecting SV2A-containing presynaptic terminal density or overall synapse count.

We cannot exclude the possibility that differential duration of exposure to antipsychotics, be that shorter or longer than 28 days, does not affect SV2A or synaptic puncta density in the rat brain. We previously reported that exposing rats to HAL and OLZ for 56 days resulted in reduced cortical volume and thickness, albeit at higher APD doses (2 mg/kg/day for HAL and 10 mg/kg/day for OLZ, respectively)[22,23]. It remains to be investigated whether these changes are related to reduced synaptic density and/or SV2A-containing terminals specifically, or whether other factors explain the apparent loss of cortical grey matter such as changes in myelin, lipid or tissue water content[42]. Conversely, at a treatment duration of 28 days we may also have missed effects on SV2A and NLGN puncta that occur during the early stage of APD treatment. One study showed that HAL and OLZ exposure reduces dopamine signalling and synaptic vesicle pool size

during the first 2-6 days of treatment, but increases synaptic vesicle pool size upon longer treatment, thereby normalising dopamine levels after ~2 weeks[43]. It is yet unknown whether this change in signalling coincides with altered levels of synaptic vesicle proteins such as SV2A. Thus, further studies are required to elucidate the impact of both short-term and chronic drug exposure on SV2A and other synaptic protein levels. Future studies could, for example, combine isolation of synaptosomes with proteomics to provide more detail on the impact of differential lengths of antipsychotic exposure on the composition of the synapse.

This study is, to our knowledge, the first in rodents to investigate the effect of chronic administration of psychotropic medications specifically on SV2A and NLGN synaptic puncta, thus providing complementary information to studies investigating the effect of these medications on overall protein levels. Our data are consistent with the findings of a recent study in monkeys that reported no change in overall synaptosomal SV2A protein levels in the superior temporal gyrus after >1 year HAL or OLZ treatment[12]. Previous studies in rats investigated the effect of APD exposure on presynaptic markers other than SV2A; of particular interest is Synaptophysin (SYN), a presynaptic vesicle protein that shows high correlation with SV2A distribution[13]. The majority of the studies that used comparable methods of antipsychotic administration (i.e. chronic exposure, defined as 2-4 weeks, to clinically relevant doses) found no statistically significant effects of either HAL or OLZ on SYN protein levels in the rat hippocampus and frontal cortex[44–47]; one study reported increased SYN levels in cortical lysates upon chronic administration of OLZ[48], however this study did not use clinically comparable dosing as defined by Kapur and colleagues[27]. The effect of APD on global postsynaptic marker expression in rats has mostly been investigated at the mRNA level, with Homer1a and PSD-95 as the most commonly reported markers. For Homer1a mRNA levels in the cortex upon HAL and OLZ administration results are inconsistent, with some studies reporting upregulation and others downregulation[49–52]. These findings have yet to be confirmed at the protein level. For PSD-95 mRNA levels in the cortex findings were likewise inconsistent[50,52], however no change was found for PSD-95 at the protein level in either striatum or frontal cortex[46,53]. Notably, both Homer1a and PSD-95 are primarily located at excitatory synapses therefore these results may not be reflective of overall synaptic protein levels.

Studies on the effects of chronic Li exposure on SYN reported no effect on either protein[47,48] or mRNA levels[54]. On the other hand, chronic Li exposure (2 weeks) was shown to increase mRNA levels of Synapsins, a class of proteins that like SV2A and SYN is located on presynaptic vesicles and is involved in

