Chronic stress exposure reduces parvalbumin expression in the rat hippocampus through an imbalance of redox mechanisms: restorative effect of the antipsychotic lurasidone

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Significance statement

Our study provides evidence for an association between the stress-induced decrease of parvalbumin, a marker of GABAergic interneurons, and the alterations of key players of the oxidative balance. Of particular interest is the observation that chronic treatment with the antipsychotic drug lurasidone is able to normalize the decrease of parvalbumin in parallel with potential anti-oxidative effects on the NRF2-KEAP1 system as well as on the enzyme NOX-2. Our data suggest a close relationship between the alterations of selected GABAergic interneurons and the redox balance that may represent an important mechanism through which lurasidone may ameliorate brain function in stress-related pathologic conditions.

Abstract

Background

Psychiatric disorders are associated with altered function of inhibitory neurotransmission within the limbic system, which may be due to the vulnerability of selective neuronal subtypes to challenging environmental conditions, such as stress. In this context, parvalbumin (PVB) positive GABAergic interneurons, which are critically involved in processing complex cognitive tasks, are particularly vulnerable to stress exposure, an effect that may be the consequence of dysregulated redox mechanisms.

Methods

Adult Male Wistar rats were subjected to the chronic mild stress (CMS) procedure for 7 weeks. After 2 weeks, both control and stress groups were further divided into matched subgroups to receive chronic administration of vehicle or lurasidone (3mg/kg/day) for the subsequent 5 weeks. Using real time RT-PCR and western blot, we investigated the expression of GABAergic interneuron markers and the levels of key mediators of the oxidative balance in the dorsal and ventral hippocampus.

Results

CMS induced a specific decrease of PVB expression in the dorsal hippocampus, an effect normalized by lurasidone treatment. Interestingly, the regulation of PVB levels was correlated to the modulation of the antioxidant master regulator NRF2 and its chaperon protein KEAP1, which were also modulated by pharmacological intervention.

Conclusions

Our findings suggest that the susceptibility of PVB neurons to stress may represent a key mechanism contributing to functional and structural impairments in specific brain regions relevant for psychiatric disorders. Moreover, we provide new insights on the mechanism of action of lurasidone, demonstrating that its chronic treatment normalizes CMS-induced PVB alterations, possibly by potentiating antioxidant mechanisms, which may ameliorate specific functions that are deteriorated in psychiatric patients.

Keywords: stress, hippocampus, parvalbumin, lurasidone, NRF2

1. Introduction

Psychiatric diseases, such as major depression and schizophrenia, are highly disabling disorders characterized by complex etiological mechanisms that lead to functional abnormalities of different neurotransmitters, including monoamines, GABA and glutamate, as well as a dysregulation of inflammation, neuroplasticity and hormonal signaling (Kupfer et al., 2012; Calabrese et al., 2016a; Owen et al., 2016; Begni et al., 2017). The multifaceted behavioral symptomatology of these disorders involves the perturbation of emotional and cognitive domains of the individual and -among the others- cognitive symptoms have a dramatic impact on the all-day life of the patients (Millan et al., 2012). In this context, at cortical and hippocampal level, the GABAergic inhibitory tone finely regulates the firing of principal glutamatergic neurons. More in details, GABAergic interneurons synchronize the firing of principal cells controlling the plasticity of excitatory synaptic inputs through dendritic inhibition, while they inhibit the output with perisomatic inhibition (Freund, 2003). Among the diverse subtypes of GABAergic interneurons populating the hippocampal formation, parvalbumin (PVB), somatostatin (SST), calbindin (CALB) and neuropeptide-Y (NPY) positive cells represent the most sensitive to stress exposure (Filipovic et al 2013; Czeh et al 2015). Specifically, the highly energized fast-spiking, parvalbumin positive (PVB+) interneurons play a pivotal role in the processing of complex information. Their contribution in cognitive decline may be fundamental, especially when dysregulation of the energy demand and/or of the oxidative balance may impair their functions (Kann, 2016). This may occur following exposure to stress, which represents a major environmental condition for mental (Pittenger and Duman, 2008; Cattaneo and Riva, 2016). Indeed, PVB+ neurons can be part of a critical loop since, while stress may lead to an impairment of this neuronal population (Zaletel et al., 2016), the suppressed function of PVB+ neurons may reduce resilience, (Perova et al., 2015).

In the present work we used the chronic mild stress (CMS) model (Willner, 2017) to investigate the detrimental effects of stress on PVB positive cells in rat hippocampus and the potential contribution of a dysregulation in redox mechanisms, which have also been associated to the pathophysiology of several psychiatric disorders (Moniczewski et al., 2015; Smaga et al., 2015; Steullet et al., 2017). We have previously shown that CMS is able to induce depressive-like behaviors such as anhedonia (Rossetti et al., 2016) as well as cognitive impairment (Calabrese et al., 2017), which are associated with alterations in key molecular players for psychiatric disorders (Luoni et al., 2014; Calabrese et al., 2016b; Molteni et al., 2016). We also investigated the effect of a chronic treatment with lurasidone in counteracting the CMS-induced alterations in rat hippocampus. Lurasidone is a multi-receptor antipsychotic drug (Tarazi and Riva, 2013) with demonstrated clinical efficacy for cognitive deficits in schizophrenia (Harvey et al., 2013) and in bipolar disorder (Yatham et al., 2017), and also depressive symptoms in schizophrenia (Nasrallah et al., 2015), and in bipolar depression (Loebel et al., 2014). We have previously demonstrated that chronic lurasidone is able to normalize the stress-induced depressive-like behaviors as well as the neuroplastic and inflammatory alterations observed in stressed rats (Luoni et al., 2014; Rossetti et al., 2016).

2. Methods

2.1 Animals

Adult male Wistar rats (Charles River, Germany) were brought into the laboratory one month before the start of the experiment. Except as described below, the animals were singly housed with food and water freely available and were maintained on a 12h light/dark cycle in a constant temperature (22 ± 2°C) and humidity (50 ± 5%) conditions. All procedures used in this study are conformed to the rules and principles of the 2010/63/EU Directive and were 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 to reduce the number of animals used (n=10 each experimental group).

2.2 Chronic mild stress procedure and pharmacological treatment

After a period of adaptation to laboratory and housing conditions, the animals (220 ± 7g - Charles River, Germany) were subjected to seven weeks of chronic mild stress (CMS), in parallel with a five-week long lasting treatment with lurasidone.

The stress regimen consisted of two periods of food or water deprivation, two periods of 45 degrees cage tilt, two periods of intermittent illumination (lights on and off every 2h), two periods of soiled cage (250ml 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 10–14h of duration and were applied individually and continuously, day and night. Control 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.

Animals were subjected to the stress procedure for 7 weeks. Following the first 2 weeks of stress, both control and stress groups were further divided into matched subgroups and for the subsequent five weeks they received oral administration (by gavage) of vehicle (hydroxy-ethyl-cellulose, HEC 1%) or lurasidone (3mg/kg daily). Our experimental design implied four groups of animals: unstressed rats that received the vehicle, used as reference control group ("No Stress/Vehicle", n=10); unstressed rats that received the drug (No Stress/Lurasidone, n=10); stressed rats that received the vehicle (Stress/Vehicle, n=10); stressed rats that received the drug (Stress/Lurasidone, n=10). After five weeks, the treatments were terminated, and all control and stressed animals were killed by decapitation 24h after the last drug administration. The brains were removed and dissected for prefrontal cortex, dorsal and ventral hippocampus as fresh tissues. All samples were then rapidly frozen in dry ice/isopentane and stored at −80◦C for the further molecular analyses.

