Effect of lurasidone treatment on chronic mild stress-induced behavioral deficits in male rats: the potential role for glucocorticoid receptor signaling.

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Abstract

Background: Stress represents one of the main precipitating factors for psychiatric diseases, characterized by an altered function of glucocorticoid receptors (GR), known to play a role in mood and cognitive function.

Aim: We investigated the ability of the antipsychotic lurasidone to modulate the involvement of genomic and non-genomic GR signaling in the behavioral alterations due to chronic stress exposure

Methods: Male Wistar rats were exposed to 7 weeks of chronic mild stress (CMS) and treated with lurasidone (3mg/kg/day) starting from the second week of stress for more five weeks. Gene expression and protein analyses were conducted in dorsal hippocampus.

Results: 7 weeks of CMS induced anhedonia and cognitive impairment, which were normalized by lurasidone. At molecular level, CMS rats showed an increase of GR protein levels by 60% ($p<0,001$ vs CTRL/VEH) in the membrane compartment, which was paralleled by an up-regulation of phosphoSINAPSYN Ia/b by 88% ($p<0,01$ vs CTRL/VEH) and of the mitochondrial marker Cox3 by 21% ($p<0,05$ vs CTRL/VEH). Moreover, while exposure to the Novel Object Recognition (NOR) test increased the nuclear translocation of GRs by 96% ($p<0,01$ vs CTRL/VEH/Naïve) and their transcriptional activity in non-stressed rats, such mechanisms were impaired in CMS

rats. Interestingly, the genomic and non-genomic alterations of GR, induced by CMS, were normalized by lurasidone.

Conclusion: Our results further support the role of glucocorticoid signaling in the dysfunction associated with stress exposure. We provide novel insights on the mechanism of lurasidone, suggesting its effectiveness on different domains associated with psychiatric disorders.

Declaration of interest: none

Introduction

Several psychiatric diseases, such as depression, addiction or post-traumatic stress disorder are defined as stress-related mental disorders due to the central role of stress in their etiopathology (Lupien et al., 2018). On this basis, different animal models have been used as surrogates of these conditions to investigate, at preclinical level, the molecular mechanisms underlying the behavioral and functional alterations associated with stress exposure and to establish the potential efficacy of a pharmacological intervention (Czéh et al., 2016).

Exposure to chronic mild stress (CMS) is probably the most studied animal model (Willner, 2017) resulting in the development of different behavioral changes that mimic clinical symptoms of depression, which may be restored by pharmacological intervention. Indeed, we demonstrated that adult rats exposed to CMS show anhedonia (Calabrese et al., 2016; Luoni et al., 2015; Rossetti et al., 2016) and cognitive deficits (Calabrese et al., 2017), which are accompanied by complex molecular alterations in selected brain regions (Calabrese et al., 2016, 2017; Luoni et al., 2015; Rossetti et al., 2016, 2018). Moreover, the reduction in sucrose intake, as well as some of the molecular abnormalities observed in CMS rats, were normalized by treatment with antidepressants as well as with the multimodal antipsychotic drug lurasidone (D_2, 5-HT_{2A} receptor antagonist, according to the NbN nomenclature)

(Calabrese et al., 2016; Luoni et al., 2015; Rossetti et al., 2016). Lurasidone acts, with high affinity, as antagonist of dopamine D_2 receptors and serotonin 5-HT_{2A} and 5-HT_7 receptors, with moderate affinity as partial agonist of the 5-HT_{1A} receptors (Tarazi and Riva, 2013).

Among the systems activated in response to stress exposure, the hypothalamic-pituitary-adrenal (HPA) axis regulates homeostasis in mammals by controlling the circulating levels of glucocorticoid hormones (GC). Indeed, GCs are involved in several fundamental physiological processes (Macfarlane et al., 2008; McEwen, 2007; Newton et al., 2017; Weger et al., 2016), whereas their malfunctioning is associated with pathological conditions, which may result from sustained and protracted activation of stress-mediated mechanisms (McEwen, 2007).

