Exposure To Chronic Stress Impairs The Ability To Cope With An Acute Challenge: Modulation By Lurasidone Treatment

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ABSTRACT

Chronic stress represents a major contributor for the development of mental illness. This study aimed to investigate how animals exposed to chronic mild stress (CMS) responded to an acute stress, as a vulnerability's challenge, and to establish the potential effects of the antipsychotic drug lurasidone on such mechanisms. Adult male Wistar rats were exposed or not (controls) to a CMS paradigm for 7 weeks. Starting from the end of week 2, animals were randomized to receive vehicle or lurasidone for 5 weeks. Sucrose intake was used to measure anhedonia. At the end, half of the animals were exposed to an acute stressor before sacrifice. Exposure to CMS produced a significant reduction in sucrose consumption, whereas lurasidone progressively normalized such alteration. We found that exposure to AS produced an upregulation of *Brain derived neurotrophic factor (Bdnf)* in the prefrontal cortex of controls animals. This response was impaired in CMS rats and restored by lurasidone treatment. While in control animals, AS-induced increase of *Bdnf* mRNA levels was specific for *Parvalbumin* cells, CMS rats treated with lurasidone show a significant upregulation of *Bdnf* in pyramidal cells. Furthermore, when investigating the activation of different brain regions, CMS rats showed an impairment in the global response to the acute stressor, that is largely restored by lurasidone treatment. Our results suggest that lurasidone treatment in CMS rats may regulate specific circuits and mechanisms, which will ultimately contribute to boost resilience under stressful challenges.

KEY WORDS: depression; antipsychotic drug; lurasidone; anhedonia; chronic mild stress; acute stress

1. INTRODUCTION

According to the World Health Organization, approximately 280 million people suffer of depression (IHME, 2019), which leads to a severe impairment in everyday life activities. Depression can also lead to suicide that represents the fourth leading cause of death in 15-29-year-old individuals (WHO, 2021). The aetiology of depression is still poorly understood, and it is related to genetic, epigenetic and environmental risk factors. With this respect, exposure to stressful events represents one of the main environmental risk factors for depression's development (Krishnan and Nestler, 2010; Malhi et Mann, 2018).
Depression is characterized by specific structural and functional changes in selected brain areas, such as the prefrontal cortex (PFC) and the hippocampus (Hipp), which play a relevant role in mood and emotion (Gass and Riva, 2007; Kang et al., 2012; Hare and Duman, 2020). At the molecular level, several studies have shown that depression is characterized by reduced neuronal plasticity that can be ameliorated by effective therapeutic intervention (Wang et al., 2022; Castrén et Monteggia, 2021). Furthermore, alterations of the Hypothalamus Pituitary Axis' (HPA) function have been reported in depressed patients, who may show altered responsiveness to an acute stressful challenge (Pariante and Lightman, 2008; Fiksdal et al., 2019; Burke et al., 2005). Indeed, the inability to cope with environmental stressors represents a main feature of depression as well as an important component for emotional control. However, there is still limited information with respect to how emotional control may be affected under a pathologic condition. While "emotional control" is a human feature, rodents can be used to investigate key pathways and systems that may be relevant in emotional control. With this respect, the use of an acute stress can be instrumental to establish if, under pathological conditions, the responsiveness to an acute stress challenge is affected and to establish the potential effect of pharmacological intervention.

On these bases, we used the chronic mild stress (CMS) model, a well-established animal model of depression, in order to investigate the coping ability of stressed rats to respond to an acute environmental challenge. Additionally, we evaluated if and how a chronic treatment with the antipsychotic drug lurasidone could modulate the response to such acute challenge. Exposure to CMS produces an array of depressive-like behavioural alterations, including anhedonia, anxiety and cognitive impairment (Willner, 2005; Hill et al., 2012; Luoni et al., 2014; Calabrese et al., 2020). We have previously demonstrated that lurasidone treatment is able to normalize the anhedonic and cognitive phenotype in CMS rats, an effect that is associated with several molecular changes in key brain regions, such as prefrontal cortex and hippocampus (Luoni et al., 2014; Calabrese et al., 2016; Rossetti et al., 2016; Rossetti et al., 2018; Calabrese et al., 2020).
With that in mind, we investigated different structural and functional markers that have been previously associated with depression and stress response, including the neurotrophin BDNF (Kozisek et al., 2008; Calabrese et al., 2010; 2011; Krishnan and Nestler, 2010; Malhi et Mann, 2018; Colucci-D'Amato et al., 2020; Wang et al., 2022), different markers of neuronal activation, such as *Cfos*, *Npas4*, *Zif-268* and *Arc*, as well as markers for HPA axis responsiveness. We carried out our analyses in different brain areas involved in mood disorders as well as in stress response, including PFC, dorsal and ventral Hippocampus (DH and VH), Amygdala (Amy) and Nucleus Accumbens (NAc) not only to analyse the specific contribution of each region, but to investigate the overall brain responsiveness to the acute stress challenge.

2. MATERIALS AND METHODS

2.1 Animals

80 male Wistar rats were purchased from Charles River, Germany one month before the start of the experiment. The animals were singly housed with food and water freely available and were maintained on: 12-h light/dark cycle (lights on at 08.00), constant temperature (22 ± 2°C) and humidity (50 ± 5%). All procedures used in this study have agreed to the rules and principles of the 86/609/EEC Directive and have been approved by the Local Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland. All efforts were made to minimize animal suffering and reduce the total number of animals used while maintaining statistically valid group numbers.

2.2 Stress Procedure

After a period of 3 weeks of adaptation, the animals were trained to consume 1% sucrose solution. Training consisted of eight 1h baseline tests, in which sucrose was presented in the home cage, following 14h food and water deprivation. The sucrose intake was measured by weighing pre-weighed bottles containing the sucrose solution, at the end of the test. Sucrose consumption was weekly monitored throughout the whole experiment.
Based on their sucrose intakes in the final baseline test, the animals were divided in two matched groups. One group of animals was subjected to the chronic mild stress CMS procedure (Willner, 2005) for a period of up to 7 consecutive weeks (n=40). Each week of stress regime consisted of: two periods of food or water deprivation, two periods of 45-degree cage tilt, two periods of intermittent illumination (lights on and off every 2h), two periods of soiled cage (250 ml water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. All stressors were 10 - 14 h of duration and were applied individually and continuously, day and night. Control animals (CTRL) were housed in separate rooms and had no contact with the stressed animals (CMS) (n=40). They were deprived of food and water for 14h preceding each sucrose test, otherwise food and water were available *ad libitum* in their home cage.

2.3 Drug administration and acute stress challenge procedure

After 2 weeks of stress paradigm exposure, both CMS and CTRL animals were furthermore divided in 2 matched subgroups, which received once daily administration of vehicle (1% hydroxy-ethylcellulose, PO) or lurasidone (3.0 mg/kg, PO) (n=20) for the subsequent 5 weeks, as previously described (Luoni et al., 2014). Three days after final vehicle or drug administration, all animals were further divided in 2 matched subgroups that were exposed or not to an acute restraint stress (n=10), consisting of a 30-minute immobilization in perforated plastic tubes (6.5 cm inner diameter) of adjustable length (Fig. 1). The restraint allowed normal breathing while limiting movements of the head and the body limbs. Finally, all animals were returned to their home cages and then sacrificed.

2.4 Brain and blood sampling

Animals were individually removed from their housing rooms for sacrifice. They were decapitated in a semi-randomized order. Following decapitation, the brains were promptly extracted from the skull and put on an ice-chilled plate. The brains were then cut in half to divide the two hemispheres. The left hemispheres were free-hands dissected on top of an ice-chilled 96-well plate to collect the following brain regions: prefrontal cortex (PFC), ventral hippocampus (VH), dorsal hippocampus (DH), amygdala (AMY) and nucleus accumbens (NAcc). Tissues were stored at -80 °C until further processing. The

right hemispheres were collected as described previously (Marchisella et al., 2021) and stored at -80°C until further processing.