2.3 RNA preparation and quantitative Real-Time PCR analyses

Total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were processed for polymerase chain reaction (PCR) as previously described (Rossetti et al., 2016) to measure the mRNA expression of parvalbumin (*Pvb*), somatostatin (*Sst*), calbindin (*Calb*), neuropeptide Y (*Npy*), NADPH oxygenase 2 (*Nox2*), nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), sulfiredoxin (*Srxn*), hemeoxigenase-1 (*Ho-1*), NAD(P)H dehydrogenase [quinone]1 (*Nqo1*), catalase (*Cat*). Primer and probes sequences are listed in Table 1.

Specifically, RNA aliquots of each sample were treated with DNase to avoid DNA contamination and then analyzed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories). The samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control (β-Actin).

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retro-transcription) 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 reaction. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression versus the control group. Specifically, fold change for each target gene relative to β-Actin was determined by the $2^{-\Delta(\Delta CT)}$ method, where ΔCT=CT(target)-CT(β-Actin); Δ(ΔCT)=ΔCT(exp. group)-ΔCT(control group); CT is the threshold cycle. For graphical clarity, the obtained data were then expressed as percentage versus control group, which has been set at 100%.

2.4 Protein extraction and western blot analyses

Brain samples were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer (containing 0.32 M sucrose, 0.1mM EGTA, 1mM HEPES solution and 0.1mM phenylmethylsulfonyl fluoride, in presence of a complete set of proteases (Roche) and phosphatase (Sigma-Aldrich) inhibitors) and then sonicated for 10s at a maximum power of 10-15% (Bandelin Sonoplus). The homogenate was clarified (1000g; 10min), obtaining a pellet (P1) enriched in nuclear components, which was resuspended in a buffer (1mM HEPES, 0.1mM dithiothreitol, 0.1mM EGTA) supplemented with protease and phosphatase inhibitors. The supernatant (S1) was then centrifuged (13000g; 15min) to obtain a clarified fraction of cytosolic proteins (S2). The pellet (P2), corresponding to the crude membrane fraction, was resuspended in the same buffer used for the nuclear fraction. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard.

Protein analyses were performed in the whole homogenate (for PVB), in the cytosolic fraction (for NRF2 and KEAP1) and in the crude membrane fraction (for NOX2). Equal amounts of protein (10µg for the homogenate, 30µg for the S2 and 15µg for the P2) were run under reducing conditions on polyacrylamide gels and then electrophoretically transferred onto polyvinylidene fluoride or nitrocellulose membranes. Unspecific binding sites were blocked with 10% non-fat dry milk and then the membranes were incubated overnight with the primary antibodies, and then for 1h at room temperature with a peroxidase-conjugated anti-rabbit or anti-mouse IgG (Table 2). Immunocomplexes were visualized by chemiluminescence using the ECL Star (Euroclone), ECL Plus (Euroclone) or ECL Clarity (Bio-Rad Laboratories). Results were standardized using β-Actin as the internal control, which was detected by evaluating the band density at 43kDa. Protein levels were calculated by measuring the optical density of the immunocomplexes using chemiluminescence (Chemidoc MP Imaging System, Bio-Rad Laboratories). To ensure that autoradiographic bands will be in the linear range of intensity, different exposure times were used.

2.5 Statistical analyses

The effects of drug treatment (lurasidone) and chronic stress exposure on the mRNA or protein levels of our molecular targets were analyzed by two-way ANalysis Of VAriance (ANOVA) followed -when appropriate- by Fisher's Least Significant Difference (LSD) *post hoc* comparisons. In addition, to evaluate the association between the modulation of the NRF2-KEAP1 system and the protein levels of PVB, Pearson product-moment correlation coefficients (r) were calculated between NRF2 or KEAP1 protein and PVB levels. Significance for all tests was assumed for $P<0.05$. Data are presented as means ± standard error (S.E.M.). SPSS (Release 24.0.0.0) was used to perform the statistical analyses.

3. Results

3.1 Analysis of the mRNA levels for different subtypes of GABAergic interneurons rats exposed to chronic mild stress and treated with lurasidone

We first investigated the mRNA levels of the GABAergic markers parvalbumin (*Pvb*), somatostatin (*Sst*), neuropeptide-Y (*Npy*) and calbindin (*Calb*) in the dorsal (D-HIP) and ventral (V-HIP) hippocampus of animals exposed to CMS and treated, or not, with the antipsychotic drug lurasidone.

The analysis of *Pvb* gene expression in the D-HIP showed a significant interaction between CMS and lurasidone treatment ($F_{3,32}$= 11.755, $P<0.01$). Indeed, stress exposure led to a significant decrease of *Pvb* mRNA levels (-18% vs. No Stress/Vehicle, $P<0.05$. Fig.1 A), which was normalized by pharmacological intervention (+19% vs. Stress/Vehicle, $P<0.05$. Fig.1 A). Of note, lurasidone administration per-se produced a significant decrease of *Pvb* when compared to control rats (-16% vs. No Stress/Vehicle, $P<0.05$; Fig.1 A). These changes appeared to be specific for the dorsal part of the hippocampus, since no significant changes were found in the ventral counterpart (Fig. 1E).

When investigating *Sst* expression in the D-HIP, we found a significant effect of CMS exposure ($F_{3,35}$= 10.023, $P<0.01$) as well as of pharmacological treatment ($F_{3,35}$= 33.850, $P<0.001$). As shown in figure 1B, *Sst* levels were increased in rats subjected to CMS (+58% vs. No Stress/Vehicle, $P<0.01$. Fig.1 B), whereas chronic lurasidone treatment up-regulated *Sst* expression in non-stressed rats (+98% vs. No Stress/Vehicle, $P<0.001$. Fig.1 B) as well as in stressed animals (+75% vs. Stress/Vehicle, $P<0.001$. Fig.1 B). In the V-HIP, we found a main effect of CMS on *Sst* mRNA levels ($F_{3,36}$= 6.687, $P<0.05$) that led to a significant decrease of this marker in stressed rats when compared to control animals (-21% vs. No Stress/Vehicle, $P<0.05$. Fig.1 F), an effect that was not modulated by the pharmacological treatment.

Conversely, the expression of the other GABAergic markers, namely *Npy* (Fig.1 C, Fig.1 G) and *Calb* (Fig.1 D and Fig.1 H), was not significantly modulated in the dorsal nor in the ventral portion of the hippocampus following CMS exposure or lurasidone treatment, providing further support to the selectivity exerted by CMS exposure on specific subpopulations of GABAergic neurons.

No significant changes were observed in the prefrontal cortex of stressed rats treated or not with chronic pharmacological treatment (Table 3).

3.2 Analysis of PVB protein levels in the hippocampus of animals exposed to chronic mild stress and treated with lurasidone

Based on the gene expression analyses of different interneuron markers, we decided to focus on parvalbumin and analyzed its protein levels in D-HIP and V-HIP of rats exposed to CMS, with or without lurasidone treatment. Within D-HIP, as depicted in figure 2A, we found a main effect of CMS exposure ($F_{3,35}$= 12.164, P<0.001) and lurasidone treatment ($F_{3,35}$= 19.860, P<0.001), as well as a significant CMS x treatment interaction ($F_{3,35}$= 7.710, P<0.01). Indeed, the levels of PVB were markedly reduced in rats exposed to CMS and treated with vehicle (-58% vs. No Stress/Vehicle, P<0.001. Fig.2 A), whereas chronic lurasidone treatment was able to normalize the CMS-induced changes of PVB levels (+67% vs. Stress/Vehicle, P<0.001. Fig.2 A).