The activities of GCs are mediated by two different types of receptors: the mineralocorticoid (MR) and the glucocorticoid (GR) that are widely distributed in the brain but particularly enriched in the hippocampus and in the paraventricular nucleus of the hypothalamus of rats (Reul and De Kloet, 1985). Interestingly, GRs exert multiple functions at cellular level (Gray et al., 2017; Levin and Hammes, 2016) since they are localized in the cytoplasm from where, upon binding with GCs, translocate into the nucleus to regulate DNA transcription (Manoli et al., 2007; Revollo and Cidlowski, 2009). GRs can also be found at membrane level where they modulate mitochondrial

function (Du et al., 2009; Manoli et al., 2007; Moutsatsou et al., 2001) and stimulate the synaptic release of neurotransmitters, such as glutamate and GABA (Hill and McEwen, 2010).

On these bases, in the present work, we investigated the contribution of GR, both at nuclear and at membrane levels, in the stress response and in the cognitive deficits observed in the CMS model (Calabrese et al., 2017). Furthermore, we aimed at established if the concomitant treatment with lurasidone is able not only to normalize the anhedonic phenotype but also the cognitive performance, and if GR-mediated functions are involved in the pharmacological effects of lurasidone.

Material and methods

Animals

Male Wistar rats (Charles River, Germany) (weight 275-300 g) were brought into the laboratory one month before the start of the experiment. The animals were singly housed with food and water freely available and were maintained on a 12-h light/dark cycle (lights on at 08.00) and in a constant temperature (22 ± 2 °C) and humidity (50 ± 5%) conditions. All procedures used in this study have conformed 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.

Stress procedure, drug administration, and behavioural tests

After a period of 3 weeks of adaptation to laboratory and housing conditions, the animals (6-10 rats for groups) were trained to consume 1% sucrose solution; training consisted of nine 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. Subsequently, sucrose consumption was monitored, under similar conditions, at

weekly intervals throughout the whole experiment.

After the sucrose training, rats were randomly divided into two groups. One group of animals was subjected to the chronic mild stress procedure for 7 consecutive weeks. During the weekly stress procedure, animals were exposed to several consecutive stressors, for 10 to 14 h periods, including: two periods of food or water deprivation, 45-degree cage tilt, intermittent illumination (with light on and off every 2hours), soiled cage (250mL of water in the sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min) and three periods of no stress. Control (CTRL) animals were housed in separate rooms and had no contact with the stressed animals. They were deprived of food and water for 14h preceding each sucrose test, but otherwise, food and water were freely available in the home cage.

The CMS paradigm is widely used by the scientific community to investigate the etiopathology of mood disorders and the effect of pharmacological treatment. Indeed, it meets the criteria of the construct, face and predictive validity, thus providing neurobiological mechanisms relevant to human depression, with a high translational potential.

On the basis of the result of the sucrose consumption test carried out following the first 2 weeks of stress, animals showing the anhedonic phenotype (starting from 58

animals, 40 develop the pathological phenotype, corresponding to 70%) were selected. Then, both stressed and control groups were further divided into two subgroups, and for subsequent five weeks they received, once a day, the administration of vehicle (1% hydroxyethylcellulose, oral gavage) or lurasidone (3 mg/kg, oral gavage) (Sumitomo Dainippon Pharma Co. Ltd, Japan). The dose of lurasidone was chosen on the basis of our previous work showing its effectiveness in normalizing the behavioural and the molecular alterations due to CMS exposure (Calabrese et al., 2016; Luoni et al., 2015; Rossetti et al., 2018). The volume of all injections was 1 ml/kg. Vehicle or lurasidone were administered at 10.00 am and the weekly sucrose tests were carried out 24h following last drug injections. After five weeks of drugs administration, all control and stressed animals (treated with vehicle or lurasidone) were randomized into two cohorts, one tested in the Novel Object Recognition (NOR) test and the second cohort (naïve) left undisturbed in their home cage (fig.1a). The NOR was performed in non-transparent open field (Calabrese et al., 2017) and the test protocol consisted in three phases: 5 minutes of training (two white cylinders), 1h of inter-trial interval in the home cage and 5 minutes of test (one white cylinder and one black prism) (fig.1a). The NOR index was calculated according to the following formula: time of novel object exploration divided by time of novel plus familiar object exploration, multiplied by 100. Immediately after the exposure to the NOR test and 24 hours after the last injection,