2.5 Corticosterone and Adrenocorticotropic hormone's plasma levels analysis

Trunk blood was collected into plastic tubes (10ml), left for clotting (30min at room temperature) and centrifuged to obtain plasma. Corticosterone's levels were detected through the enzyme-linked immunosorbent assay (ELISA) technique, by using the *Corticosterone (Human, Rat, Mouse) ELISA* (RE52211, TECAN, IBL International GmbH). Adrenocorticotropic hormone's levels were detected through *ELISA technique, by using Mouse/Rat ACTH SimpleStep ELISA® Kit* (adrenocorticotropic hormone) (ab263880, Abcam). Both procedures were done according to the manufacturers protocol.

2.6 qRT-PCR Analysis of mRNA Levels

For gene expression analyses, total RNA was isolated from the different areas by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l., Segrate, Italy), and the mRNA levels of different genes were detected by using reverse transcriptase real-time polymerase chain reaction (qRT-PCR), as previously described (Luoni et al., 2014; Begni et al., 2021). Relative target gene expression was calculated according to the Δ(ΔCt) method. For some genes TaqMan gene expression assays were purchased from Eurofins MWG-Operon (Germany) and are summarized in Table 1, while for other genes both probe and primer were purchased from Life Technologies (Monza, Italy) (Table 2).

2.7 RNAscope in situ hybridization assay

Fixed-frozen brains were cut with a microtome (thickness: 30 μm). One out of six coronal sections were mounted on positively charged microscopic glass slides (Thermo Fisher Scientific). *Bdnf* (Rn-317541) was used as target RNA probe. Rat-Peptidylprolyl Isomerase B (Rn-313921) was used as a positive control probe for *Bdnf* expression. All probes were used on separated brain slices. All staining steps were performed following the RNAscope multiplex protocol, and immunofluorescence was performed immediately after in situ hybridization as described previously (Marchisella et al., 2021). Primary antibodies were used as follows: 1:1000 rabbit anti-parvalbumin (#NB120-1142, Novus Bio) and 1:100 rabbit anti-calcium calmodulin kinase II (M A1048, Thermo Fischer). Detection was obtained with Alexa dye-conjugated antibodies 1:500. Sections were cover-slipped with fluorescent mounting medium ProLong Gold Antifade reagent (Thermo Fisher Scientific) containing DAPI for nuclei visualization.

Images were acquired with an LSM-900 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a 10x objective to navigate the area of interest and 60x to snap the image. Images shown are from maximum projections of 10-12 z-planes. *Bdnf* images, signal detection and unbiased quantification were performed using ImageJ (National Institute of Health) software.

2.8 Statistical analysis

The data of sucrose intake were analyzed (GraphPad Prism 9) by analyses of variance (ANOVA) with three between-subject factors (stress/control, drug treatments and successive sucrose tests). The Tukey's test comparison was used for post-hoc comparisons of means. Molecular and RNAscope data were analyzed with 2-way ANOVA with stress (CMS sham/AS vs CT sham/AS) and treatment (Vehicle vs Lurasidone) as independent factors. Tukey's test comparison was used for post-hoc comparisons of means for qRT-PCR data and Sidak's for RnaScope analysis.

2.8.1 Integrated analysis of brain activation

For the integrated analysis of brain activation, statistical analyses were performed using R, including packages: Hmisc, rcorr, corrplot, qgraph. Using the z-score of Immediately Early Genes (IEGs: Npas4, Zif-268, Cfos and Arc) as "activity-brain region indicator", correlation matrices for regional z-score of IEGs were computed using nonparametric Sperman Rank-Order correlations. To compare z-score-based activity networks between each group without acute stress and its counterpart with acute stress, the correlation coefficients were subjected to z-transformation to generate z-scores, by using the following formula:

$$z - score(rho) = 0.5 * \{[ln(1 + rho)] - [ln(1 - rho)]\}$$

where rho is the Sperman correlation's coefficient.

The differences between z-scores were calculated as Z-observed (Z_{obs}), with the following formula:

$$Z_{obs} = \frac{(Z_1 - Z_2)}{\sqrt{[(\frac{1}{n_1 - 3}) + (\frac{1}{n_2 - 3})]}}$$

Z-observed were plotted using the R package "qgraph" which represents an increase or decrease in correlation (color) and intensity of the difference (line thickness).

3. RESULTS

3.1 An anhedonic phenotype induced by CMS paradigm exposure in rats is normalized by chronic lurasidone treatment

Exposure to CMS induced an anhedonic phenotype, as indicated by the decrease in sucrose consumption. Indeed, after two weeks of stress, CMS animals showed a significant reduction in sucrose intake (-46% after two weeks, $p<0.0001$). The animals were then randomized to receive vehicle (VEH) or lurasidone (LUR) at 3 mg/kg for five weeks while continuing CMS exposure. As shown in Fig. 1, CMS rats treated with vehicle maintained the anhedonic phenotype throughout the five-week period. Conversely, in accordance with previous work performed in our laboratory (Luoni et al., 2014), lurasidone treatment produced a gradual improvement of the anhedonic phenotype of CMS rats until complete normalization.

3.2 CMS exposure alters the peripheral response to an acute stress challenge

The main goal of our study was to investigate the responsiveness of CMS rats to an acute stress challenge (AS) and to establish the potential of lurasidone on such mechanisms. First, we analyzed the plasma levels of ACTH and CORT, as a measure of the HPA axis responsiveness to the acute challenge. As shown in Fig. 2a, we found a significant effect of stress on ACTH levels ($F=5.141$; $p<0.01$). Although there was no significant "stress x treatment" interaction, exploratory Tukey's post-hoc comparison showed a significant increase of ACTH levels in CMS animals after AS ($p<0.05$), which was not present in control animals. Conversely, while CMS animals treated with lurasidone showed increased ACTH levels after the AS, this effect did not reach statistical significance. Regarding corticosterone levels (Fig. 2b), we found a significant effect of stress ($F=7.405$; $p<0.001$) and treatment ($F=17.92$; $p<0.0001$). Based on exploratory Tukey's post-hoc analysis, control animals exposed to AS showed a significant elevation of corticosterone levels ($p<0.05$ vs stressed CTRL), an effect that was similar in CMS rats treated with vehicle. Moreover, CMS rats treated with lurasidone showed only a

slight, and non-significant elevation of plasma corticosterone levels when exposed to the acute challenge.

3.3 Modulation of activity-dependent genes in the prefrontal cortex following acute stress exposure

We next wanted to establish if CMS exposure may alter the functional activation of specific brain regions after the acute challenge. First, we focused on the PFC that represents a key brain region in stress-related disorders and that is important in coordinating the response to environmental challenges. To achieve this purpose, we analyzed the expression of different activity regulated genes, known as *immediately early genes* (*IEGs: c-Fos. Arc, Zif-268* and *Npas-4*), which represent a proxy for the activation of different cellular phenotypes within a given brain region. As shown in Fig. 3, all activity regulated genes showed a similar profile of modulation, although the magnitude of the observed changes was different. In fact, while we did not observe any significant change in the expression of all *IEGs* under resting conditions, the exposure to the acute stress produces a significant up-regulation of their mRNA levels in control animals as well as in CMS rats, regardless of the treatment (see Table 3, for the statistical analyses). These results suggest that the 'overall' activation of the PFC in response to the acute challenge is not altered as a consequence of CMS exposure. This was confirmed by the analyses of the 'global' activation by using the Z score approach, as shown in Fig. 3e, that indicated a similar increase in all the experimental groups exposed to the acute stress challenge.

3.4 Modulation of Bdnf expression in the prefrontal cortex following acute stress exposure

Although the overall activation of the PFC in response to the acute challenge was not altered in CMS rats, we decided to investigate the expression of *Bdnf*, a hallmark of plasticity that may serve as a link between neuronal activation and downstream mechanisms coordinating the response to the external challenge. As shown in Fig. 4, we found a significant effect of stress ($F=28.37$; $p<0.0001$) and a significant "stress x treatment" interaction ($F=2.852$; $p<0.05$) in the modulation of total Bdnf expression. Exposure to AS produced a significant up-regulation of total *Bdnf* mRNA levels in control animals ($p<0.0001$ vs CONT VEH without AS), an effect that was not observed in CMS rats exposed to AS. Interestingly, chronic lurasidone treatment was able to restore the modulation of *Bdnf* following the AS, with a significant upregulation of its mRNA levels ($p<0.001$ vs CMS LUR without AS). A similar pattern of changes was also observed for the pool of *Bdnf* transcripts with *long 3' UTR*, which is known to undergo dendritic targeting, as well as for *Bdnf exon IV*, which represent the main activity dependent transcript of the neurotrophin, and for *Bdnf isoform IX.* Conversely, the expression of *Bdnf exon I* and *exon VI* (Fig. 4c,e) did not show major changes in response to the acute challenge, suggesting that the altered *Bdnf* modulation following AS and the 'restoring' properties of lurasidone may be due to specific transcriptional mechanisms governing the neurotrophin expression (see Table 3, for details on the statistical analyses).

