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Chronic Stress and Antidepressant Agomelatine Induce Region-Specific Changes in Synapsin I Expression in the Rat Brain

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The antidepressant agomelatine acts as a melatonergic receptor (MT_1/MT_2) agonist and 5-HT_{2C} receptor antagonist. Agomelatine has demonstrated efficacy in treating depression, but its neurobiological effects merit further investigation. Preclinical studies reported that agomelatine enhances adult hippocampal neurogenesis and increases expression of several neuroplasticity-associated molecules. Recently, we showed that agomelatine normalizes hippocampal neuronal activity and promotes neurogenesis in the stress-compromised brain. To characterize further the effects of this antidepressant in the stressed brain, here we investigated whether it induces changes in the expression of synapsin I (SynI), a regulator of synaptic transmission and plasticity. Adult male rats were subjected to daily footshock stress and agomelatine treatment for 3 weeks. Their brains were subsequently stained for total and phosphorylated SynI. Chronic footshock and agomelatine induced region-specific changes in SynI expression. Whereas chronic stress increased total SynI expression in all layers of the medial prefrontal cortex, agomelatine treatment abolished some of these effects. Furthermore, chronic agomelatine administration decreased total SynI expression in the hippocampal subregions of both stressed and nonstressed rats. Importantly, chronic stress decreased the fraction of phosphorylated SynI in all layers of the medial prefrontal cortex as well as selectively in the outer and middle molecular layers of the hippocampal dentate gyrus. These stress effects were at least partially abolished by agomelatine. Altogether, our data show that chronic stress and agomelatine treatment induce region-specific changes in SynI expression and its phosphorylation. Moreover, agomelatine partially counteracts the stress effects on SynI, suggesting a modulation of synaptic function by this antidepressant.

Key words: affective disorders; chronic footshock stress; depression; synaptic plasticity

The novel antidepressant agomelatine acts as a melatonergic receptor (MT_1/MT_2) agonist (Audinot et al., 2003) and serotonergic receptor (5-HT_{2C}) antagonist (Millan et al., 2003). Its antidepressant activity results from a potential synergy between these sets of receptors (de Bodinat et al., 2010). Agomelatine has shown efficacy in treatment of major depressive disorder (Kennedy and Emsley, 2006; Olie and Kasper, 2007; Goodwin et al., 2009).

Preclinical studies have demonstrated a variety of agomelatine-mediated changes in the brain, including increased hippocampal neurogenesis, enhanced expression of brain-derived neurotrophic factor (BDNF), and activation of several cellular signals implicated in the action of antidepressant drugs (Banasr et al., 2006; Conboy et al., 2009; Soumier et al., 2009). Moreover, agomelatine was shown to be effective in the stress-compromised brain, in which it normalized hippocampal neuronal activity and promoted neurogenesis (Dagyte et al., 2010, 2011a). Also, agomelatine was reported to reverse altered hippocampal neurogenesis and BDNF expression in glucocorticoid receptor-impaired mice, a transgenic animal model of depression (Paizanis et al., 2010).

Stress is a significant risk factor for development of many psychiatric disorders, including depression (Kendler et al., 1999). Thus, animal models of chronic stress provide a valuable tool to investigate neurobiological changes underlying stress-related psychopathologies and therapeutic actions of antidepressant drugs (Fuchs et al., 2001; Nestler et al., 2002). Such models revealed that

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stress is associated with impaired neurotrophin signaling (Rasmusson et al., 2002; Gronli et al., 2006), neuronal and dendritic atrophy (Magarinos and McEwen, 1995; Radley et al., 2004; Bessa et al., 2009), and altered synaptic plasticity (Cerqueira et al., 2007; Goldwater et al., 2009; Joels et al., 2009) in the hippocampus and prefrontal cortex. On the other hand, antidepressants were shown to increase neurotrophin signaling and promote neuronal and synaptic remodeling in these limbic brain regions (Duman, 2004; Castren et al., 2007; Sairanen et al., 2007; Bessa et al., 2009). Enhanced neuronal connectivity and strengthening of specific synapses are hypothesized to underlie the mechanism of antidepressant action; however, the precise changes at the synaptic level are largely unknown.