the animals were decapitated and prefrontal cortex and hippocampus (dorsal and ventral) were collected and stored at -80°C. In detail, prefrontal cortex, corresponding to the plates 6-10 according to the atlas of Paxinos and Watson (Paxinos and Watson, 2004), was dissected from 2 mm thick slices. The hippocampus was dissected from the whole brain and specifically, the dorsal hippocampus corresponds to the plates 25-33, whereas the ventral hippocampus corresponds to the plates 34-43 according to the atlas of Paxinos and Watson (Paxinos and Watson, 2004).

RNA preparation and gene expression analysis by quantitative Real-time PCR

Total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (RT-PCR) to assess Growth Arrest and DNA Damage-inducible protein (*Gadd45β*), Serum/Glucocorticoid Regulated Kinase 1 (*Sgk-1*), Nuclear Receptor Subfamily 4 Group A Member 1 (*Nr4a1*), Mitogen-Activated Protein Kinase Phosphatase 1 (*Dusp1*), FK506 binding protein 5 (*Fkbp5*), S100A10 (*P11*), Mitochondrially Encoded Cytochrome c Oxidase I (*Cox1*), Mitochondrially Encoded Cytochrome c Oxidase III (*Cox3*) mRNA levels (primer and probe sequences are listed in

the supplementary table 1a/b). An aliquot of each sample was treated with DNase (Thermoscientific, Italy) to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories, Italy). Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (*36B4*).

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression.

Protein extraction and western blot analysis

Western blot analysis was used to investigate GR, phospho SYNAPSIN Ia/b Ser603 and SYNAPSIN I in the subcellular fractions. Tissues were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM EGTA, 1 mM HEPES solution in the presence of a complete set of proteases (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at

2,500 rpm for 10 min at 4°C to obtain a pellet enriched in nuclear components, which was suspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA) with protease and phosphatase inhibitors. The supernatant obtained was further centrifuged at 10,000 g for 15 min at 4°C to obtain the pellet corresponding to the membrane fraction which was re-suspended in the same buffer prepared for the nuclear fraction. The purity of the fraction obtained was previously reported (Brivio et al., 2019). Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as a calibration standard. Equal amounts of protein were run under reducing conditions on 10% SDS-polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The blots for GR were blocked with bovine serum albumin (BSA) in TBS+0,2% sodium azide, while the ones for the other proteins with 10% nonfat dry milk and then were incubated with the primary antibodies summarized in supplementary table 2. Membranes were then incubated for 1 h at room temperature with the opportune secondary antibody (see supplementary table 2). Immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (Euroclone) and the Chemidoc MP imaging system (Bio-Rad Laboratories). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa.

Statistical analysis

All the statistical analyses were conducted by using “IBM SPSS Statistics, version 24”. The analyses of the sucrose consumption were performed with two-way analysis of variance (ANOVA) with repeated measures, followed by Fisher’s Protected Least Significant Difference (PLSD). The results of the NOR test were analyzed with the two-way ANOVA. When appropriate, further differences were analyzed by the PLSD. Moreover, the molecular results were analyzed with the Unpaired t-test or two-way ANOVA followed by PLSD. Each experimental group consists of 6-10 rats. The complete statistical analyses are listed in supplementary table 3. In addition, Pearson correlation coefficients (r) were evaluated between nuclear GR of single animals and the corresponding expression of its target genes. Significance for all tests was assumed for $p<0.05$. Data are presented as means ± standard error (SEM).