318 neurotransmitter release[55], and is reduced in BD[47]. In cell culture, Li induced enhanced recruitment of both
319 SYN and PSD95 to synapses[56], and enhanced clustering of both gephyrin and PSD95[57], indicative of an
320 involvement in synaptic plasticity and synaptogenesis at both inhibitory and excitatory synapses. Indeed,
321 multiple studies confirm that Li treatment enhances dendritic branching and synapse formation, both *in vitro*
322 and *in vivo*[24,58]. The neurotropic effect of Li, and particularly the increased clustering of postsynaptic scaffold
323 protein, are consistent with the increase in postsynaptic NLGN puncta observed in our study, as well as with
324 the reported neurotrophic effects of this drug in humans[25]. At the resolution of our data we are unable to say
325 whether the increase in NLGN puncta is due to an increase in the number of postsynaptic specialisations
326 (such as increased spine density), or whether an increase in the size of pre-existing puncta due to
327 postsynaptic protein accumulation[59] leads to inclusion of puncta that were previously below detection level.
328 More research is required to understand the underlying molecular mechanism, and should include analysis of
329 electrophysiological recordings in brain slices of Li-treated animals to understand the functional
330 consequences of increased postsynaptic puncta in absence of a change in presynaptic puncta density.

331 Collectively, our data are thus consistent with previous observations in studies that used comparable
332 methods of APD exposure in rats. Specifically, our results for SV2A parallel earlier studies on the effect of
333 APDs and Li on SYN, whereas the effect of Li on increased NLGN puncta reflects the oft-reported
334 neurotropic effect of Li. It should be taken into account, however, that direct comparisons are difficult to
335 make as these studies are heterogeneous in terms of measures reported (protein *vs* mRNA), synaptic markers
336 studied, regimens used to establish chronic exposure (daily injections, administration via food intake, or
337 constant exposure via mini-pumps), length of exposure, as well as concentrations of drugs administered.
338 Minor differences in methods of drug administration may well underlie different outcomes of these studies.
339 Furthermore, due to slightly lower blood levels of Li in our samples, which fell just below the therapeutic
340 range and earlier observed plasma levels[23] the effect of Li treatment may not have been as robust in our study
341 compared to other investigations, and the effect on overlapping synaptic puncta count upon Li exposure may
342 be underestimated.

343 Our data do not exclude the possibility that chronic APD or lithium exposure would affect synaptic
344 proteins other than those assessed here, or cause more subtle effects in synapse rearrangement that cannot be
345 detected at the resolution of light microscopy. For example, electron microscopy studies in the rat PFC after

up to 1 year of exposure to HAL (3 mg/kg) showed a shift towards an increased number of inhibitory synapses without affecting total synapse number or synaptic vesicle density[60,61]. In agreement with this observation, autoradiography studies from our lab and others revealed increased $GABA_A$ receptor binding in the rat ACC upon chronic HAL exposure[62,63]. Future studies combining SV2A staining with specific pre- and postsynaptic markers for inhibitory and excitatory synapses, such as vGAT/Gephyrin or vGlut/PSD95, respectively, may further our understanding of differential effects of drug exposure on these two populations. As it is currently unknown whether synaptic changes as observed in SCZ occur globally, or primarily on either inhibitory or excitatory synapses, it remains to be investigated whether a putative shift towards enhanced inhibition induced by APD treatment ameliorates symptoms or rather causes unwanted side-effects. Importantly, our work also did not assess whether treatment leads to functional changes in synaptic transmission. Since accumulation of APD in synaptic vesicles has autoinhibitory effects on synaptic transmission during early stages of treatment, but efficacy decreases over time despite stable levels of postsynaptic dopamine receptor occupancy[43,64,65], elucidating the functional impact of both short-term and chronic drug exposure on synaptic function, such as vesicle pool size, neurotransmitter release and signalling, and how these correlate with putative changes in synaptic protein levels, remains a priority for future work.

It is also worth reflecting here that the number of synapses detected is highly dependent on the synaptic marker, method of detection, and method of quantification used. It is not uncommon to see different numbers for pre- and postsynaptic markers, which will also be reflected by a lower number of overlapping (total) synapses as compared to the individual markers[66] (Fig.2 and S2). Notably, our reported density of 0.1-0.2 SV2A puncta per μm^3 is in the same order of magnitude as observations by Rasakham et al.[67], who reported an approximate synaptic puncta density of 0.5 puncta per μm^3 in the PFC of rats in a similar age range, using Synaptophysin and PSD95 immunostaining with fluorescence microscopy. At higher resolution, an electron microscopy study detected up to 6 synaptic puncta per μm^3 in the rat PFC[68], suggesting that only a subset of synaptic puncta is detected at the resolution of fluorescence microscopy. Thus, our study should be considered to reflect a percentage of change rather than a change in absolute synapse numbers.