In line with the gene expression data, these alterations show anatomical selectivity. Indeed, within V-HIP (Fig.2 B), despite a main effect of CMS exposure ($F_{3,35}$= 9.486, P<0.01), we only found a trend toward a decrease of PVB levels in stressed animals (-16% vs No Stress/Vehicle P=0.054), which was not influenced by the pharmacological treatment.

3.3 Analysis of NADPH oxidase-2 gene and protein expression in the dorsal hippocampus of animals exposed to chronic mild stress and treated with lurasidone

PVB neurons show a sustained firing activity that requires a high demand of energy, which may expose them to an increased susceptibility toward the detrimental effects of oxidative stress. On these bases, we investigated if the effects of CMS exposure in D-HIP could be associated with alterations of molecules involved in the complex machinery regulating the oxidative balance in the brain. We analyzed the gene expression of NADPH oxidase 2 (*Nox2*), an enzyme responsible for the production of reactive oxygen species (ROS) by activated macrophages, including microglia. The analysis of *Nox2* mRNA levels in D-HIP revealed a significant interaction between stress and lurasidone administration ($F_{37,3}$= 4.521, P<0.05). Indeed, the direct comparisons between groups showed that *Nox2* gene expression was increased in rats exposed to CMS (+33% vs. No Stress/Vehicle, P<0.05. Fig.3 A), an effect that was not present in CMS rats chronically treated with lurasidone. We then investigated the protein levels of $gp91^{PHOX}$, the main membrane bound subunit of the enzyme. As depicted in figure 3B, stress exposure had a main effect on NOX2 protein levels ($F_{33,3}$= 7,121 P<0.05), since CMS rats showed an increase of NOX2, when compared to control animals (+98% vs. No Stress/Vehicle, P<0.01. Fig.3 B), an effect that was attenuated by chronic lurasidone administration.

3.4 Analysis of the NRF2-KEAP1 antioxidant system in the dorsal hippocampus of CMS of animals exposed to chronic mild stress and treated with lurasidone

The Nuclear factor (erythroid-derived 2)-like 2 (*NRF2*) and the Kelch-like ECH-associated protein 1 (KEAP1) have a pivotal role in the control of the cellular antioxidant response. Indeed, upon nuclear translocation NRF2 binds to its consensus sequences (the so-called antioxidant responsive elements - AREs) to promote the transcription of several enzymes involved in the cellular mechanisms of detoxification. The transcription factor interacts in the cytosol with KEAP1, a chaperon protein that prevents its translocation into the nucleus thus inhibiting its transcriptional antioxidant activity.

When considering the expression of the transcription factor *Nrf2* we found a statistically significant interaction between CMS and lurasidone treatment ($F_{3,36}$= 11.616, P<0.01). Indeed, as shown in figure 4A, while CMS exposure produced a slight, non-significant decrease of the transcription factor (-11% vs. No Stress/Vehicle, P>0.05), lurasidone was able to increase its mRNA levels only when administered to CMS animals (+24% vs. Stress/Vehicle, P<0.01). Based on gene expression analyses, we decided to deepen our investigation by assessing the protein levels of NRF2 as well as of its inhibitor KEAP1 in the D-HIP.

The analysis of the protein levels of NRF2 revealed a significant stress*lurasidone interaction ($F_{3,32}$= 4.195, P<0.05). Indeed, as shown in figure 4B, the protein levels of NRF2 were decreased by CMS (-40% vs. No Stress/Vehicle, P<0.05), an effect that was, at least in part, restored by lurasidone treatment considering that the levels of NRF2 protein in Stress/Lurasidone group did not differ from sham rats.

Interestingly, KEAP1 levels were strongly modulated by chronic lurasidone treatment ($F_{3,30}$= 15.226, P<0.001). Indeed, although CMS exposure did not affect KEAP1 levels, chronic lurasidone administration significantly reduced its protein levels in sham (-48% vs. No Stress/Vehicle, P<0.01. Fig.4 C) as well as in CMS rats (-55% vs. Stress/Vehicle, P<0.05. Fig.4 C).

3.5 Pearson correlation analysis between NRF2/KEAP1 protein levels and PVB in the dorsal hippocampus of animals exposed to chronic mild stress and treated with lurasidone

Next, in order to establish a potential relationship between the effects of stress exposure and pharmacological treatment on PVB expression with the levels of NRF2/KEAP1 antioxidant system, we performed a Pearson product-moment correlation coefficient analysis between the protein levels of NRF2 or KEAP1 and the protein levels of PVB in the D-HIP. As presented in figure 5, NRF2 showed a significant positive correlation with the GABAergic marker (r= 0.414, P<0.05. Fig.5 A), while a negative correlation was observed between the chaperone protein KEAP1 and PVB (r= -0.501, P<0.01. Fig.5 B).

3.6 Analysis of the transcriptional effects of NRF2 in the dorsal hippocampus of animals exposed to chronic mild stress and treated with lurasidone

Based on the changes in the functional interplay between NRF2 and KEAP1 after CMS exposure and/or lurasidone treatment, we decided to investigate the expression of some genes downstream from the transcriptional activity of

NRF2, namely the enzymes sufiredoxin 1 (*Srnx1*), heme oxygenase-1 (*Ho-1*), NAD(P)H dehydrogenase [quinone]1 (*Nqo1*) and catalase (*Cat*).

As depicted in figure 6, the expression of *Srxn1* (panel A) and *Ho-1* (panel B) were not modulated by CMS exposure or chronic lurasidone treatment. However, we found that the mRNA levels of *Nqo1* showed a significant stress x treatment interaction ($F_{3,37}$= 7.806, P<0.01). Indeed, chronic treatment with lurasidone was able to up-regulate its expression, only when administered to CMS animals (+23% vs. Stress/Vehicle, P<0.01. Fig.6 C). Moreover, we found a significant main effect of lurasidone treatment ($F_{3,38}$= 6.334, P<0.05) on *Cat* gene expression. Specifically, the pharmacological treatment increased the levels of the enzyme, but only when it was administered to sham animals (+21% vs. No Stress/Vehicle, P<0.05. Fig.6 D).

4. Discussion

In recent years the interest on GABAergic interneurons has gained much attention, due to their key role in functions that are altered in different psychiatric disorders, including major depression and schizophrenia (Luscher and Fuchs, 2015; Owen et al., 2016). Within this context, our data point out that the restorative effect of pharmacological treatment on PVB expression may be mediated by the drug-mediated regulation of the oxidative balance within the brain.