Results

Lurasidone treatment normalized the behavioral alterations produced by CMS exposure

In line with our previous results (Calabrese et al., 2016), exposure to CMS, as well as lurasidone treatment, affected the hedonic phenotype as indicated by the significant effect found in the two-way ANOVA with repeated measures ($F_{3,76}$= 27.42, $p<0.001$) (fig. 1b). Specifically, exposure to CMS produced a significant reduction of sucrose consumption starting from the first week of stress, an effect that was stable for the duration of the whole procedure (Supplementary table 4). As described in the methods, after 14 days of CMS, animals showing anhedonia were randomized into two groups to receive vehicle or lurasidone for five weeks, while continuing the stress exposure. As expected, lurasidone, starting from the third week of treatment, gradually increased the sucrose intake in stressed animals, with a complete recovery at the end of the 5-week treatment period (fig. 1b). As shown in figure 1c, by quantifying the overall result at the end of the 7 weeks of CMS, we found a significant effect of stress (F_{1-80}=19.256, $p<0.001$), lurasidone treatment (F_{1-80}=4.322, $p<0.05$) as well as a significant stress X treatment interaction (F_{1-80}=16.724, $p<0.001$). Indeed, stress exposure significantly reduced sucrose consumption by 49% ($p<0.001$ vs CTRL/VEH), an

effect that was normalized by chronic lurasidone administration by 70% ($p<0.001$ vs CMS/VEH).

Exposure to CMS was also associated with a significant impairment of cognitive performance, measured as NOR index. Indeed, we observed a significant effect of CMS ($F_{1-40}=9.869$, $p<0.01$), of treatment ($F_{1-40}=9.688$, $p<0.01$) as well as a significant stress X treatment interaction ($F_{1-40}=6.866$, $p<0.05$). In agreement with our previous findings (Calabrese et al., 2017), CMS exposure produced a significant cognitive impairment, as indicated by the reduction of the NOR index by 30% ($p<0.001$ vs CTRL/VEH), while chronic lurasidone treatment normalized the stress-induced cognitive deficits by 42% ($p<0.001$ vs CMS/VEH) (fig. 1d).

Interestingly, lurasidone did not influence the sucrose intake and the cognitive performance in control rats.

CMS exposure impaired the translocation of GR to the nucleus associated with the cognitive performance: restorative effect of chronic lurasidone treatment

Since glucocorticoids are involved in cognitive processes (De Quervain et al., 2016), we investigated the role of GR translocation into the nucleus, as a proxy of glucocorticoid signaling activation, during the cognitive performance in the NOR test. Hence, we analyzed nuclear GR protein levels under resting condition or following the NOR test in

normal animals, as compared to rats exposed to CMS. In the dorsal hippocampus, we found that GR protein levels were significantly increased after the NOR test in the nuclear fraction of control animals by 96% ($p<0.01$ vs CTRL/VEH/Naïve), but not in CMS rats (fig. 2b). Interestingly, chronic treatment with lurasidone, which was effective in normalizing the behavioral performance (fig.1c), was able to correct the CMS-induced alterations of GR signaling in response to the NOR test. Indeed, lurasidone administration restored the increase of nuclear GR levels upon exposure to the test by 50% ($p<0.05$ vs CMS/LUR/Naïve), as found in control animals (fig. 2b). Actually, the increase of nuclear GR is specific for animals tested in the NOR, since we did not observe any modulation in the groups of rats exposed to the open field (empty arena) (supplementary table 5). The up-regulation of nuclear GR levels observed after the NOR test in control animals is specific for the dorsal part of the hippocampus since we did not observe any significant change in the ventral hippocampus as well as in the prefrontal cortex (Supplementary figure 1).