Synapsins are the most abundant synaptic vesicle-associated proteins and play multiple roles in synaptic transmission and plasticity (De Camilli et al., 1983; Greengard et al., 1993; Cesca et al., 2010). Synapsins tether synaptic vesicles to the cytoskeleton in a phosphorylation-dependent manner and thereby regulate neurotransmitter release. Also, synapsins are suggested to participate in the formation of new nerve terminals, maturation of synaptic contacts, neurite elongation, and synthesis of other synaptic vesicle proteins (Ferreira and Rapoport, 2002). Three distinct synapsin genes give rise to several differentially spliced isoforms whose expression is developmentally regulated (Sudhof et al., 1989; Ferreira et al., 2000). Synapsins Ia and Ib, collectively referred to as *synapsin I* (SynI), are the most abundant isoforms in mature neurons, accounting for 6% of total vesicle proteins (Huttner et al., 1983).

The present study examined SynI expression and its phosphorylation pattern in forebrain regions of rats exposed to chronic stress and treated with the antidepressant agomelatine. We used a chronic footshock stress model, which was previously shown to induce multiple changes in stress-related brain areas (Trentani et al., 2002, 2003; Kuipers et al., 2003, 2006; Westenbroek et al., 2004; Dagyte et al., 2009). Our recent study using this model revealed that chronic treatment with agomelatine normalizes stress-compromised neuronal activity in the dentate gyrus and promotes hippocampal neurogenesis (Dagyte et al., 2010). Here we investigated whether chronic stress and agomelatine treatment induce distinct changes in SynI expression in several brain regions, to characterize further the effects of this novel antidepressant in the stressed brain. We hypothesized that chronic footshock impairs SynI expression in stress-related brain areas and that treatment with agomelatine abolishes this effect.

MATERIALS AND METHODS

Animals and Housing

The experiments were performed using adult (10–12 weeks of age) male Wistar rats (Harlan, Horst, The Netherlands) weighing 300–350 g at the beginning of the experiment. The animals were housed individually in a climate-controlled room with constant temperature (21°C ± 1°C) and a 12-hr light/12-hr dark cycle (lights on at 0600 hr). Food and water were available ad libitum. The experiments were approved by the ethical committee for the use of experimental animals of the University of Groningen and carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental Protocol

Four groups of rats were used for the present experiment: two groups of home-cage control rats (CTR), which were treated either with vehicle (CTR Veh) or with agomelatine (CTR Ago), and two groups of chronically stressed rats (STR), which were also treated either with vehicle (STR Veh) or with agomelatine (STR Ago). Each group consisted of eight animals. The home-cage control rats were sacrificed concomitantly with the stressed rats, which were euthanized 24 hr after the last footshock exposure. A 24-hr delay between the last footshock session and animal sacrifice was chosen with the intention to avoid confounding effects of the acute stress response and in an attempt to focus on lasting effects of chronic stress.

Stress Procedure

During a 2-week acclimatization period before the start of the experiment, all rats were weighed and handled daily. A chronic footshock stress model was used in this study to dissect the prolonged stress-evoked synaptic changes that might predispose to psychopathology. This chronic stress model was previously shown to induce effects on the hypothalamic-pituitary-adrenal axis and behavior (Trentani et al., 2002, 2003; Kuipers et al., 2003; Westenbroek et al., 2003, 2005; Dagyte et al., 2009). Footshock stress was applied in a footshock box with a grid floor connected to a shock generator and scrambler. Stress-group rats were subjected to a daily session of footshock stress for 21 consecutive days. During the session in the footshock box, rats received five uncontrollable and inescapable footshocks (0.8 mA in intensity and 8 sec in duration). All footshock sessions took place during the light phase. To increase unpredictability and minimize habituation, both timing and duration of footshock sessions as well as intervals between shocks within a session varied randomly (session starting time between 0800 and 1300 hr, session duration 15–80 min, shock interval 1–15 min). Control rats stayed undisturbed in their home cages throughout the experiment.