Most of PVB expressing cells are present in the central nervous system as interneurons, particularly within selected brain structures, including cerebral cortex, hippocampus, cerebellum and spinal cord (Zaletel et al., 2016). In the hippocampus, a critical brain area involved in the control of emotional states, stress response and cognitive function (Fanselow and Dong, 2010), PVB positive cells are mostly fast spiking GABAergic interneurons that control the circuitry activity of pyramidal cells through their inhibitory activity. The reduction of PVB expression found in the D-HIP of CMS exposed rats, is in line with the detrimental effects of stress or other adverse manipulations on the GABAergic system reported in other preclinical studies. For example, Czeh and collaborators have shown that 5 weeks of psychosocial stress were able to impair PVB expression in specific subregions of treeshrew hippocampus (Czeh et al., 2005). Similar results were obtained with other stress paradigms in rats, such as chronic immobilization (Hu et al., 2010) and social isolation (Filipović et al., 2013). Interestingly, in our experimental setting, the D-HIP appears to be more vulnerable to the effects of CMS, an effect that is in line with the results of Czeh and co-workers (Czéh et al., 2015). Considering that functional alterations of this hippocampal subregion have a major role in cognitive dysfunctions, we hypothesize that reduced PVB expression may contribute to the impaired cognitive function we have recently shown in rats exposed to the CMS paradigm (Calabrese et al., 2017). It is likely that the expression of PVB may be due to a decrease of protein expression than to cell loss. Indeed, as demonstrated by others, chronic stress exposure does not increase caspase-3 expression in GABAergic interneurons (Filipović et al., 2013). Interestingly, we found a strong induction of SST expression in the D-HIP following stress exposure. In both hippocampus and neocortex, PVB-positive interneurons target the soma and the perisomatic dendrites of pyramidal neurons, controlling the output signaling of principal excitatory neurons. In parallel, SST interneurons generally target the more distal dendrites, gating the excitatory signals (Horn and Nicoll, 2018). Moreover, the activity of PVB and SST interneurons is strictly interconnected as part of complex inhibitory microcircuits involved in the control of behavior and learning (Caroni, 2016). Considering that SST decrease has been causally related to anxiety/depression-like behaviors (Lin and Sibille, 2015), our result may seem counterintuitive. However, the increased expression of SST may represent a compensatory mechanism to limit the impaired somato-dendritic inhibition of pyramidal neurons after CMS-induced PVB loss. In parallel, the increase of SST observed after lurasidone treatment may be related to a specific mechanism induced by the drug, that is independent from the model analyzed. Indeed, we have previously shown a similar effect in serotonin transporter knockout rats treated with lurasidone (Luoni et al., 2013). In this sense, further functional studies are demanded to better clarify the impact of pharmacological treatment with psychotropic drugs on these interneuron populations.

The opposite effects of CMS on these two markers, may also explain why the protein levels of the glutamic acid decarboxylase (GAD)67 were not modulate by stress exposure in the D-HIP (data not shown). Indeed, we may speculate that -in our experimental setting- this enzyme is differentially modulated in the diverse interneuron sub-populations.

While the precise detrimental mechanisms triggered by stress exposure on PVB interneurons are not well clarified, the selective vulnerability of PVB positive interneurons to chronic stress may be due to their peculiar fast spiking activity. The firing of PVB interneurons requests high amount of energy, and the increased metabolic activity under certain conditions, such as stress, may expose PVB neurons to potentially toxic effects of reactive oxygen and nitrogen species, which alter the redox balance of the cell (Kann, 2016).

With this respect, the NOX family represents a very important group of enzymes that, especially in the injured nervous system, is a major source of ROS (Cooney et al., 2013). The upregulation of NOX2 expression after stress suggests an increase of the pro-oxidative activity in rats exposed to CMS. Increased levels of NOX2 have also been observed in brain areas of animals exposed to prolonged social isolation from weaning (Schiavone et al. 2009) as well as in a model of post-traumatic stress disorder (PTSD), where NOX2 upregulation was paralleled by a decrease of PVB expression (Sun et al., 2016). Considering that NOX2 has been reported as the primary phagocytic oxidase (Bermudez et al., 2016), its altered expression may be associated to the increased activity of microglial cells under stressful conditions. In parallel, the production of pro-oxidative agents from NOX2 induce the activation of microglia, triggering a detrimental inflammatory loop, potentially harmful for neurons (Vilhardt et al., 2017). This hypothesis is supported by previous data from our laboratory showing increased expression of hippocampal CD11b, a marker of microglia activation, in animals exposed to 7 weeks of CMS (Rossetti et al., 2016). Although in the present study we measured the levels of $gp190^{PHOX}$, the principal membrane subunit of NOX2 enzyme, without evaluating other subunits of the enzymatic complex, we believe that the expression of the fundamental enzymatic subunit may reflect an increased response of the pro-oxidative system within the hippocampus. The detrimental effects of oxidative stress on PVB expression observed in response to CMS may also be the result of a glucocorticoid receptor (GR)-dependent extragenomic mechanism, which may affect the firing activity of these interneurons. Indeed, it has been proposed that the activation of membrane bound GR may induce the production of nitric oxide (NO), a small neurotransmitter responsible of the activation of PVB positive interneurons (Hu et al., 2010). The sustained activation of GR during chronic stressful condition may decrease PVB expression by sensitizing interneuron activation and, possibly, through a toxic effect due to NO release. Indeed, NO may be converted in different reactive nitrogen species thus leading to oxidative damage of proteins, lipids and DNA that are able to alter neuronal homeostasis (Moniczewski et al., 2015). We also showed that prolonged stress exposure alters the NRF2-KEAP-ARE system, a master regulator of the anti-oxidative response (Sandberg et al., 2014), with a decrease of NRF2 expression that may impair the activation of anti-oxidant mechanisms. Our results are in line with previous studies showing the negative effects of stress on NRF2. For example, a decreased expression of NRF2, with a parallel increase of oxidative stress, was found in social defeat-vulnerable animals exposed to chronic stress (Bouvier et al., 2016) as well as in rats exposed to 4 weeks of chronic stress (Omar and Tash, 2017).

Chronic treatment with lurasidone, an antipsychotic drug approved for the treatment of schizophrenia and bipolar depression, was able to normalize CMS-induced decrease of PVB expression in D-HIP. In this sense, other studies support the idea that antipsychotic drugs, such as clozapine (Filipovic et al., 2017) and risperidone (Piontkewitz et al., 2012), may regulate the function of GABAergic interneurons through the modulation of specific markers. While these drugs share high affinity for 5-HT7 receptors, it is difficult to ascertain if this is the unique mechanism through which these agents modulate GABAergic function, considering the vast heterogeneity in their receptor profiles. Indeed, we believe that the observed effects represent adaptive mechanisms following prolonged drug administration regulating complex neuronal circuits that will eventually lead to changes in selective GABAergic subtypes. Lastly, the effect of lurasidone closely resembles what we have recently observed in the hippocampus of adult mice exposed to prenatal immune challenge (Luoni et al., 2017).

Chronic treatment with lurasidone, an antipsychotic drug approved for the treatment of schizophrenia and bipolar depression, was able to normalize CMS-induce decrease of PVB expression in D-HIP, in line with data showing that psychotropic drugs administered in animals exposed to psychosocial stress normalized the alterations of PVB expression (Czeh et al., 2005; Filipović et al., 2017). Moreover, the effect of lurasidone closely resembles what we have recently observed in the hippocampus of adult mice exposed to prenatal immune challenge (Luoni et al., 2017).