Chronic lurasidone treatment restored the transcription of GR-responsive genes altered in CMS rats

To further investigate if the changes in the nuclear translocation of GRs were associated with a differential activation of downstream pathways, we measured the mRNA levels of genes, such as *Gadd45β, Sgk-1*, *Nr4a1, Dusp1, Fkbp5,* and *P11*, whose transcription depends on GR. These genes, known to play a role in learning and memory processes as well as in the stress response, are significantly affected in psychiatric disorders (Hawk and Abel, 2011; Leach et al., 2012; Peña De Ortiz et al., 2000; Tsai et al., 2002; Von Hertzen and Giese, 2005) and may, therefore, contribute to the cognitive alterations found as a consequence of chronic stress exposure. Interestingly, the alteration of nuclear GR levels following NOR exposure was mirrored by similar effects on the transcription of *Gadd45β, Sgk-1,* and *Nr4a1* (fig. 3a, b, c). Indeed, when compared to naïve rats, control animals exposed to the NOR test showed a significant increase of the mRNA levels for *Gadd45β* by 30% ($p<0.001$ vs CTRL/VEH/Naïve) (fig. 3a), Sgk1 by 31% ($p<0.001$ vs CTRL/VEH/Naïve) (fig. 3b) and Nr4a1 by 26% ($p<0.01$ vs CTRL/VEH/Naïve) (fig. 3c), an effect that was not observed in CMS rats exposed to the cognitive test. Moreover, in line with its ability to restore the impaired translocation of GRs in CMS rats, chronic lurasidone treatment was able to normalize the activity-dependent transcription of GR-responsive genes. In fact, we

found that the mRNA levels of *Gadd45β* (fig. 3a), *Sgk1* (fig. 3b) and *Nr4a1* (fig. 3c) were significantly up-regulated by 39% ($p<0.01$ vs CMS/LUR/Naïve), 49% ($p<0.001$ vs CMS/LUR/Naïve) and 46% ($p<0.01$ vs CMS/LUR/Naïve) respectively upon exposure to the test in CMS animals chronically treated with lurasidone.

It is important to point out that not all GR-dependent genes investigated show this pattern of changes. Indeed, *Dusp1* mRNA levels were increased by 38% after the NOR test in control rats ($p<0.001$ vs CTRL/VEH/Naïve) as well as by 29% ($p<0.01$ vs CMS/VEH/Naïve) in vehicle/CMS rats (fig. 3d), and by 19% ($p<0.04$ vs CMS/LUR/Naïve) in lurasidone/CMS rats, whereas the expression of *Fkbp5* and *P11* was not significantly modulated by the NOR test or by lurasidone administration (fig 3e-f).

Interestingly, as revealed by the Pearson product-moment correlation coefficient analysis, nuclear GR levels positively correlated with the expression of *Gadd45β* ($R^2= 0.09351$, $p<0.05$) (fig. 4a), *Sgk1* ($R^2= 0.08702$, $p<0.05$) (fig. 4b), *Nr4a1* ($R^2=0.09582$, $p<0.05$) (fig. 4c) but not with *P11* ($R^2= 0.02902$, $p>0.05$) (fig.4d) suggesting that the transcription of these genes does represent a direct consequence of the ability of GR to translocate upon exposure to the cognitive test.

Membrane-bound GR levels are altered after exposure to CMS: restoration by lurasidone treatment

As mentioned in the introduction, glucocorticoid receptors may also localize at membrane level. Thus, we investigated if stress exposure may influence the membrane-bound GRs levels and what was the impact of the pharmacological intervention with lurasidone.

As shown in fig. 5a, we observed a significant effect of stress ($F_{1\text{-}25}$=5.880, $p<0.05$), treatment ($F_{1\text{-}25}$=4.879, $p<0.05$) as well as a stress X treatment interaction ($F_{1\text{-}25}$=5.880, $p<0.05$) on the levels of membrane-bound GR proteins. Indeed, exposure to CMS produced a significant increase of GR levels in the membrane fraction (+60% $p<0,001$ vs CTRL/VEH), an effect that was normalized by chronic lurasidone treatment (-36%, $p<0,01$ vs. CMS/VEH).