Drug Treatment

Hydroxyethylcellulose (HEC) 1% was used as a vehicle for agomelatine delivery. Agomelatine solution was prepared every day by dissolving agomelatine powder in 1% HEC at a concentration of 40 mg/ml. The choice of agomelatine dose was made on the basis of its activity at this concentration in animal models of depression and anxiety (Papp et al., 2003; Millan et al., 2005) and on neuroplasticity (Banasr et al., 2006; Soumier et al., 2009; Dagyte et al., 2010). Rats were injected intraperitoneally either with agomelatine (40 mg/kg)

or with vehicle daily at 1600 hr (2 hr prior to the dark phase) for 21 days. All injections were done after footshocks had been applied.

Brain Collection and Immunohistochemistry

At the end of the experiment, 24 hr after the last stress session and 18 hr after the last agomelatine injection, rats were anesthetized with sodium pentobarbital and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer. Brains were dissected and postfixed in the same solution overnight at 4°C and subsequently cryoprotected by immersion in a 30% buffered sucrose solution for up to 48 hr. Coronal serial sections of 30 μm were cut using a cryostat and stored in 0.01 M phosphate-buffered saline (PBS) with 0.1% sodium azide at 4°C until immunohistochemistry was performed.

Effects of chronic footshock stress and agomelatine treatment on synaptic function were assessed by immunohistochemistry for total and phosphorylated SynI, a marker of synaptic vesicles. The immunostainings were performed on free-floating sections under continuous mild agitation. Brain sections were preincubated in 5% normal serum and 0.3% Triton X-100 and then were incubated with one of the following antibodies for 40 hr at 4°C: primary rabbit anti-SynI (1:600; HPA000397; Sigma-Aldrich, Stockholm, Sweden) or rabbit antiphospho-Ser^{549}-SynI (1:100; p1560-549; Phosphosolutions, Aurora, CO). Subsequently, sections were rinsed in 0.01 M PBS and incubated for 2 hr at room temperature with secondary biotinylated goat anti-rabbit antibody (1:500; Jackson Immunoresearch, Suffolk, United Kingdom). Then, avidin-biotin complex (1:500; Vector ABC kit; Vector Laboratories, Burlingame, CA) was added for 2 hr, after which the staining was visualized with 1 mg/ml diaminobenzidine and 0.003% H_2O_2. Thereafter, sections were rinsed, mounted on slides, dehydrated, and coverslipped for microscopic analysis.

To control for cross-reactivity resulting from nonspecific binding, negative controls were included by incubating several sections and performing the immunostainings without one of the antibodies (primary or secondary). All of these reactions yielded immunonegative results, confirming that the obtained immunostainings with our primary antibodies were due solely to immunodetection of these primary antibodies.