Next, we used RNAscope to better characterize the differential modulation of *Bdnf* expression in the PFC following AS exposure. More specifically, we investigated its mRNA levels in Parvalbumin (PV), as compared to Calcium Calmodulin Kinase II (CAMKII) positive neurons, which represent the major inhibitory and excitatory neurons in this brain region. Representative images of the analyses are shown in figure 5a. Regarding the expression of total *Bdnf* within PV+ interneurons, the 2-way ANOVA analysis (Fig. 5b) showed a significant main effect of treatment ($F(2, 390) = 16{,}87$; $p<0.0001$) and a significant "stress x treatment" interaction ($F(2, 390) = 6{,}168$; $p<0.01$). Sidak's post hoc analysis revealed that exposure of control animals to the AS produced a statistically significant increase of *Bdnf* puncta in PV^+ neurons ($p<0.0001$), an effect that was not observed in CMS rats, regardless of the treatment (see Fig. 5b).

When analyzing *Bdnf* mRNA levels in CAMKII$^+$ pyramidal neurons, the 2-way ANOVA analysis showed a significant treatment effect ($F(2, 159) = 3.351$; $p<0.05$) as well as a significant "stress x treatment" interaction ($F(2, 159) = 9{,}479$; $p<0.0001$) (Fig. 5b). While we did not observe any significant change in control animals exposed to the AS, Sidak's post hoc analysis showed that exposure of CMS rats to the AS reduced *Bdnf* mRNA levels in CAMKII$^+$ neurons ($p<0.001$), whereas a significant increase was observed when CMS rats treated with LUR were exposed to the acute challenge ($p<0.001$).

3.5 Modulation of glucocorticoid-related genes in the Prefrontal Cortex after acute stress exposure

In order to establish if the altered responsiveness of the PFC to the acute challenge in CMS rats was the consequence of a glucocorticoid-related mechanism, we analyzed the mRNA levels of different genes known to depend upon glucocorticoid receptor activation, including *Nr3c1*, *Fkbp5*, *Sgk1*, *Dusp1*, *Gadd45β*, *Nr4a1* (see Supplementary, fig.1). For *Nr3c1* we found a significant interaction "stress x treatment" effect ($F=3.080$; $p<0.05$), although Tukey's post-hoc comparison didn't show any relevant difference among the experimental groups. While for *Fkbp5* there was no significant main effect, the 2-way ANOVA showed significant effects of stress ($F=50.48$; $p<0.0001$), treatment ($F=4.755$; $p<0.05$) as well as "stress x treatment" interaction ($F=3.779$; $p<0.05$) for Sgk1 mRNA levels . Tukey's post-hoc comparison showed a significant upregulation of *Sgk1*'s expression because of AS exposure, in all experimental groups (+49% CTRL VEH sham vs AS; +40% CMS VEH sham vs AS; +79% CMS LUR sham vs AS). Concerning *Dusp1*, we found a significant main stress effect ($F=67.74$; $p<0.0001$), whereas *Gadd45β* we found a significant main effect of stress ($F=35.54$; $p<0.0001$) and treatment ($F=13.25$; $p<0.001$). With respect to *Nr4a1,* the 2-way ANOVA revealed a significant main stress effect ($F=79.42$; $p<0.0001$) and a significant "stress x treatment" interaction ($F=3.597$, $p<0.05$); Tukey's post-hoc comparison revealed a significant upregulation of *Nr4a1* expression following the acute stress exposure, in all experimental groups, with some differences in terms of magnitude of such effect (+122% CTRL VEH sham vs AS; +163% CMS VEH sham vs AS; +89% CMS LUR sham vs AS).

3.6 Modulation of activity-dependent genes in other brain regions after acute stress exposure

Considering that exposure to stress may produce specific effects on different brain regions (Malhi et Mann, 2018), we examined the degree of activation of other brain structures, including ventral and dorsal hippocampi, amygdala and nucleus accumbens, which are also known to play an important role in mood disorders as well as in stress-related conditions. In broad terms, we found that the expression of *Arc*, *Zif-268*, *Npas4* and *c-Fos* was significantly elevated in all these areas because of AS exposure (see Supplementary fig. 2, 3, 4 and 5), with limited differences between the experimental groups (please refer to Supplementary Table 1 for further details and to Table 3 for the statistical analysis of each gene). Based on the expression profile of these different *IEGs*, we next calculated the Z-activation score for each brain region. As shown in figure 6a, in the Ventral Hippocampus (VH) we found a main effect of stress ($F=37.83$, $p<0.0001$) and treatment (6.824; $p<0.05$): AS exposure produced an increase in the activation of this region in all experimental groups, regardless of CMS or treatment. Regarding Amygdala (AMY), 2-way ANOVA revealed a main effect of stress ($F=149.8$; $p<0.0001$) and a significant "stress x treatment" interaction ($F=2.780$; $p<0.05$) (fig. 6b). Tukey's post-hoc comparison showed a significant increase of the z-score after the acute challenge in CTRL, CMS VEH as well as CMS LUR ($p<0.0001$ vs their counterparts without AS), with a significant difference between CMS VEH with AS and CMS LUR with AS ($p<0.05$). Considering the Dorsal Hippocampus (DH), we found a main effect of stress ($F=28.13$; $p<0.0001$) and treatment ($F=4.452$; $p<0.05$), with a significant increase of the activation after the acute stress in all the experimental groups, regardless of CMS and lurasidone

treatment (fig. 6c). Such pattern was also found in the Nucleus Accumbens (NAc) with a main effect of stress ($F=58.42$; $p<0.0001$) and treatment ($F=4.956$; $p<0.05$) (fig. 6d).

3.7 Investigation of overall brain response to an acute stress challenge: an integrated approach

Since the stress response is a complex event that recruits different brain regions through specific circuits, we used the z-score of the *IEGs* as a marker for brain activity to investigate potential changes in the interconnectivity between brain regions, following the exposure to the AS. Using this strategy, we aimed to achieve a global picture of the effects that the AS challenge may produce in terms of simultaneous and/or reciprocal modulation of different brain regions under physiological or pathologic (CMS) conditions.

We used Spearman correlation matrices of IEGs z-score values (based on $\Delta(\Delta Ct)$ of *IEGs* across all investigated brain regions (Supplementary data, fig. 7), to identify the coactivation between two different areas. The correlation matrices between different areas were computed for all experimental groups, exposed or not to AS. Considering that our main purpose was to evaluate the acute stress response among experimental groups, we compared each paired group exposed to AS to the same pair without AS, by calculating the z-observed (Z_{obs}) values.

When comparing control rats with or without AS (Fig. 7a), we can observe how AS exposure produced a wide reduction in the degree of co-activation between all areas, as shown by the red lines. This indicates that the relationship of activation of the first group (control group exposed to AS) is lower when compared to the control group not exposed to AS. As an example, control rats showed a marked reduction of the degree of co-activation of couples PFC/AMY ($Z_{obs}=-3.32$), PFC/VH ($Z_{obs}=-3.5$); NAc/AMY ($Z_{obs}=-3.64$), Nac/VH ($Z_{obs}=-2.2$), AMY/VH ($Z_{obs}=-2.29$).