Since this pool of receptors may be localized on cellular and mitochondrial membranes, we measured SYNAPSIN-I protein levels (Hill and McEwen, 2010; Revest et al., 2010) as marker of its activity at synaptic levels, and we assessed the expression of Cox1 and Cox3 as a proxy of mitochondrial function (Du et al., 2009; Manoli et al., 2007; Moutsatsou et al., 2001).

As shown in fig. 5b, in line with the increase of 'membrane-bound' GR levels in CMS rats, we found that the levels of phospho SYN-I (Ser603) were significantly increased in

CMS animals by 78% ($p<0.05$ vs CTRL/VEH), an effect that was normalized by chronic lurasidone treatment ($p<0.05$ vs CMS/VEH), as supported by the significant stress X treatment interaction ($F_{1-21}=4.748$, $p<0.05$). Conversely, neither CMS exposure nor lurasidone treatment were able to modulate the total form of SYN-I (fig. 5c). Interestingly, the activation of this pathway due to stress exposure is supported by the significant upregulation of 51% of the ratio between the phosphorylated and the total form of SYN-I (fig. 5d) ($p<0.05$ vs CTRL/VEH) found in CMS rats. Moreover, as indicated by the significant stress X treatment interaction ($F_{1-21}=5.967$, $p<0.05$), this effect is prevented by lurasidone ($p<0.05$ vs CMS/VEH).

With respect to the mitochondrial component, we found a significant effect of lurasidone treatment on *Cox1* expression ($F_{1-40}=6.668$, $p<0.05$), that was significantly down-regulated by 21% by the treatment in control animals ($p<0.05$ vs CTRL/VEH) (fig. 5e). However, the mRNA levels of *Cox3* were significantly affected by CMS exposure ($F_{1-39}=4.434$, $p<0.05$) as well as by drug treatment ($F_{1-39}=7.334$, $p<0.05$) (fig. 5f). Indeed, its expression was significantly increased in CMS rats by 21% ($p<0.05$ vs CTRL/VEH), and was normalized following chronic lurasidone treatment ($p<0.01$ vs CMS/VEH). Moreover, exposure to the NOR test did not produce any significant modification of the membrane-bound GR protein levels as well as of the downstream synaptic and mitochondrial effectors (Supplementary table 6).

Discussion

Dysfunction of glucocorticoids is a hallmark of psychiatric and stress-related disorders and represents an important target for pharmacological intervention. Indeed GCs, by interacting with their receptors, have profound effects on cognition and emotion, two key domains for mental illnesses, and regulate different molecular pathways that are associated with psychiatric disorders.

In the present study, we demonstrate that exposure to CMS, a well validate experimental model of depression (Willner, 2017) is associated with significant changes in the subcellular localization of GR and of the functional activity of GR-mediated pathways, which may contribute to the behavioral defects produced by CMS exposure. Interestingly, we found that chronic treatment with the antipsychotic lurasidone was able to normalize the behavioral alterations induced by the adverse experience and to correct the abnormalities of GR-related signaling.

Cognitive impairment represents a consolidated consequence of chronic stress exposure, which is due to an altered function of different brain regions as well as to the impairment of different intracellular mediators (Bibb et al., 2010; Calabrese et al., 2017; De Quervain et al., 2016) . Our results provide evidence for a potential contribution of GR signaling in the negative cognitive outcome associated with prolonged stress exposure. Indeed, while exposure of control animals to a cognitive

task produces an increased translocation of GRs to the nuclear compartment, we found that such mechanism is impaired in CMS rats that show a deficit in the NOR test. Interestingly, increased nuclear translocation of GRs during the cognitive performance is specifically observed in the dorsal hippocampus, the sub-region of the hippocampus mainly involved in cognition and spatial learning (Fanselow and Dong, 2010; Kheirbek et al., 2013) and highly sensitive to the negative structural and functional effects of chronic stress exposure (Cameron and Schoenfeld, 2018; Sapolsky et al., 2000).