Due to the peculiar receptor profile of lurasidone, it is difficult to enlighten a molecular mechanism responsible for the effects of the pharmacological treatment on GABAergic interneurons. Our results, however, suggest that the ability of lurasidone to modulate the oxidative stress balance may be part of its restorative effect. Indeed, chronic lurasidone treatment was able to induce the gene expression of NRF2 only in stressed animals, suggesting an anti-oxidative effect of the pharmacological treatment only under adverse conditions. The positive effect of lurasidone was also found at translational level, since the CMS-induced decrease of NRF2 was partially normalized in animals treated with the antipsychotic drug. Interestingly lurasidone is also able to regulate KEAP1, a chaperone protein that segregates NRF2 into the cytosol and promotes its proteasome-mediated degradation (Sandberg et al., 2014). Indeed, while KEAP1 protein levels in the cytosolic fraction were not altered by CMS exposure, we found that lurasidone treatment was able to reduce its levels, suggesting that the drug not only increases the expression of NRF2, but may also promote its activity through a negative modulation of KEAP1.

Our results suggest that, in addition to synaptic and neuroplastic mechanisms (Tarazi and Riva, 2013; Luoni et al., 2014), lurasidone is able to modulate the brain oxidative balance, which may contribute to its therapeutic effects and eventually enhance neuronal resiliency. Interestingly, similar mechanisms have also been described for other psychotropic drugs, including antidepressants (Martín-Hernández et al., 2016; Omar and Tash, 2017) and antipsychotics (MacDowell et al., 2016).It's interesting to note that the modulation of NRF2 and KEAP1 showed a significant Pearson correlation (positive and negative, respectively) with PVB protein levels, providing further support to the notion that the alterations of the GABAergic system following CMS exposure may be causally linked to a dysregulation of the oxidative balance in the D-HIP. In addition, the pharmacological treatment with lurasidone was able to induce the expression of key antioxidant enzymes related to NRF2 transcriptional activity. The specific increased levels of *Nqo1* and *Catalase* support the idea of an anti-oxidative activity of the drug. This is in line with previously published data, showing that -despite stress exposure did not impair the transcription of antioxidant enzymes- the administration of an atypical antipsychotic increased antioxidant response in stressed animals (MacDowell et al., 2016).

In summary, the susceptibility of PVB neurons to stress may represent a key mechanism contributing to functional and structural deterioration in specific brain regions, such as the D-HIP, associated with psychiatric illness. The ability to counteract PVB alterations, for example with antioxidants/redox regulators (Steullet et al., 2017), or to promote the activity of PVB neurons (Chen et al., 2017) may represent a novel and important strategy to promote resilience. With this respect, our data provide new insights on the mechanism of action of lurasidone in the context of stress-related hippocampal dysfunction, suggesting that its pharmacological profile, which can improve neuronal/synaptic plasticity in hippocampus and cortex through both protective (antioxidant) and functional (BDNF) (Luoni et al., 2014) mechanisms, should supports clinical efficacy reported in schizophrenia and bipolar disorder.

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Statement of Interest

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Captions

Table 1 Sequences of forward and reverse primers and probes used in qRT-PCR analysis and purchased from Eurofins MWG Operon (Germany)* and Applied Biosystem (Italy)**

Table 2 Primary and secondary antibodies used in western blot analyses.

(o/n, overnight; o/2n, over two nights; RT, room temperature; BSA, bovine serum albumin)

Table 3 Gene expression analysis of interneuron markers *Pvb, Sst, Calb, Npy* in the prefrontal cortex of rats exposed to chronic mild stress.

The mRNA levels of parvalbumin (*Pvb*), somatostatin (*Sst*) neuropeptide y (*Npy*) and calbindin (*Calb*) were measured in the prefrontal cortex of rats exposed to CMS, in combination with chronic treatment with vehicle or lurasidone. The data expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle set at 100%) are the mean +/- SEM of at least 7 independent determinations.

Figure 1 Gene expression analysis of interneuron markers *Pvb, Sst, Calb, Npy* in the dorsal and ventral hippocampus of rats exposed to chronic mild stress: modulation by lurasidone treatment

The mRNA levels of parvalbumin (*Pvb*), somatostatin (*Sst*) neuropeptide y (*Npy*) and calbindin (*Calb*) were measured in the dorsal (A, B, C, D) and ventral (E, F, G, H) hippocampus of rats exposed to CMS, in combination with chronic treatment with vehicle or lurasidone. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle set at 100%) are the mean +/- SEM of at least 7 independent determinations.

*P<0.05, **P<0.01, ***P<0.001 vs. No Stress/Vehicle; ##P<0.01, ###P<0.001 vs. Stress/Vehicle (Two-way ANOVA with PLSD).

Figure 2 Protein expression of PVB in the hippocampus of rats exposed to chronic mild stress: modulation by lurasidone treatment

The protein levels of parvalbumin (PVB) were measured in the dorsal (A) and in the ventral (B) hippocampus of rats exposed to CMS, in combination with chronic treatment with vehicle or lurasidone. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%) are the mean +/- SEM of at least 6 independent determinations. Representative Western blot bands of PVB are shown under the respective graphs.

***P<0.001 vs. No Stress/Vehicle; ###P<0.001 vs. Stress/Vehicle (Two-way ANOVA with PLSD).

Figure 3 Gene expression and protein analyses of Nox2 in the dorsal hippocampus of rats exposed to chronic mild stress: modulation by lurasidone treatment

The mRNA (A) and the protein levels (B) of NADH oxidase-2 (NOX2) were measured in the dorsal hippocampus of rats exposed to CMS in combination with chronic treatment with vehicle or lurasidone. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%) are the mean +/- SEM of at least 8 independent determinations. Representative Western blot bands of NOX2 are shown under the respective graph.

*P<0.05, **P<0.01 vs. No Stress/Vehicle (Two-way ANOVA with PLSD).

Figure 4 Analysis of NRF2-KEAP1 expression in the dorsal hippocampus of rats exposed to chronic mild stress: modulation by lurasidone treatment

The mRNA (A) and the protein levels (B) of nuclear factor-E2-related factor-2 (NRF2) and the protein levels of the chaperon protein kelch like ECH associated protein-1 (KEAP1, C) were measured in the cytosolic fraction of the dorsal hippocampus of rats exposed to CMS in combination with chronic treatment with vehicle or lurasidone. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%) are the mean +/- SEM of at least 7 independent determinations. Representative Western blot bands of NRF2 and KEAP1 are shown under the respective graphs.

*P<0.05, **P<0.01 vs. No Stress/Vehicle; #P<0.05, ##P<0.01 vs. Stress/Vehicle (Two-way ANOVA with PLSD).

Figure 5 Pearson correlation analysis between NRF2 or KEAP1 and PVB protein levels in the dorsal hippocampus

The Pearson moment-product correlation (r) between NRF2 (A), KEAP1 (B) and PVB protein levels were measured in the dorsal hippocampus of rats exposed to CMS in combination with chronic treatment with vehicle or lurasidone. The statistical significance was assumed with P< 0.05.

Figure 6 Analysis of NRF2-induced transcriptional response in the dorsal hippocampus of rats exposed to chronic mild stress: modulation by lurasidone treatment

The mRNA levels of Sufiredoxin (*Srxn1*, A), Heme oxigenase-1 (*Ho-1*, B), NAD(P)H dehydrogenase [quinone]-1 (*Nqo1*, C) and Catalase (*Cat*, D) were measured in the dorsal hippocampus of rats exposed to CMS in combination with chronic treatment with vehicle or lurasidone. The data, expressed as a percentage of unstressed rats treated with vehicle (No Stress/Vehicle, set at 100%) are the mean +/- SEM of at least 8 independent determinations.