Some limitations of our study should be noted. Whereas patients typically start psychotropic treatment in young adulthood, the animals exposed to olanzapine in our study were considered adolescent at the age of

treatment onset. The younger age and lighter weight of these animals was required to dissolve the drugs at a concentration that would results in clinically relevant levels. Hence, additional studies in older animals are required to eliminate any interaction between OLZ exposure and the normal maturational changes in either synaptic marker. Furthermore, drug treatment was performed in otherwise healthy animals, therefore the interaction between drug exposure and illness was not assessed. Interestingly, OLZ partially rescued stress-induced reduction of SYN and PSD-95 protein levels in the rat prefrontal cortex[46], as well as PCP-induced reduction of SYN and PSD-95 protein levels and neurite outgrowth in neuronal culture[69], and PCP-induced reduction in spine density[70], without affecting these measures in control condition. Future research should therefore include investigating the effect of APD and mood stabilising drugs on SV2A protein levels and synaptic puncta density in animal models relevant for the study of SCZ and mood disorders. Finally, future studies in animals should assess the effect of psychotropic medication in both male and female rodents. This will provide further important insight into both medication and gender effects in treatments that are relevant for the study of human psychiatric disorder and the interpretation of SV2A neuroimaging studies.

In summary, our study shows that chronic APD exposure (up to 28 days, at the doses indicated) does not alter presynaptic SV2A or postsynaptic NLGN puncta density in the prefrontal and anterior cingulate cortex of rats. In contrast, chronic Li exposure (up to 28 days, at the dose indicated) enhances postsynaptic puncta, although we saw no effect on the presynaptic terminal. Our data provide useful insight into the impact of psychotropic treatment on SV2A and synaptic puncta density, which may aid in the interpretation of clinical studies.

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Experimental Investigation and Data Analysis: EFH, ACV, RM, CJO
Contribution of Unique Resources: ODH, ACV
Funding: ODH, ACV
Writing – original draft: EFH, ACV
Writing – editing and revision: EFH, ACV, RM, ODH, SN, DPS
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Data availability: Data generated for this study will be available from the corresponding author upon request.

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Figure legends

Figure 1. SV2A and NLGN immunostaining in rat PFC and ACC upon 28 days exposure to Haloperidol or Lithium. A. Fluorescent image (automatically stitched) of rat PFC (left) and ACC (right), stained with SV2A, NLGN, or DAPI as indicated. White squares indicate the positions where higher resolution confocal images (panel B,C) were acquired. **B,C.** Confocal images of the PFC (**B**) or ACC (**C**) showing synaptic staining with SV2A (red, top), NLGN (green, middle) or merged including DAPI (blue, bottom). Treatments are indicated at the top (VEH, Vehicle; HAL, 0.5 mg/kg/day Haloperidol; Li, 2 mM eq/kg/day Lithium). Yellow boxes indicate the position of the zoomed insets. Arrows indicate examples of co-localisation. Scalebar 15 μm (entire image) or 4 μm (zoomed insets).

Figure 2. Quantification of SV2A and NLGN puncta upon 28 days exposure to Haloperidol or Lithium. A-B. Scatter plots of puncta count (left), area (middle) and staining intensity (right) for rat PFC and ACC based on staining for SV2A (**A**), or NLGN (**B**). Treatments are indicated at the top (VEH, Vehicle, grey circles; HAL, 0.5 mg/kg/day Haloperidol, orange squares; Li, 2 mM eq/kg/day Lithium, blue triangles). **C**. Scatter plot of overlapping SV2A/NLGN puncta count.