On the other hand, when comparing CMS rats with or without AS (Fig. 7b), we found the AS response was weaker as compared to control rats. For example, while CMS rats still showed a reduction in the degree of co-activation between AMY and NAc after AS, this effect is much smaller (Z_{obs} is -0.27) than in control rats ($Z_{obs}=-3.64$). In some cases, the pattern of changes following AS was completely different. Indeed, CMS rats exposed to AS show increased co-activation of some areas' pair, such as PFC/VH ($Z_{obs}=+0.09$) or VH/DH ($Z_{obs}=+0.86$), an effect that was opposite with respect to control animals. Lastly, the analysis of CMS rats chronically treated with lurasidone (Fig.7c) showed a mixed pattern of changes. To some extend the AS response of CMS rats treated with lurasidone is closer to control rats, as compared to CMS rats treated with vehicle. As an example, the reduction in the degree of co-activation between NAc and AMY was largely restored after chronic lurasidone treatment ($Z_{obs}=-1.52$). Lurasidone treatment also restored the reduction of co-activation of PFC/NAc ($Z_{obs}=-0.7$) and AMY/VH ($Z_{obs}=-0.82$). Nevertheless, there are some differences in CMS rats treated with lurasidone when compared to controls, following AS exposure, as shown for PFC/VH or PFC/AMY. Considering this more global view of the AS response, our findings suggest that CMS exposure produces significant alterations in the response to the acute challenge, while lurasidone treatment can restore, largely but not entirely, such AS response.

4. DISCUSSION

This study examined the responsiveness to an acute stress as a proxy for emotional control in order to establish potential alterations as a consequence of chronic stress exposure and how such changes could be modulated by lurasidone treatment.

At the behavioral level, in line with our previous work (Luoni et al., 2014; Calabrese et al., 2016; Rossetti et al., 2016; Rossetti et al., 2018; Calabrese et al., 2020), we show that CMS produces an anhedonic phenotype, which was normalized by chronic lurasidone administration.

Although limited to a single time point, the investigation of the HPA axis responsiveness following the acute challenge suggests that CMS rats may have a protracted elevation of plasma ACTH levels, although the overall response in terms of CORT levels does not differ from control animals. Interestingly, chronic treatment of CMS animals with lurasidone appears to alter this pattern, buffering the elevation of ACTH and, more prominently, of CORT levels.

The most relevant finding of our study is represented by the differential ability of the PFC from CMS rats in responding to the acute challenge. Indeed, based on the analyses of activity-regulated genes, we found that the PFC is similarly recruited following the acute stress, since we observed a significant upregulation of IEGs' expression in all the experimental groups. However, we show that CMS exposure prevents the acute up-regulation of total Bdnf expression within the PFC following an AS. The changes of total *Bdnf* mRNA levels appear to be sustained by the modulation of some isoforms, including the activity-dependent *exon IV* and *IX*, as well as the pool of transcripts with *long 3'UTR* that may undergo dendritic targeting (Vicario et al., 2015; An et al., 2008). Interestingly, the modulation of Bdnf following the acute challenge activity was normalized by chronic lurasidone treatment, although this may occur in different cellular phenotypes, when compared to control rats. Indeed, using RNAScope we found that CTRL rats exposed to AS show an up-regulation of Bdnf mRNA levels within Parvalbumin positive interneurons, whereas lurasidone treated CMS rats show a significant upregulation of in pyramidal neurons. We believe that the differential modulation of Bdnf expression following the acute stress is not strictly due to a glucocorticoid-dependent mechanism, since genes whose transcription is dependent on glucocorticoid receptor activation are up regulated in all experimental groups after AS. Although speculative, the impaired modulation of Bdnf transcription may be the consequence of the altered release of different neurotransmitters that participate in the functional regulation and responsiveness of the PFC and in the modulation of Bdnf expression (Mora et al., 2011). Moreover, our results suggest that chronic antipsychotic treatment may alter the Excitatory/Inhibitory balance within the PFC through a recruitment of pyramidal glutamatergic neurons, which may have a differential impact on downstream circuits, also considering that *Bdnf* exerts an acute effect on synaptic transmission and plasticity, by increasing the presynaptic release of Glutamate as well as its post-synaptic response (Poo, 2001; Lu, 2003).

When investigating other brain areas that may contribute to the AS response, we found a significant activation that was largely similar among experimental groups, regardless CMS exposure or pharmacological treatment. However, when an integrated approach was used to investigate the reciprocal activation of key brain regions, we found significant differences in the co-activation state between control and CMS rats, under resting conditions or following AS. This suggests that the chronic stressful experience affects different brain circuits that, speculatively, may contribute to the manifestation of specific psychopathologic domain as well as to an enhanced susceptibility under challenging conditions. Interestingly, lurasidone was able to normalize some of these alterations, in line with its ability to improve different functional domains in CMS rats (Luoni et al., 2014; Rossetti et al., 2016; Calabrese et al., 2020).

Although further studies are needed to better characterize the neuronal pathways whose activation may be altered because of chronic stress exposure, our results suggest that exposure to AS is able to unmask functional deficits that can be relevant to cope under the adverse condition. Indeed, it has been demonstrated that increased glutamatergic input from VH to NAcc is associated with stress vulnerability, whereas its reduction may lead to resiliency (Bagot at al., 2015). Conversely, increased glutamatergic transmission from medial PFC and AMY to NAcc is linked to stress resiliency, while its reduction may lead to a vulnerable phenotype (Bagot et al., 2015).

These alterations represent an important target for pharmacological intervention. Our results using the antipsychotic drug lurasidone suggest that such intervention not necessarily restore the 'original' condition, found in control animals, but lead to the activation of alternative mechanisms and pathways that may ultimately contribute to functional improvement and enhanced resilience. Such activity may depend upon the ability of lurasidone to interact and regulate different neurotransmitter receptors (Tarazi & Riva). Future studies will be required to identify the specific receptors or intracellular mechanisms that may alter the functional activity of the circuits that are involved in stress responsiveness.

In summary, the present study provides new insights on the ability of lurasidone treatment in modulating stress-related dysfunction. Indeed, lurasidone is not only able to promote adaptive mechanisms that are crucial for the ability to improve specific psychopathological domains, but regulates specific circuits and mechanisms, which will ultimately contribute to boost resilience under stressful challenges.

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FIGURES AND TABLES' LEGENDS

***Table 1*. Sequences of Forward and Reverse Primers and Probes Used in qRT-PCR Analyses.** Abbreviations: qRT-PCR, reverse-transcriptase real-time polymerase chain reaction. Purchased from Eurofins MWG Operon (Germany).

***Table 2*. assays ID of probe used in qRT-PCR Analyses.** Abbreviations: qRT-PCR, reverse-transcriptase real-time polymerase chain reaction. Purchased from Life Technologies (Monza, Italy).

***Table 3.* Summary of the statistical results with the main effects of Stress, Treatment and the "Stress x Treatment" interaction, as derived from 2-way ANOVA analyses of gene expression data.**

Fig. 1 ***Effect of Chronic Mild Stress (CMS) exposure on sucrose consumption as a measure of anhedonia: modulation by sub-chronic Lurasidone Treatment.***
The data represent the average of sucrose consumption for each group expressed in grams (g) ± SEM and are the average of n=10. A three-way ANOVA shows a main effect of time (Weeks) ($F=11.09$; $p<0.0001$), CMS ($F=582.5$; $p<0.0001$), Treatment ($F=33.97$; $p<0.0001$) as well as a significant interaction for "Weeks x Chronic stress x Treatment" ($F=5.038$; $p<0.0001$), "Weeks x Chronic stress" ($F=12.87$; $p<0.0001$), and "Weeks x Treatment" ($F=3.819$; $p<0.001$); significant "Chronic stress x Treatment" interaction effect ($F=34.87$; $p<0.0001$) (3-way ANOVA).. $^{****}p<0.0001$ vs CONT VEH; $^{\#\#}p<0.01$ vs CMS VEH; $^{\#\#\#\#}p<0.0001$ vs CMS VEH.

Fig.2 Effects of Acute stress on Corticosterone and Adrenocorticotropic hormone (ACTH) plasma levels in CMS rats: modulation by lurasidone treatment.
The data represent the average of Corticosterone and ACTH plasma levels for each experimental group (n=6-10) expressed in ng/ml ± SEM. For corticosterone, the 2-way ANOVA shows a main effect of stress ($F=7.405$; $p<0.001$) and treatment ($F=17.92$; $p<0.0001$). For ACTH, the 2-way ANOVA shows a main effect of stress ($F=5.141$; $p<0.01$).