*P<0.01 vs. No Stress/Vehicle; ##P<0.01 vs. Stress/Vehicle (Two-way ANOVA with PLSD).

Table 1

Gene	Forward Primer	Reverse Primer	Probe
Pvalb*	CTGGACAAAGACAAAAGTGGC	GACAAGTCTCTGGCATCTGAG	CCTTCAGAATGGACCCCAGCTCA
Sst*	ACTCCGTCAGTTTCTGCAG	CAGGGCATCGTTCTCTGTC	AGTTCCTGTTTCCCGGTGGCA
Calb*	AGAACTTGATCCAGGAGCTTC	CTTCGGTGGGTAAGACATGG	TGGGCAGAGAGATGATGGGAAAATAGGA
Npy*	GACAGAGATATGGCAAGAGATCC	CTAGGAAAAGTCAGGAGAGCAAG	CCCCAGAACAAGGCTTGAAGACCC
Nrf2**	Rn00582415_m1		
Keap1**	Rn01448220_m1		
Nox2**	Rn00675098_m1		
Srnx**	Rn04337926_g1		
Ho-1**	Rn00561387_m1		
Nqo1**	Rn00566528_m1		
Cat**	Rn00560930_m1		
ß Actin*	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	TCTGGGTCATCTTTTCACGGTTGGC

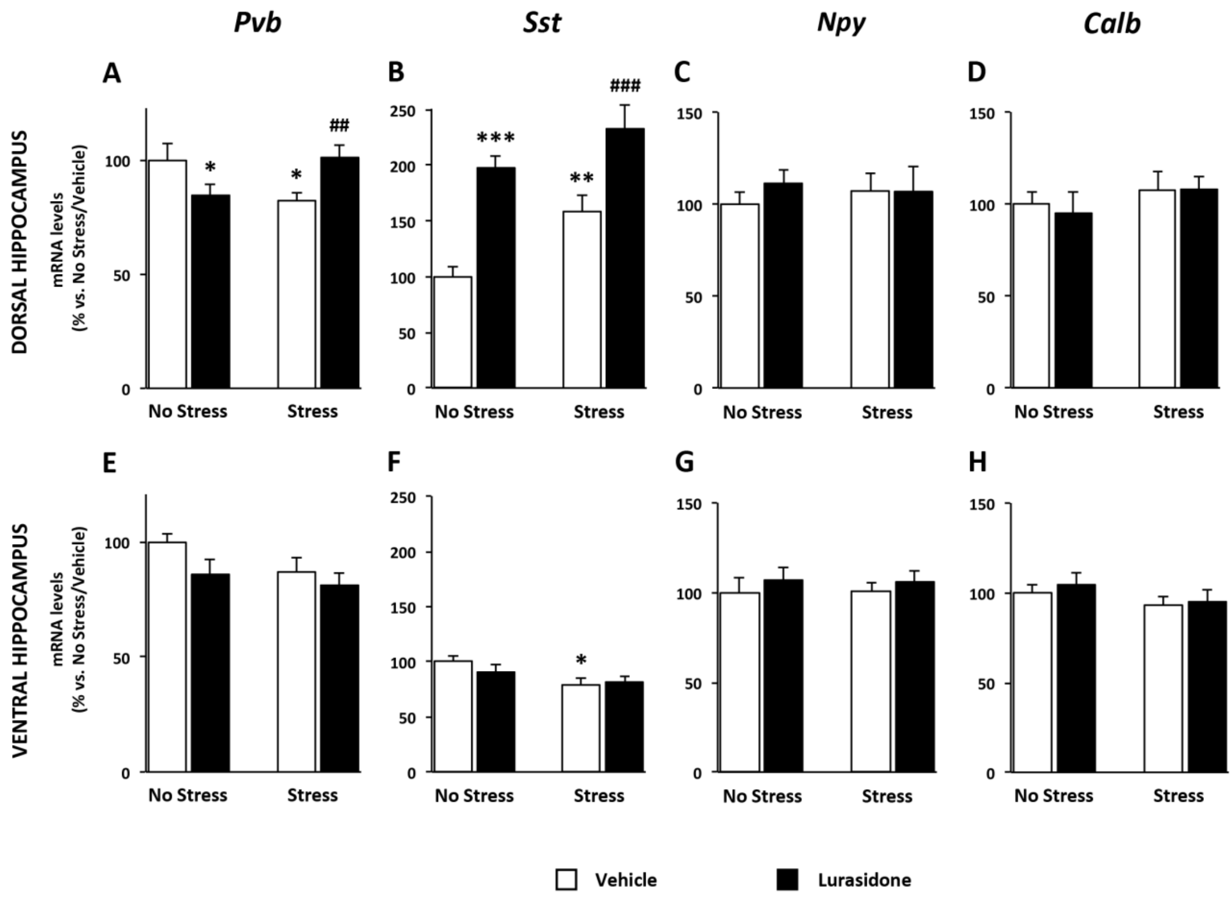
Table 2

Primary antibody	Primary antibody condition	Secondary antibody condition
PVB - 10 kDa (Abcam)	1:2500 in 5% non-fat dry milk 4°C o/n	1:2500, anti-rabbit (Cell Signaling) 5% non-fat dry milk, 1h, RT
NRF2 - 110 kDa (R&D System)	1:500 in 5% BSA 4°C o/2n	1:500, anti-mouse (Sigma-Aldrich) 5% BSA, 1h, RT
KEAP1 - 66 kDa (R&D System)	1:250 3% non-fat dry milk 4°C o/n	1:500, anti-mouse (Sigma-Aldrich) 5% non-fat dry milk, 1h, RT
NOX2 - 58 kDa (BD)	1:500 3% non-fat dry milk 4°C o/n	1:500, anti-mouse (Sigma-Aldrich) 3% non-fat dry milk, 1h, RT
β-ACTIN 43 kDa (Sigma-Aldrich)	1:2000 in 3% non-fat dry milk 1h, RT	1:20000, anti-mouse (Sigma-Aldrich) 3% non-fat dry milk, 1h, RT