Average value represents mean; two-way ANOVA with Bonferroni's correction; *$p<0.05$.

589 *Table 1. Statistical analysis (2-way ANOVA) of SV2A and NLGN staining in rat frontal cortex upon HAL and*

590 *Li treatment*

Variable	Region	Treatment	Region x Treatment
SV2A puncta count	F(1,28)=10.40; **p<0.01**; η_p^2=0.27	F(2,28)=2.072; ns[1]; η_p^2=0.38	F(2,28)=2.442; ns
SV2A puncta area	F(1,28)=7.570; **p<0.05**; η_p^2=0.21	F(2,28)=0.5517; ns; η_p^2=0.22	F(2,28)=0.4628; ns
SV2A staining intensity	F(1,28)=7.354; **p<0.05**; η_p^2=0.21	F(2,28)=0.2032; ns; η_p^2=0.09	F(2,28)=0.0268; ns
NLGN puncta count	F(1,28)=4.376; **p<0.05**; η_p^2=0.14	F(2,28)=4.203; **p<0.05**; η_p^2=0.64	F(2,28)=0.1007; ns
NLGN puncta area	F(1,28)=4.157; p=0.051; η_p^2=0.13	F(2,28)=3.580; **p<0.05**; η_p^2=0.57	F(2,28)=0.1245; ns
NLGN staining intensity	F(1,28)=8.478; **p<0.01**; η_p^2=0.23	F(2,28)=1.986; ns; η_p^2=0.50	F(2,28)=0.5509; ns
Synaptic puncta count	F(1,28)=5.506; **p<0.05**; η_p^2=0.16	F(2,28)=2.026; ns; η_p^2=0.34	F(2,28)=0.1408; ns

591 [1]ns, not significant

592

593 *Table 2. Statistical analysis (2-way ANOVA) of SV2A and NLGN staining in rat frontal cortex upon OLZ*

594 *treatment*

Variable	Region	Treatment	Region x Treatment
SV2A puncta count	F(1,14)=1.480; ns; η_p^2=0.10	F(1,14)=0.1845; ns; η_p^2=0.059	F(1,14)=0.4974; ns
SV2A puncta area	F(1,14)=0.0268; ns; η_p^2=0.002	F(1,14)=1.222; ns; η_p^2=0.26	F(1,14)=0.0013; ns
SV2A staining intensity	F(1,14)=0.148; ns; η_p^2=0.01	F(1,14)=1.518; ns; η_p^2=0.36	F(1,14)=0.1523; ns
NLGN puncta count	F(1,14)=0.0605; ns; η_p^2=0.004	F(1,14)=0.7205; ns; η_p^2=0.28	F(1,14)=0.5051; ns
NLGN puncta area	F(1,14)=0.0063; ns; η_p^2<0.001	F(1,14)=0.9316; ns; η_p^2=0.29	F(1,14)=0.9005; ns
NLGN staining intensity	F(1,14)=0.1431; ns; η_p^2=0.01	F(1,14)=0.9287; ns; η_p^2=0.26	F(1,14)=2.076; ns
Synaptic puncta count	F(1,14)=0.235; ns; η_p^2=0.02	F(1,14)=0.8453; ns; η_p^2=0.28	F(1,14)=0.1283; ns