Quantification

All analyses were carried out by an observer blind to the group assignment. The immunoreactivity (ir) of total and phosphorylated SynI was quantified by measuring optical density (OD) of each staining. Images were taken using a Leica charged-coupled device digital camera mounted on a microscope (DMIRB; Leica, Cambridge, United Kingdom) at ×50. The OD of total and phosphorylated SynI expression was quantified in multiple brain regions of interest (ROIs) and corrected for nonspecific background labeling, measured in the corpus callosum [the final corrected OD (COD) = the OD measured in the ROI – the OD measured in the corpus callosum]. ROIs included the medial prefrontal cortex (mPFC; layer I–VI of the prelimbic area: Bregma +3.70 to +2.20), the hippocampal dentate gyrus [DG; outer, middle, and inner molecular layers (OML, MML, and IML, respectively) and hilus: Bregma –2.80 to –4.30], the CA3 area [stratum radiatum (SR), stratum lucidum (SL), and stratum oriens (SO): Bregma –2.80 to –4.30], the CA1 area [stratum lacunosum-moleculare (SLM), stratum radiatum (SR) and stratum oriens (SO): Bregma –2.80 to –4.30], and the lateral posterior thalamic nucleus (its mediorostral and laterorostral parts, LPMR and LPLR, respectively: Bregma –3.80 to –4.30). The latter ROI was chosen as a control region, because LPMR and LPLR are not directly involved in processes associated with chronic stress and antidepressant treatment. Bregma coordinates were determined according to the rat brain atlas (Paxinos and Watson, 2007). The COD, quantified in each ROI, was expressed in arbitrary units corresponding to gray levels using a Quantimet 550 image analysis system (Leica).

The ratio between phosphorylated and total SynI was determined by dividing the COD of phospho-SynI by the COD of total SynI in each ROI. Although we did not prove colocalization of phospho-SynI and total SynI stainings, nonetheless such presentation of the results is meaningful insofar as it helps in interpreting our findings. Because the COD was measured in arbitrary units corresponding to the intensity of the staining, certain ROIs with a relatively high phospho-SynI expression had ratio values higher than 1.

Statistical Analysis

Two-way ANOVA was used to determine the effects of stress and agomelatine treatment on SynI expression and its phosphorylation. When significant effects were found, post hoc analysis was performed using the Fisher LSD test. The level of significance was set at $P < 0.05$.

RESULTS

mPFC

Figure 1 shows representative examples of SynI and phospho-SynI immunostainings in the mPFC. Changes in total SynI expression and its phosphorylation in different layers of the mPFC resulting from chronic stress and agomelatine treatment are shown in Figure 2. Two-way ANOVA revealed a significant stress effect on total SynI-ir in all layers of the mPFC (Fig. 2A; layer I: $F_{1,28} = 4.99$, $P = 0.03$; layer II: $F_{1,28} = 5.22$, $P = 0.03$; layer III: $F_{1,28} = 6.41$, $P = 0.02$; layer V: $F_{1,28} = 7.02$, $P = 0.01$; layer VI: $F_{1,28} = 9.82$, $P = 0.004$). Post hoc analysis showed a significant chronic stress-induced increase in total SynI-ir in all layers of the mPFC in vehicle-treated animals (CTR Veh vs. STR Veh: layer I, $P = 0.01$; layer II, $P = 0.03$; layer III, $P = 0.03$; layer V, $P = 0.04$; layer VI, $P = 0.01$). This stress effect was not present in animals concomitantly treated with agomelatine. A statistically significant difference was found in layer I of the mPFC (STR Veh vs. STR Ago: $P = 0.03$). In other layers of the mPFC, a similar trend was observed; however, it did not reach statistical significance (STR Veh vs. STR Ago: layer II, $P = 0.066$; layer III, $P = 0.11$; layer V, $P = 0.15$; layer VI, $P = 0.076$). Figure 2B shows the expression of phospho-SynI in different layers of the mPFC. Two-way ANOVA