Fig. 3 Effects of Acute stress on Arc, Zif, Npas4 and Cfos expression in the prefrontal cortex (PFC) of chronically stressed (CMS) rats: modulation by lurasidone treatment.

The mRNA levels of *Arc*, *Zif-268*, *Npas4* and *c-Fos* were measured in the PFC of unstressed control (CONT) and CMS rats treated with vehicle or Lurasidone, under resting conditions or following an acute restraint stress (AS) (n=6-10). The data are expressed as a percentage of unstressed control animals treated with vehicle [unstressed CONT VEH]) (set at 100%). The z-activation score is also shown, the data being expressed as mean of z-scores of all IEGs, based on mean (set at 0) and SD of unstressed control animals treated with vehicle [unstressed CONT VEH]). Error bars in all graphs represent SEM. The complete statistical analysis is reported in **Table 3**. Tukey's post-hoc comparison: ***Arc***: $^{****}p<0.0001$ vs CONT VEH without AS; $^{\$\$\$\$}p<0.0001$ vs CMS VEH without AS; $^{@@@}p<0.001$ vs CMS LUR without AS; ***c-Fos***: $^{****}p<0.0001$ vs CONT VEH without AS; $^{\$\$\$\$}p<0.0001$ vs CMS VEH without AS; $^{@@@@}p<0.0001$ vs CMS LUR without AS).

Fig. 4 Effect of Acute stress on Bdnf expression in the prefrontal cortex (PFC) of chronically stressed (CMS) rats: modulation by lurasidone treatment.

The mRNA levels of total Bdnf as well as of its different transcripts (long 3'-UTR, exon I, IV, VI and IX) were measured in the PFC of unstressed control (CONT) and CMS rats treated with vehicle or Lurasidone, under resting conditions or following an acute restraint stress (AS). The data represent the average of n=6-10 animals and are expressed as a percentage of unstressed control animals treated with vehicle [unstressed CONT VEH]) (set at 100%). The complete statistical analysis is reported in **Table 3**. Tukey's post-hoc comparison: ***Total Bdnf***: $^{****}p<0.0001$ vs CONT VEH; $^{**}p<0.01$ vs CMS VEH with AS; $^{###}p<0.001$ vs CMS VEH; ***3'-UTR Long Bdnf***: $^{*}p<0.05$ vs CONT VEH; $^{###}p<0.001$ vs CMS VEH.

Fig. 5 RNAscope analysis of Bdnf mRNA levels within specific neuronal sub-population in the prefrontal cortex of chronically stressed (CMS) rats: modulation by lurasidone treatment.

(5a) Representative images of RNAscope analysis. DAPI signal is represented by the blue; Parvulbimin signal is represented by the green; CamKII signal is represented by the reddish-purple; red dots represent the mRNA *Bdnf* signal. Scale bars of 10 μm are also reported. (5b) In situ hybridization analysis of *Bdnf* gene expression in parvalbumin and CAMKII positive cells. Data represent mean ± SEM.

Bdnf in PV^{+} cells: 2-way ANOVA main effects: treatment ($F(2, 390) = 16,87$; $p<0.0001$) and "stress x treatment" interaction ($F(2, 390) = 6,168$; $p<0.01$). $^{*}p<0.05$ vs CONT VEH without AS; $^{****}p<0.0001$ vs CONT VEH with AS; $^{####}p<0.0001$ vs CONT VEH with AS (Sidak's post-hoc analysis).

Bdnf in $CAMKII^{+}$ cells: 2-way ANOVA main effects: treatment ($F(2, 159) = 3.351$; $p<0.05$) and "stress x treatment" interaction ($F(2, 159) = 9,479$; $p<0.0001$); $^{***}p<0.001$ vs CMS VEH without AS, $^{****}p<0.0001$ vs CMS VEH without AS., $^{###}p<0.001$ vs CMS LUR without AS (Sidak's post-hoc analysis).

Fig. 6 Effects of Acute stress on z-Score of IEGs in Ventral and Dorsal Hippocampi, Amygdala and Nucleus Accumbens of chronically stressed (CMS) rats: modulation by lurasidone treatment.

The z-activation score based on the mRNA levels of *Arc*, *Zif-268*, *Npas4* and *c-Fos* in different brain regions is reported. The data are expressed as mean of z-scores of all IEGs, based on mean (set at 0) and SD of unstressed control animals treated with vehicle [unstressed CONT VEH]) (n=6-10). Error bars in all graphs represent SEM. The complete statistical analysis is reported in **Table 3**. Tukey's post-hoc comparison: **Amygdala**: $^{****}p<0.0001$ vs CONT VEH without AS; $^{\$\$\$\$}p<0.0001$ vs CMS VEH without AS; $^{@@@@}p<0.0001$ vs CMS LUR without AS; $^{\#}p<0.05$ vs CMS VEH with AS.

Fig. 7 Comparison of correlated brain activity among groups (z-score of IEGs among all brain regions).

The z-scores were used to calculate the z-observed (Z_{obs}), that indicates the difference between two z-scores. The difference between two z-scores could be: Positive, indicating that the first group had a stronger or more positive relationship of co-activation between the two considered regions than the second group, illustrated by blue lines; Negative, indicating that the first group had a weaker or less positive relationship of co-activation between the two considered regions than the second group, illustrated by red lines.

Abbreviations: CTRL= controls, CMS= chronic mild stress, VEH= vehicle; LUR= lurasidone, AS= acute stress, SHAM= no acute stress. PFC= Prefrontal Cortex; DH= Dorsal Hippocampus; VH= Ventral Hippocampus; AMY= Amygdala and NAc= Nucleus Accumbens. Color code= Red means Z_{obs} negative values; blue means Z_{obs} positive values.

Gene	Forward Primer	Reverse Primer	Probe
Bdnf Total	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Bdnf isoform IX	GCTGTCCCCGAGAAAGAAAGT	GTCATCACTCTTCTCACCTGG	CTGTTCTGTGTCTGTCTCTGCTTCCT
Bdnf isoform I	GGGAGACAAGATTTTAAGACACTG	GTCATCACTCTTCTCACCTGG	TTGTGGCTTTGCTGTCCTGGAGA
c-Fos	TCCTTACGGACTCCCCAC	CTCCGTTTCTCTTCCTCTTCAG	TGCTCTACTTTGCCCCTTCTGCC
Npas4	TCATTGACCCTGCTGACCAT	AAGCACCAGTTTGTTGCCTG	TGATCGCCTTTTCCGTTGTC
Sgk1	GACTACATTAATGGCGGAGAGC	AGGGAGTGCAGATAACCCAAG	TGCTCGCTTCTACGCAGC
Fkbp5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAAG	TGTCCATCTCCCAGGATTCTTTGGC
Dusp1	TGTGCCTGACAGTGCAGAAT	ATCTTTCCGGGAAGCATGGT	ATCCTGTCCTTCCTGTACCT

Gene	assay ID
long 3'-UTR (untranslated region) *Bdnf*	Rn02531967_s1
Bdnf* isoform *IV	Rn01484927_m1
Bdnf* isoform *VI	Rn01484928_m1
Arc	Rn00571208_g1
Zif-268	Rn00561138_m1
Nr3c1	Rn00561369_m1
Nr4a1	Rn01533237_m1
Gadd45β	Rn01452530_g1