Cognition is an active process that requires the integrative action of different mediators. Here we show that in control animals exposed to the NOR test GRs translocate into the nucleus following the retrieval phase of the cognitive task and, in turn, regulate the transcription of specific GR responsive genes (Gray et al., 2017), including *Gadd45β*, *Sgk1,* and *Nr4a1,* which may play a role in the cognitive performance. Indeed, it has been shown that Gadd45β KO mice exhibit deficits in the hippocampal long-term memory (Leach et al., 2012), whereas *Sgk1* and *Nr4a1* levels are increased in the rat hippocampus after learning tasks (Peña De Ortiz et al., 2000; Tsai et al., 2002; Von Hertzen and Giese, 2005). Moreover, transfection of Sgk-1 in the CA1 hippocampal subfield can facilitate spatial memory performance in rats (Tsai et al., 2002).

The inability of CMS rats to activate GR signaling upon exposure to the cognitive test is reminiscent of what is known as “glucocorticoid resistance”, a feature common to different psychiatric conditions, which may contribute to HPA axis hyperactivity as well as to impaired transcriptional mechanisms associated with GR activation (Pariante, 2009). Interestingly, over the years, elegant works from Pariante and coworkers have shown that antidepressant treatment may correct “glucocorticoid resistance” thus leading to an amelioration of downstream mechanisms affected in mood disorders (Carvalho and Pariante, 2008; Mason and Pariante, 2006; Pariante et al., 2003; Zhang et al., 2005). Here, we show that chronic treatment of CMS rats with the multi-receptor antagonist lurasidone was not only able to normalize the anhedonic phenotype, as previously demonstrated by our group (Calabrese et al., 2016; Luoni et al., 2015; Rossetti et al., 2016), but was also effective in correcting the cognitive impairment originating from prolonged stress exposure. Interestingly, lurasidone was also able to normalize the alteration of GR signaling by restoring the nuclear translocation following the cognitive test and promoting the transcription of some GR-responsive genes during ongoing cognitive activity. Indeed, according to the multimodal mechanism of action of lurasidone and seen the complex cross-talk between neurotransmitters and HPA axis (Joëls and Baram, 2009), several mechanisms may be responsible for the effects exerted by this drug on GR function. These may

include the regulation of CRH transcription and release, as a consequence of the activity on the dopaminergic system (Kelly and Fudge, 2018), as well as the modulation of complex intracellular signaling pathways bridging the simultaneous activity on different neurotransmitter receptors (Aringhieri et al., 2018).

We also found that exposure to chronic stress increases the levels of membrane-bound GRs, which may underlie alterations in synaptic mechanisms as well as in mitochondrial function. Indeed, this increase was paralleled by a significant up-regulation of the active form of SYNAPSIN I, a marker of the functional activity of the receptor in the synaptosomal membranes (Revest et al., 2010). Our results are in line with a previous study demonstrating that exposure to chronic mild stress increases the density of SYNAPSIN I immunoreactive synaptic buttons in the CA3 subfield of the rat hippocampus, an effect that was restored by the treatment with the GR antagonist Mifepristone (Wu et al., 2007).

Glucocorticoids may also act through GRs located on the mitochondrial membrane, thus regulating the transcription of genes involved in the biosynthesis of respiratory enzymes (Tsiriyotis et al., 1997), such as *Cox1* and *Cox3*, the catalytic subunits of cytochrome c oxidase, the last enzyme in the respiratory electron transport chain (Demonacos et al., 1996; Liang et al., 2006). In line with previous results (Adzic et al., 2009; Hunter et al., 2016), we found that CMS exposure increases the expression of

Cox3 mRNA levels in the dorsal hippocampus. Although it is difficult to provide a functional interpretation of our results, elevation of GR at mitochondrial levels may induce apoptosis (Sionov et al., 2006), while changes in the functionality of Cox enzyme may reflect alterations in energy production or modifications of the cellular energy demand (Liang et al., 2006).

Interestingly, it has been reported the existence of a cross-talk between nuclear and mitochondrial GR in cardiomyocytes (Goldenthal et al., 2005) and it has been shown that in liver cells cortisol is up-taken following a similar kinetics (very rapid) in mitochondrial and in the nuclei (Beato et al., 1969). So, while speculative, it may be inferred that the increased GR levels observed at membrane level in CMS rats may reduce the functional activity of GR in the nucleus.