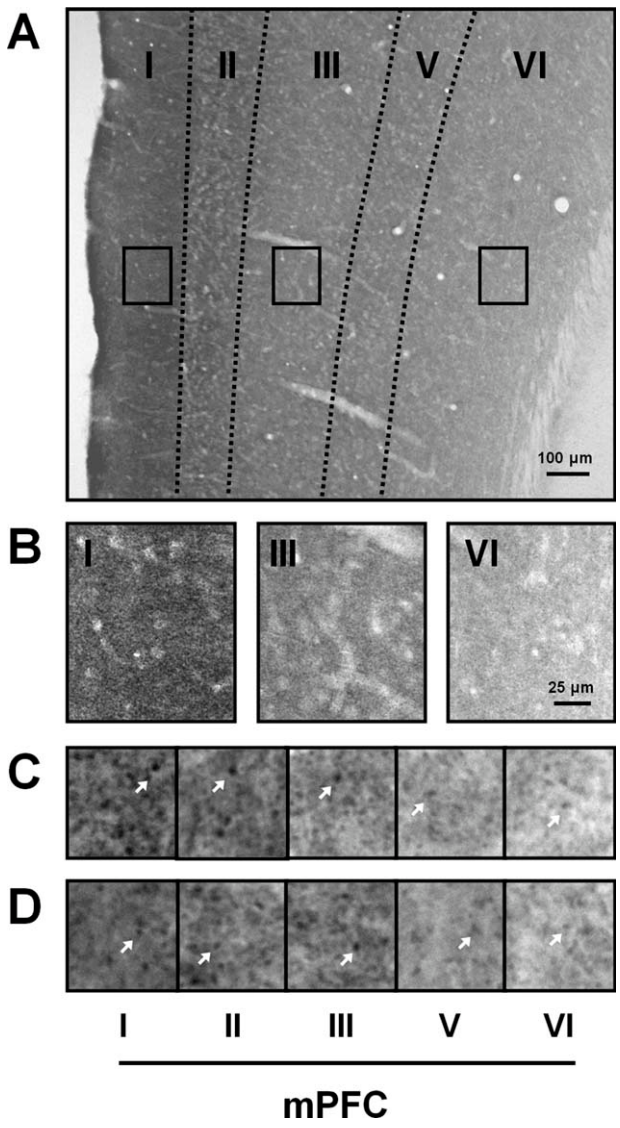

Fig. 1. Representative photomicrograph of SynI immunostaining in the mPFC of nonstressed vehicle-treated animals (**A**). B show higher magnification of SynI expression in layers I, III, and VI. Arrows point to total SynI (**C**) and phospho-SynI (**D**) labeling in different layers (I–VI) of the mPFC. One square with detailed overview (C,D) = 15 × 15 µm.

revealed no significant effects. A significant stress effect emerged when ratio between phosphorylated and total SynI was calculated (Fig. 2C). This stress effect was found in all layers of the mPFC (layer I, $F_{1,28} = 6.60$, $P = 0.02$; layer II, $F_{1,28} = 10.04$, $P = 0.004$; layer III, $F_{1,28} = 17.08$, $P = 0.0003$; layer V, $F_{1,28} = 13.15$, $P = 0.001$; layer VI, $F_{1,28} = 8.70$, $P = 0.006$). Post hoc analysis showed that chronic stress significantly decreased the ratio of SynI phosphorylation in all layers of the mPFC in vehicle treated animals (CTR Veh vs. STR Veh: layer I, $P = 0.046$; layer II, $P = 0.01$; layer III, $P = 0.002$; layer V, $P = 0.006$; layer VI, $P = 0.03$).