Brain Area	*Gene*	STRESS	TREATMENT	STRESS X TREATMENT
PREFRONTAL CORTEX	*Arc*	F=55.16; p<0.0001	---	F=4.140; p<0.01
	Zif-268	F=60.84; p<0.0001	---	---
	Npas4	F=15.03; p<0.0001	---	---
	c-Fos	F=105.5; p<0.0001	F=6.010; p<0.05	F=2.813; p<0.05
	z-Score IEGs	F=59.60; p<0.0001	---	---
	Total Bdnf	F=28.37; p<0.0001	---	F=2.852; p<0.05
	3' UTR Long Bdnf	F=23.09; p<0.0001	---	F=3.120; p<0.05
	Bdnf isoform IV	F=25.40; p<0.0001	---	---
	Bdnf isoform IX	F=17.00; p<0.0001	---	---
	Bdnf isoform VI	F=5.515; p<0.01	---	---
	Bdnf isoform I	---	F=8.241; p<0.01	---
	Nr3c1	---	---	F=3.080; p<0.05
	Sgk1	F=50.48; p<0.0001	F=4.755; p<0.05	F=3.779; p<0.05
	Fkbp5	---	---	---
	Dusp1	F=67.74; p<0.0001	---	---
	Gadd45β	F=35.54; p<0.0001	F=13.25; p<0.001	---
	Nr4a1	F=79.42; p<0.0001	---	F=3.597, p<0.05
VENTRAL HIPPOCAMPUS	*Arc*	F=39.12; p<0.0001	F=19.92; p<0.0001	F=7.066; p<0.001
	Zif-268	F=17.24; p<0.0001	---	F=3.060; p<0.05
	Npas4	F=6.001; p<0.01	---	---
	c-Fos	F=44.20; p<0.0001	---	---
	z-Score IEGs	F=37.83; p<0.0001	F=6.824; p<0.05	---
AMYGDALA	*Arc*	F=108.3; p<0.0001	---	F=5.645; p<0.01
	Zif-268	F=70.69; p<0.0001	F=6.058; p<0.05	---
	Npas4	F=49.23; p<0.0001	---	---
	c-Fos	F=136.6; p<0.0001	F=4.452; p<0.05	F=5.964; p<0.01

	z-Score IEGs	F=149.8; p<0.0001	---	F=2.780; p<0.05
DORSAL HIPPOCAMPUS	*Arc*	F=12.59; p<0.0001	---	---
	Zif-268	F=29.46; p<0.0001	F=6.092; p<0.05	---
	Npas4	F=3.239; p<0.05	---	---
	c-Fos	F=18.41; p<0.0001	---	---
	z-Score IEGs	F=28.13; p<0.0001	F=4.452; p<0.05	---
NUCELUS ACCUMBENS	*Arc*	F=63.97; p<0.0001	---	---
	Zif-268	F=50.51; p<0.0001	F=6.038; p<0.05	---
	Npas4	F=21.62; p<0.0001	---	---
	c-Fos	F=39.31; p<0.0001	---	---
	z-Score IEGs	F=58.42; p<0.0001	F=4.956; p<0.05	---

FIG1 TIF

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FIG2 TIF

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FIG3 TIF

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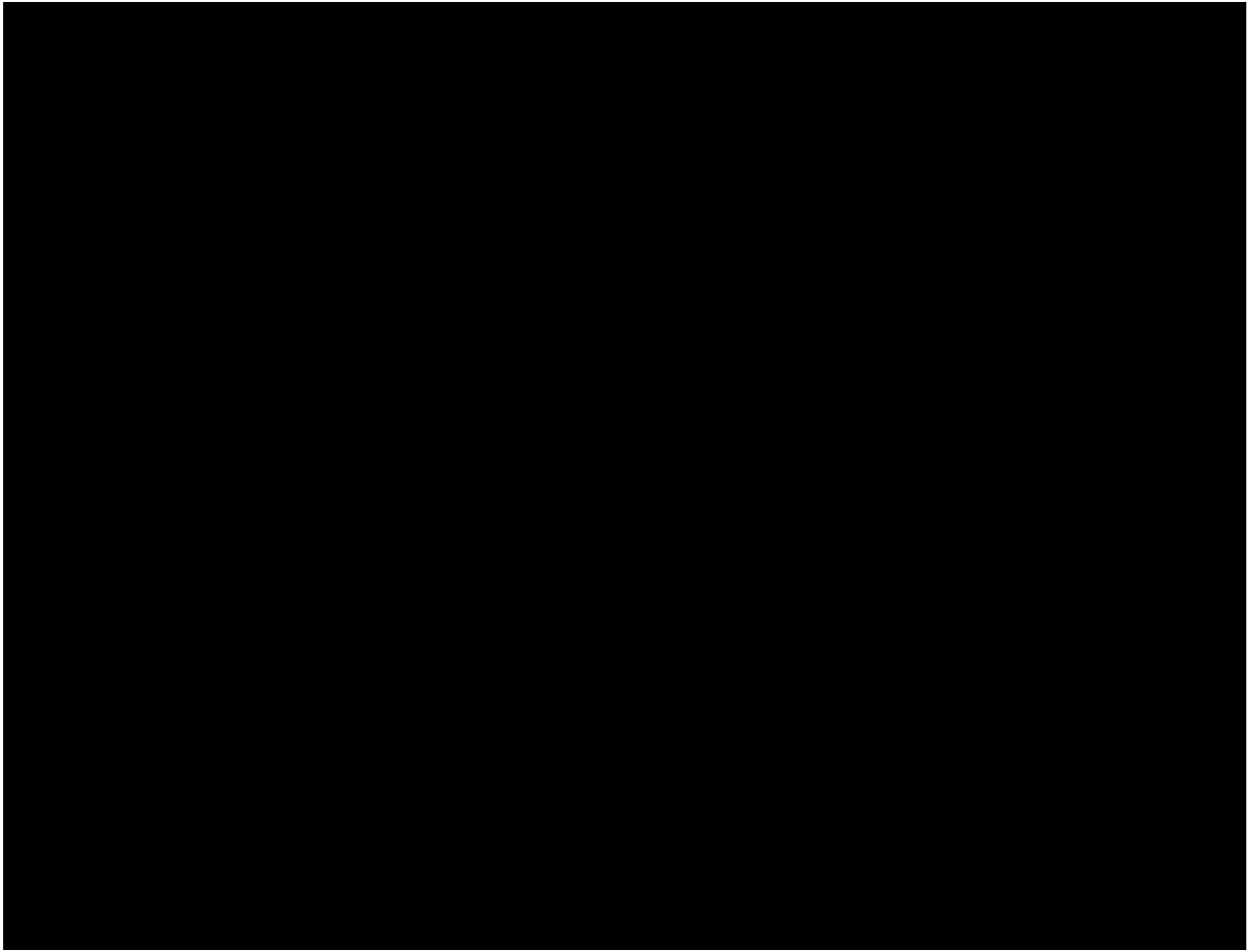

FIG4 TIF

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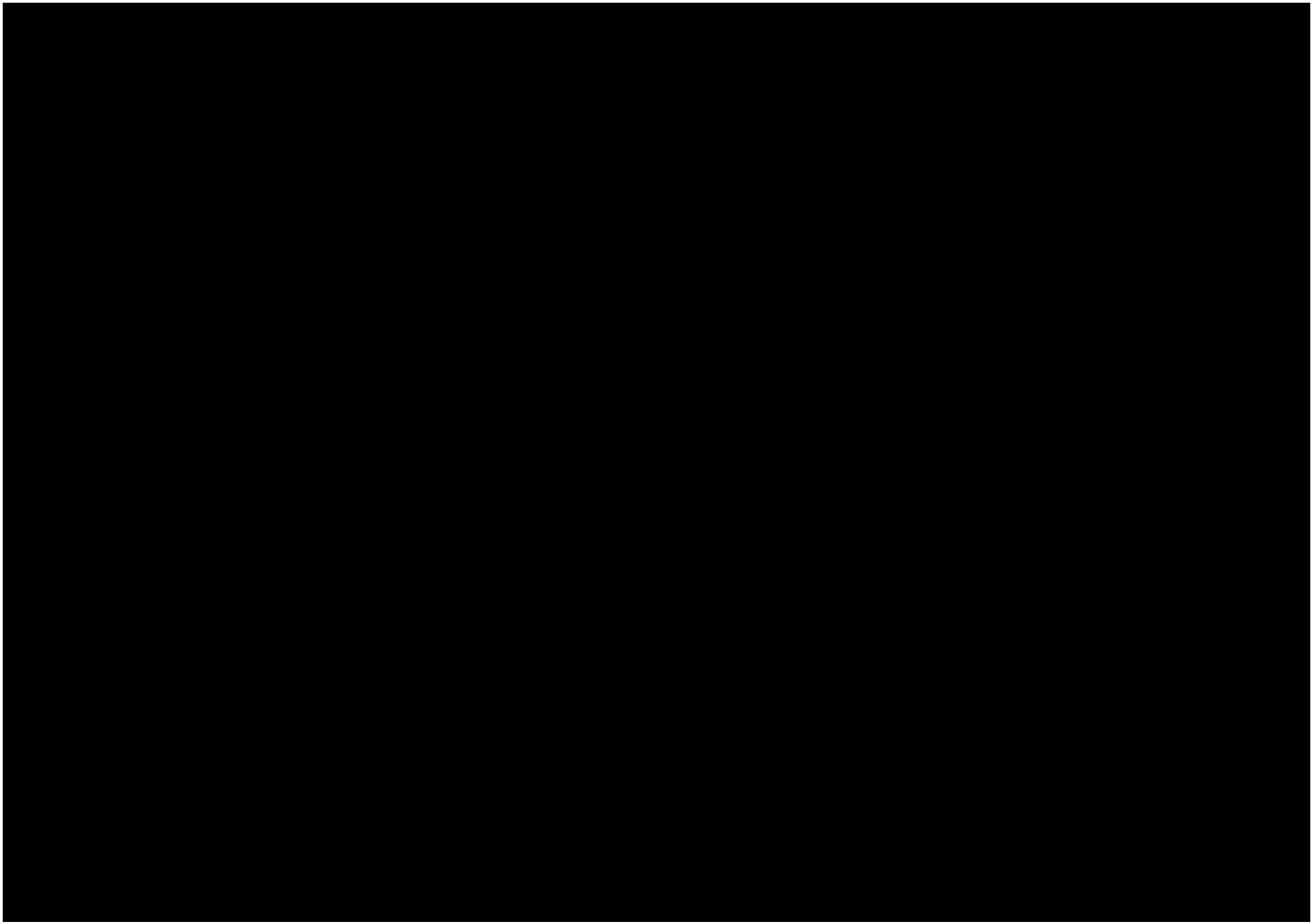