Table 3

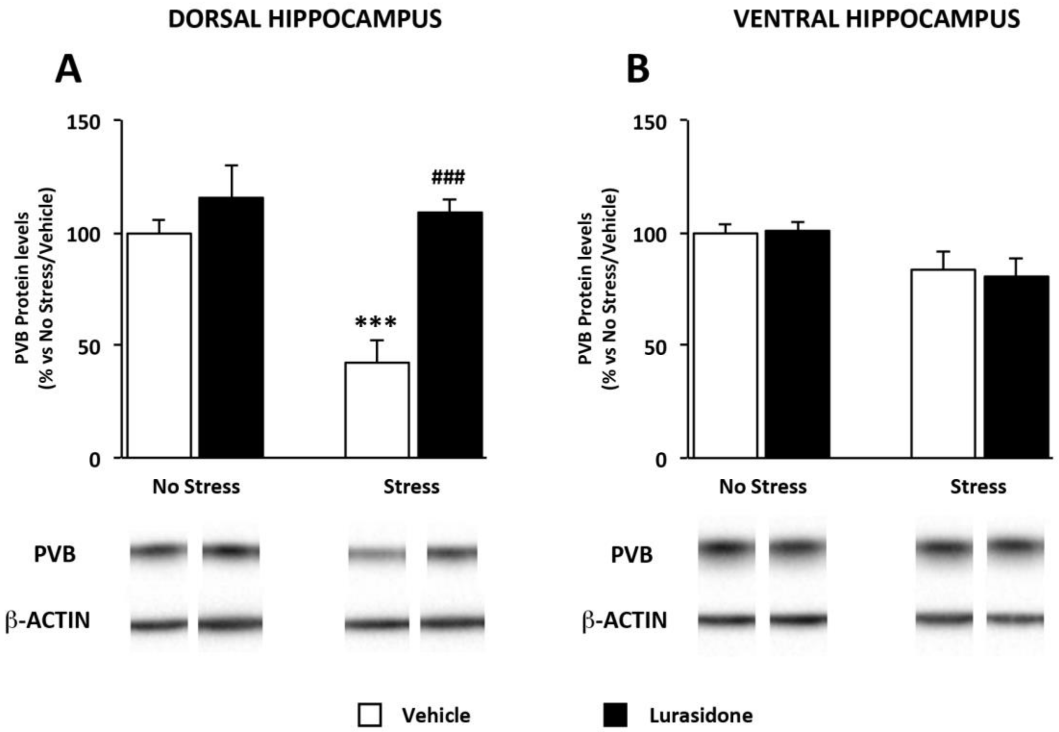
	No Stress	Lurasidone	Stress	Lurasidone/Stress
Pvb	100 ± 5	92 ± 6	95 ± 5	90 ± 3
Sst	100 ± 6	117 ± 8	103 ± 5	110 ± 8
Calb	100 ± 5	99 ± 8	97 ± 4	99 ± 3
Npy	100 ± 4	96 ± 3	96 ± 6	97 ± 4

Figure 1.



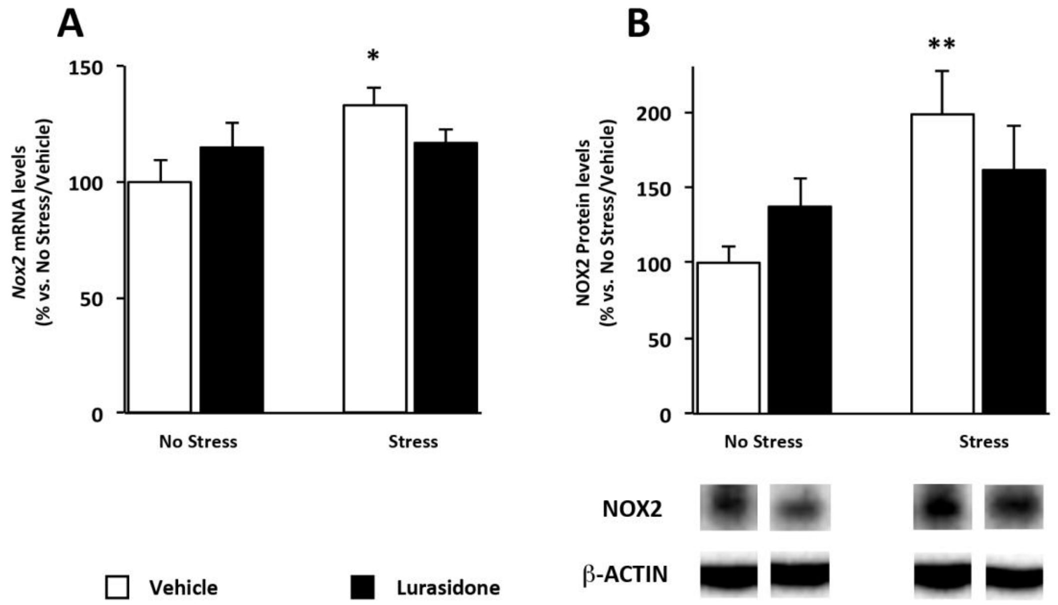
Pvb
Sst
Npy
Calb
A
B
C
D
DORSAL HIPPOCAMPUS
mRNA levels
(% vs. No Stress/Vehicle)
No Stress
Stress
E
F
G
H
VENTRAL HIPPOCAMPUS
Vehicle
Lurasidone

Figure 2.



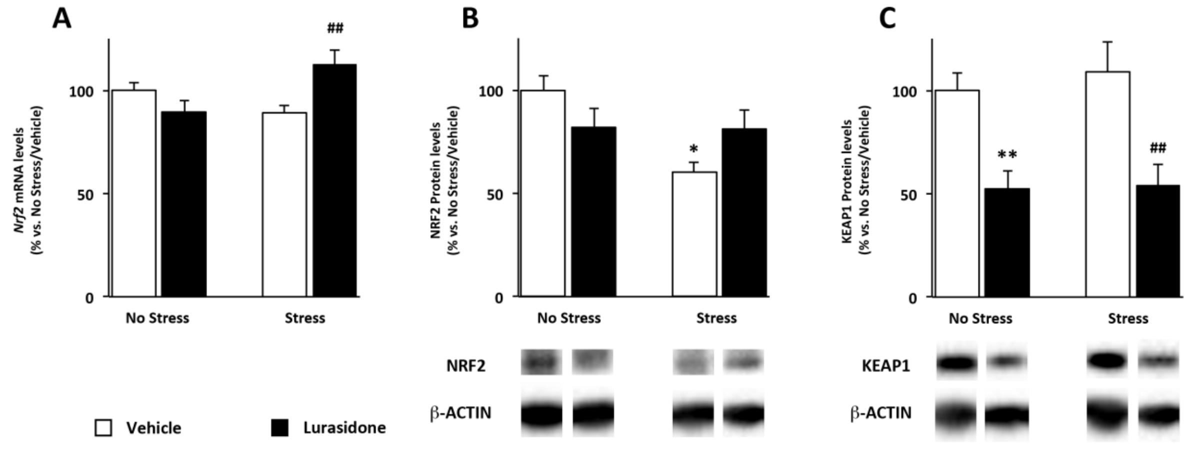
DORSAL HIPPOCAMPUS
VENTRAL HIPPOCAMPUS
A
B
150
100
50
0
PVB Protein levels
(% vs No Stress/Vehicle)
***
###
No Stress
Stress
PVB
β-ACTIN
Vehicle
Lurasidone

Figure 3.



A
B
150
100
50
0
*
**
Nox2 mRNA levels
(% vs. No Stress/Vehicle)
No Stress
Stress
200
150
100
50
0
NOX2 Protein levels
(% vs. No Stress/Vehicle)
No Stress
Stress
NOX2
β-ACTIN
Vehicle
Lurasidone

Figure 4.



A
B
C
Nrf2 mRNA levels (% vs. No Stress/Vehicle)
NRF2 Protein levels (% vs. No Stress/Vehicle)
KEAP1 Protein levels (% vs. No Stress/Vehicle)
100
50
0
No Stress
Stress
##
*
**
NRF2
β-ACTIN
KEAP1
Vehicle
Lurasidone

Figure 5.