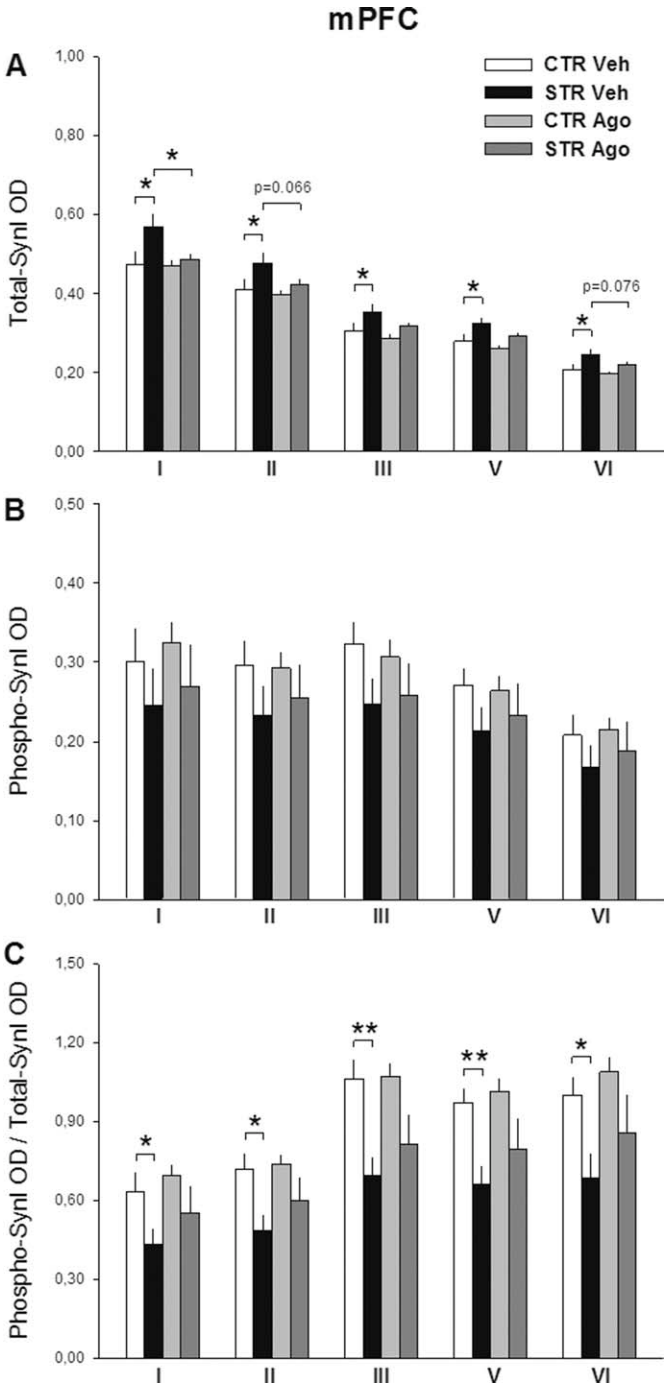

Fig. 2. Effects of chronic stress and agomelatine treatment on total and phosphorylated SynI expression in different layers (I–VI) of the mPFC. Chronic stress increased total SynI-ir in all layers of the mPFC, and agomelatine treatment partially abolished these changes (**A**). Although no significant effects were found on the levels of phospho-SynI-ir (**B**), the ratio between phosphorylated and total SynI revealed that chronic stress significantly decreased the fraction of phospho-SynI (**C**). $*P < 0.05$, $**P < 0.01$.