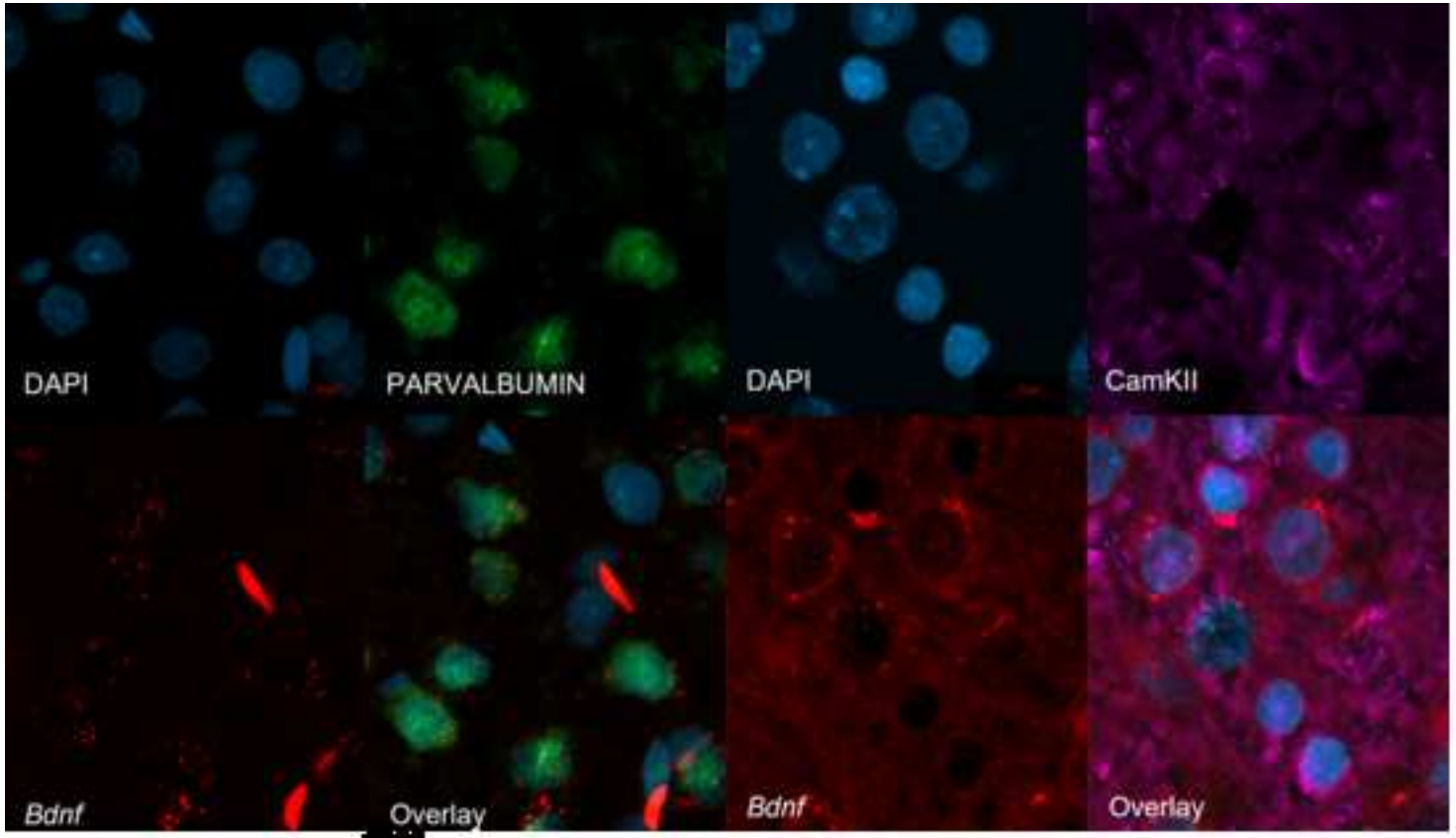

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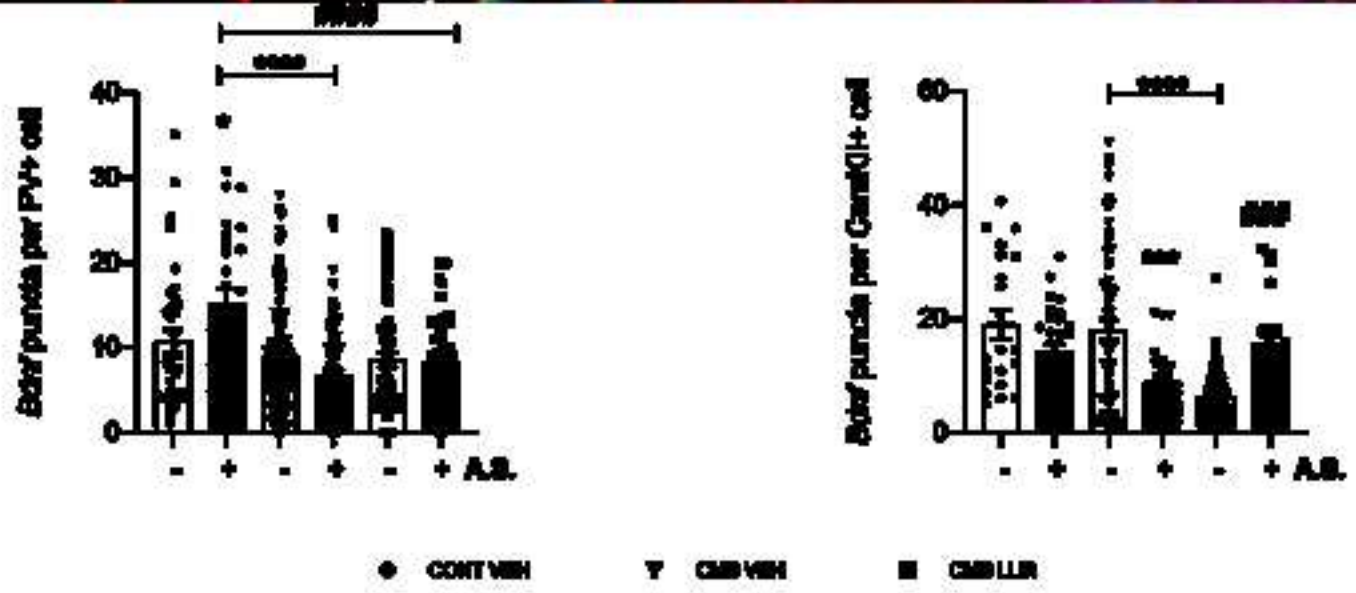
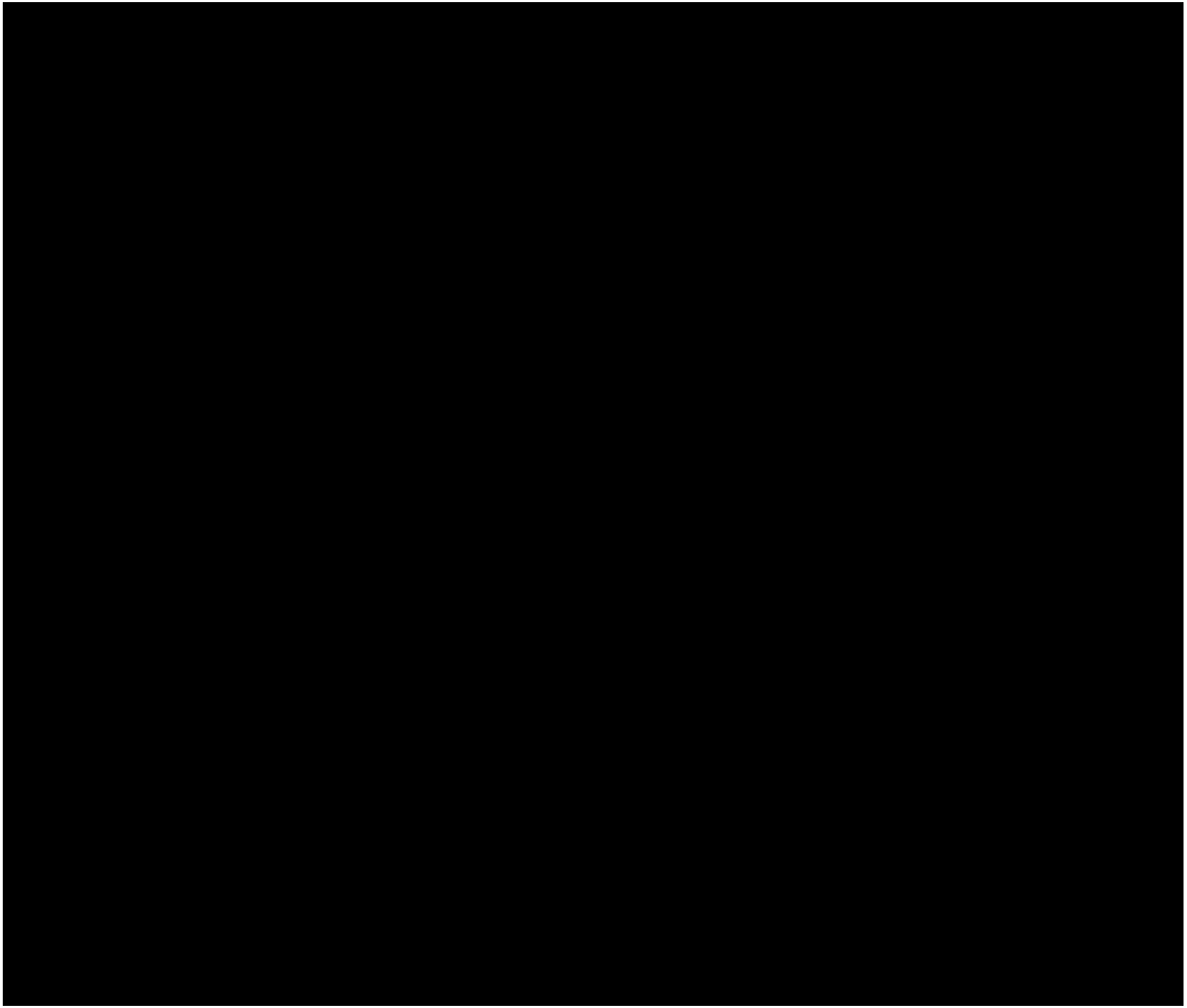
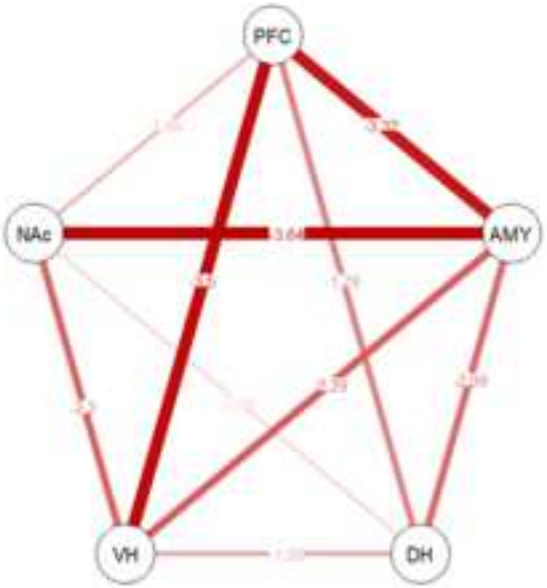


(b)

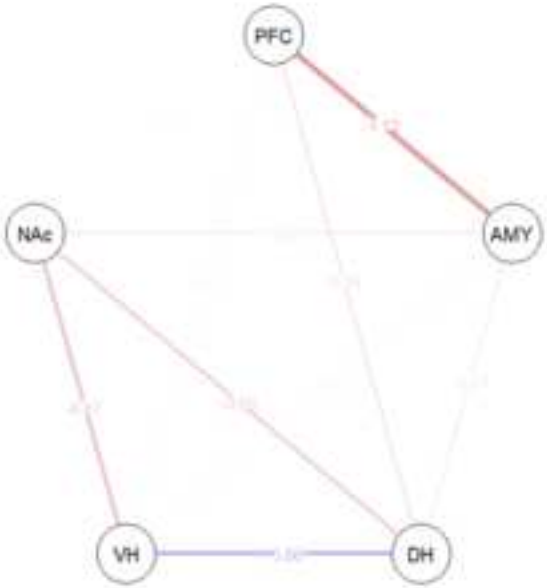
FIG6 TIF

Click here to access/download;Figure(s);FIG6.TIF.tif

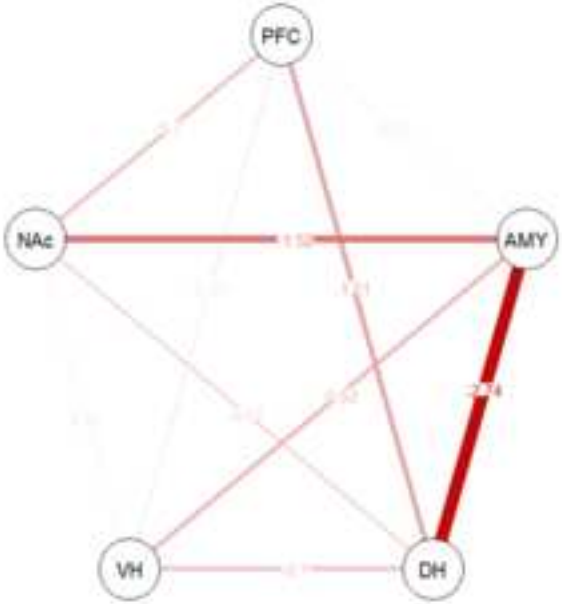




CTRL VEH AS vs CTRL VEH SHAM



CMS VEH AS vs CMS VEH SHAM



CMS LUR AS vs CMS LUR SHAM

ROLE OF FUNDING SOURCE

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CONTRIBUTORS

M.A Riva and M. Papp designed the study and wrote the protocol; I.Pisano managed the literature searches and molecular analyses and their statistical analyses and interpretation and wrote the first draft of the manuscript. P. Gruca and E. Litwa and M. Papp performed the in vivo experiment and the behavioral analyses; V. Begni, K.C. Creutzberg, F. De Rosa and F. Marchisella performed the molecular analyses and undertook the statistical analysis. M. Marizzoni and A. Cattaneo managed and undertook the statistical analyses. All authors contributed to and have approved the final manuscript.

CONFLICT OF INTEREST

M.A.R. has received compensation as speaker/consultant from Angelini, Iqvia, Lundbeck, Otzuka, Sumitomo Dainippon Pharma and Sunovion, and he has received research grants from Sumitomo Dainippon Pharma and Sunovion. All other authors declare that they have no conflicts of interest.

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