595

Figure 1

Click here to access/download;Figure;SV2A-puncta-Fig1_Revised.tif

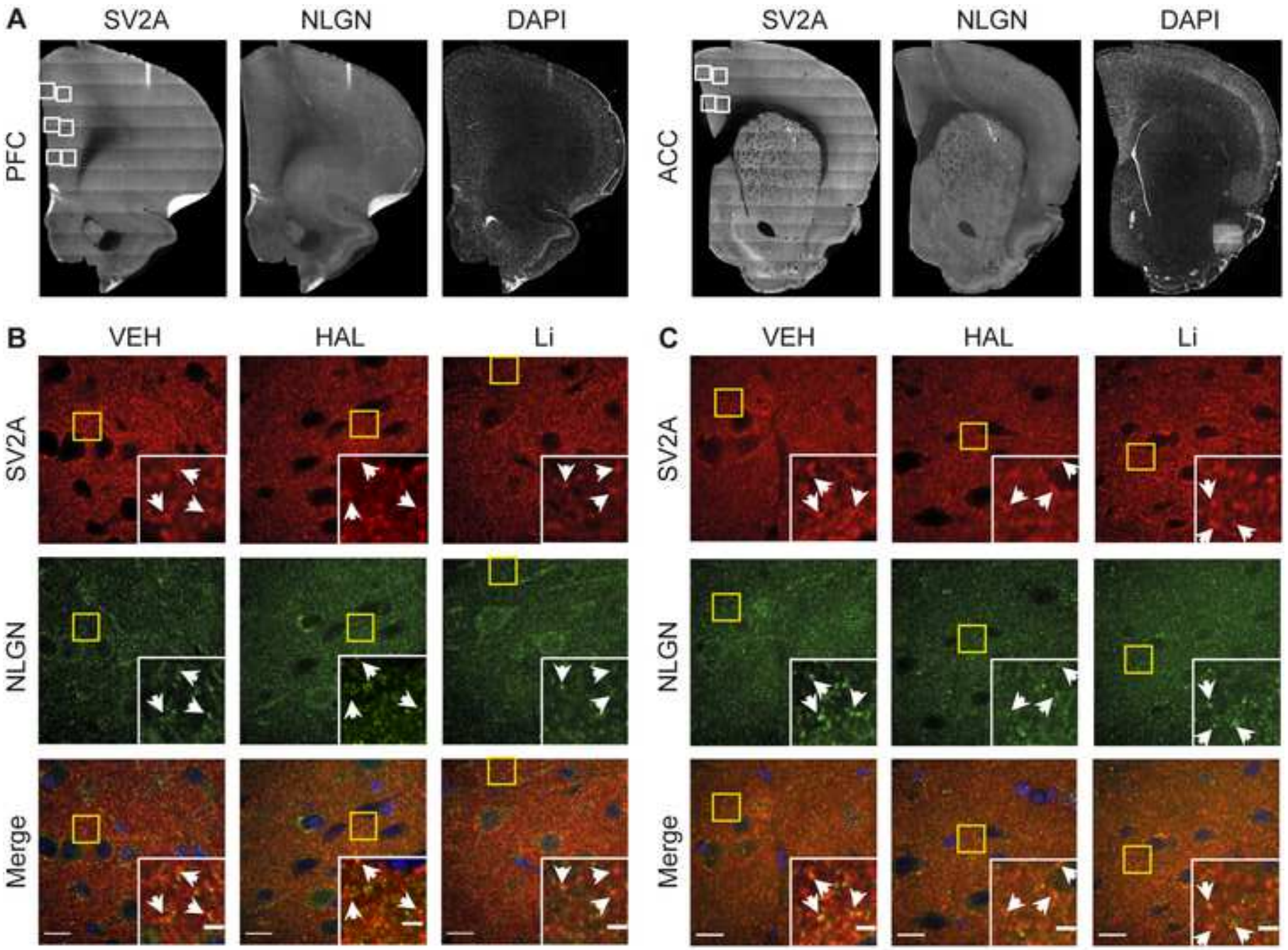

Figure 2

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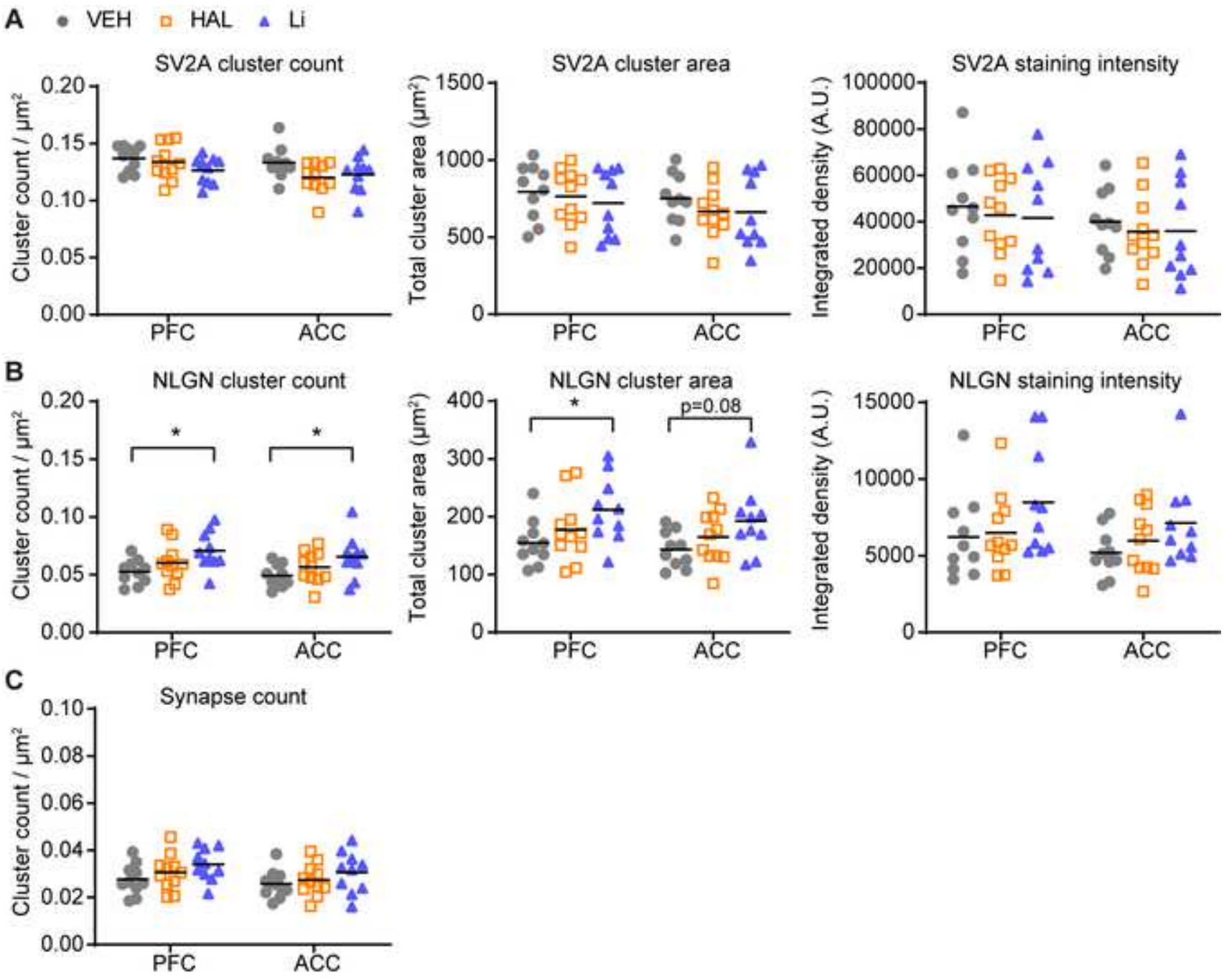

CRediT author statement:

EFH: Methodology, Investigation, Formal analysis, Visualisation, Writing – Original Draft, Writing – Review & Editing; **MCC**: Methodology; **SN**: Conceptualisation, Methodology, Supervision, Writing – Review & Editing; **RM**: Methodology, Investigation, Writing – Review & Editing; **CJO**: Methodology, Investigation; **DPS**: Writing – Review & Editing; **ODH**: Conceptualisation, Supervision, Funding acquisition, Writing – Review & Editing; **ACV**: Conceptualisation, Methodology, Resources, Supervision, Funding acquisition, Writing – Original Draft, Writing – Review & Editing.

Supplementary Figures S1-S2