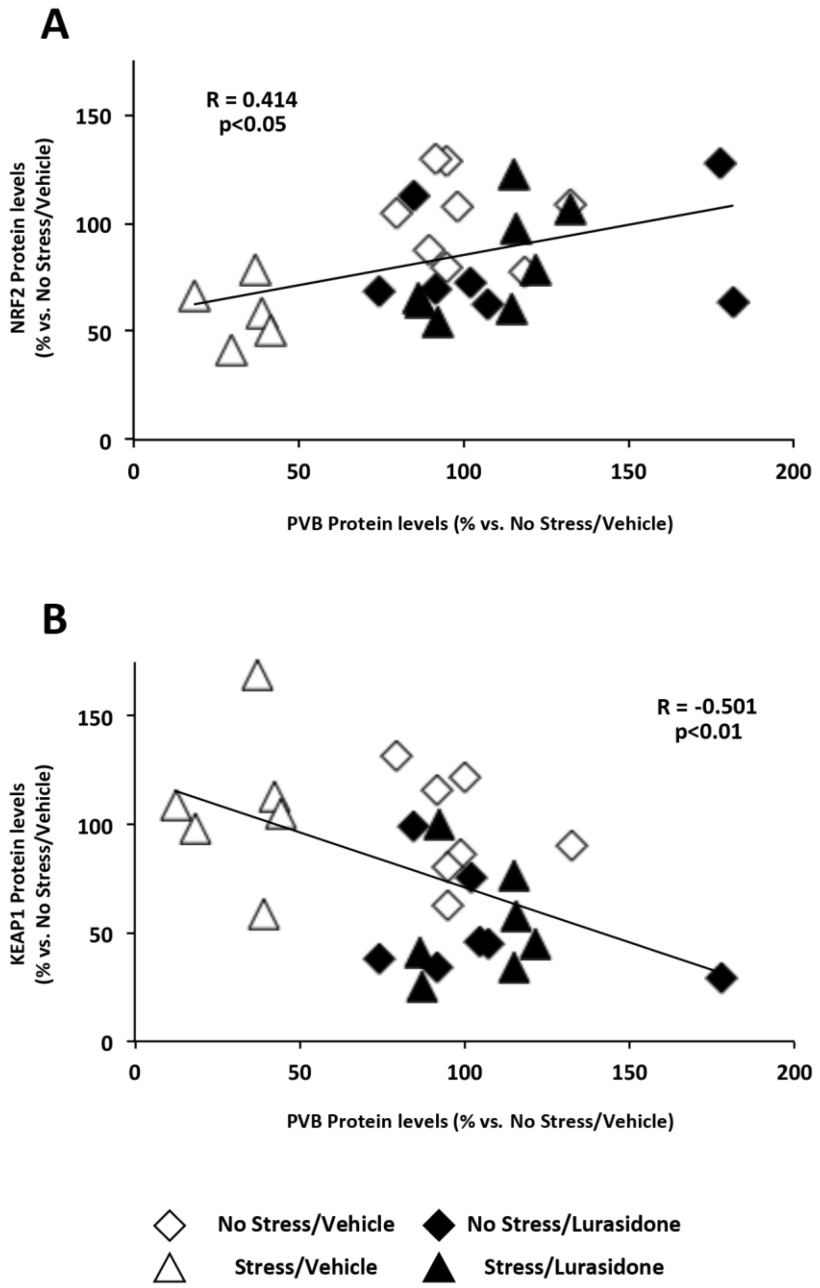
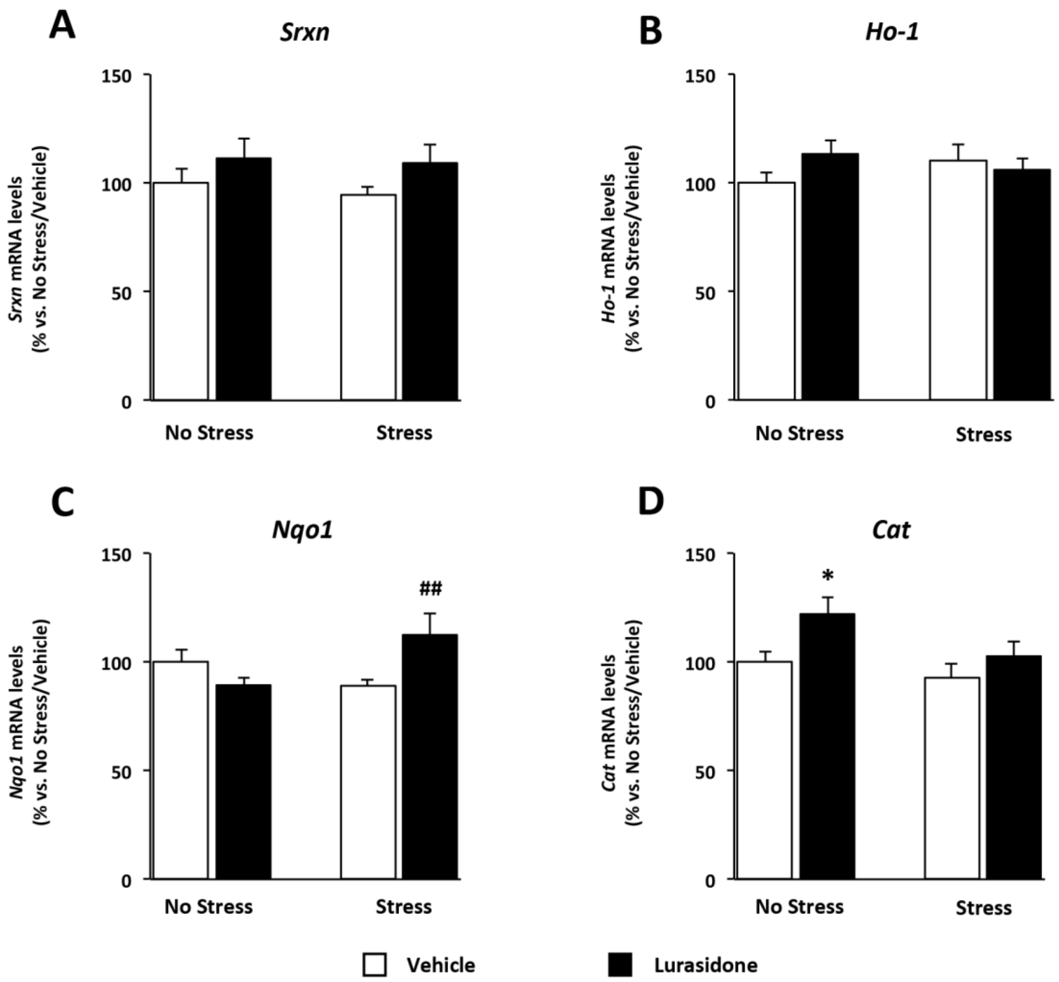

A
R = 0.414
p<0.05
NRF2 Protein levels
(% vs. No Stress/Vehicle)
150
100
50
0
0
50
100
150
200
PVB Protein levels (% vs. No Stress/Vehicle)
B
R = -0.501
p<0.01
KEAP1 Protein levels
(% vs. No Stress/Vehicle)
150
100
50
0
0
50
100
150
200
PVB Protein levels (% vs. No Stress/Vehicle)
No Stress/Vehicle
No Stress/Lurasidone
Stress/Vehicle
Stress/Lurasidone

Figure 6.


A
Srxn
150
100
50
0
Srxn mRNA levels
(% vs. No Stress/Vehicle)
No Stress
Stress
B
Ho-1
150
100
50
0
Ho-1 mRNA levels
(% vs. No Stress/Vehicle)
No Stress
Stress
C
Nqo1
150
100
50
0
##
Nqo1 mRNA levels
(% vs. No Stress/Vehicle)
No Stress
Stress
D
Cat
150
100
50
0
*
Cat mRNA levels
(% vs. No Stress/Vehicle)
No Stress
Stress
Vehicle
Lurasidone