All in all, our data provide further support for a role of glucocorticoids and their receptor in the dysfunction associated with chronic stress exposure. We also provide new insights on the mechanism of action of lurasidone in the context of stress-related disorders, suggesting that it may promote adaptive mechanisms that are crucial for the ability to modulate different pathological domains associated with psychiatric disorders.

Figure legends:

Fig. 1*: Behavioural characterization of animals exposed to chronic mild stress and treated with lurasidone*.

Panel a: schematic representation of the experimental paradigm and schedule of the behavioral tests; panel b: sucrose intake was measured at weekly intervals in control (CTRL) or stressed (CMS) animals treated with vehicle (VEH) or lurasidone (LUR); panel c: analysis of sucrose intake measured at the end of the experiment (Week 7) in CTRL or CMS rats treated with VEH or LUR; panel d: Novel object recognition (NOR) index evaluated at the end of the experiment (Week 7) in CTRL or CMS animals treated with VEH or LUR. The data are the mean ± SEM of at least 10 independent determinations. ***p<0.001 vs CTRL/VEH; #p<0.05, ##p<0.01; ### p<0.001 vs CMS/VEH) (two-way ANOVA with repeated measures panel b; two-way ANOVA with PLSD panel c-d).

Fig. 2: *Analysis of nuclear GR protein levels in the dorsal hippocampus.*

Panel a: representative Western blot analysis of nuclear GR. β-actin was used as internal standard.

Panel b: GR protein levels in chronically stressed (CMS) rats treated with vehicle (VEH) or lurasidone (LUR), under resting conditions (Naive) or after exposure to the novel object recognition test (NOR). The data, expressed as mean ± SEM of at least 6 independent determinations, represent the percentage of CTRL/VEH/Naive (set at 100%). ** p<0.01 vs CTRL/VEH/Naïve; # p<0.01 vs CMS/LUR/Naïve (Unpaired *t*-test).

Fig. 3*: Analysis of GR-responsive genes expression in the dorsal hippocampus*.

The data, expressed as mean ± SEM of at least 6 independent determinations, represent the percentage of CTRL/VEH/Naive (set at 100%). * p<0.05, **p<0.01, *** p<0.001 vs CTRL/VEH/Naïve; §§ p<0.01 vs CMS/VEH/Naïve; # p<0.05, ## p<0.01, ### p<0.001vs CMS/LUR/Naïve (Unpaired *t*-test).

Fig. 4*: Correlation analysis between the levels of GR in the nucleus and the expression of the GR-regulated genes.*

The figure summarizes the analyses by Pearson's product-moment correlation (R^2) between nuclear GR levels and the expression of *Gadd45*β (panel a), *Sgk-1* (panel b), *Nr4a1* (panel c) and *P11* vs GR (panel d).

Fig. 5*: Analysis of membrane GR levels and its associated pathways in the dorsal hippocampus.*

The protein levels of membrane-bound GR (panel a), phospho SYNAPSIN-I Ser603 (panel b), total SYNAPSIN-I (panel c) are measured in the membrane fraction of chronically stressed (CMS) rats treated with lurasidone (LUR). panel d showed the ratio of the protein levels between the phosphorylated and total form of SYNAPSIN-I. Panel e-f showed the gene expression of Mitochondrially Encoded Cytochrome c Oxidase I and III (*Cox1-3*). The data are expressed as the percentage of CTRL/Naive (set at 100%) and are the mean ± SEM of at least 4

independent determinations. * $p<0.05$, ***$p<0.001$ vs CTRL/VEH; # $p<0.05$, ## $p<0.01$ vs CMS/LUR (two-way ANOVA with PLSD). Representative Western blot bands of GR, phospho SYN-I Ser603 and total SYN-I are shown under the respective graphs. β-actin was used as internal standard.

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