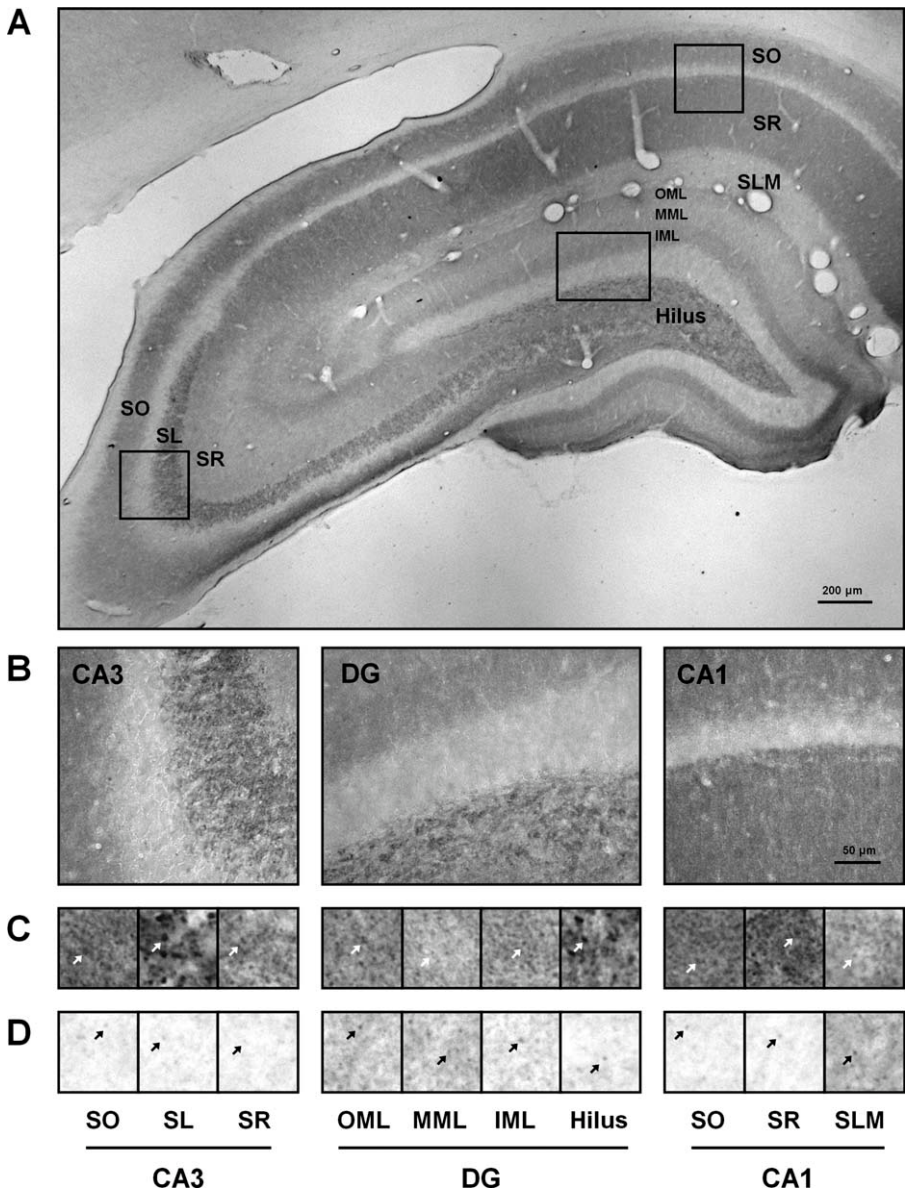

Fig. 3. Representative photomicrograph of SynI immunostaining in the hippocampus of nonstressed vehicle-treated animals (**A**). B shows higher magnification of SynI expression in the DG, CA3, and CA1 areas. Arrows point to total SynI (**C**) and phospho-SynI (**D**) labeling in different layers of the hippocampal subregions. DG, dentate gyrus; OML, outer molecular layer; MML, middle molecular layer; IML, inner molecular layer; SO, stratum oriens; SL, stratum lucidum; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. One square with detailed overview (C,D) = 15 × 15 µm.

Although treatment with agomelatine seemed to reverse this stress-induced decrease in the ratio of SynI phosphorylation partially, none of its effects reached statistical significance.

Hippocampus

Figure 3 shows representative examples of SynI and phospho-SynI immunostainings in the hippocampus. Changes in total SynI expression and its phosphorylation in the hippocampal subregions resulting from chronic stress and agomelatine treatment are depicted in Figures 4–6.

Dentate gyrus. Two-way ANOVA revealed a significant drug effect on total-SynI-ir in the DG of the hippocampus (Fig. 4A). Whereas stress had no significant effect on total SynI expression, agomelatine decreased total SynI-ir in all the molecular layers of the DG and in the hilus of both control and stressed rats (OML: $F_{1,28} = 9.99$, $P = 0.004$; MML: $F_{1,28} = 17.59$, $P = 0.0003$; IML: $F_{1,28} = 12.01$, $P = 0.002$; hilus: $F_{1,28} = 10.65$, $P = 0.003$). Analysis of phospho-SynI expression in the

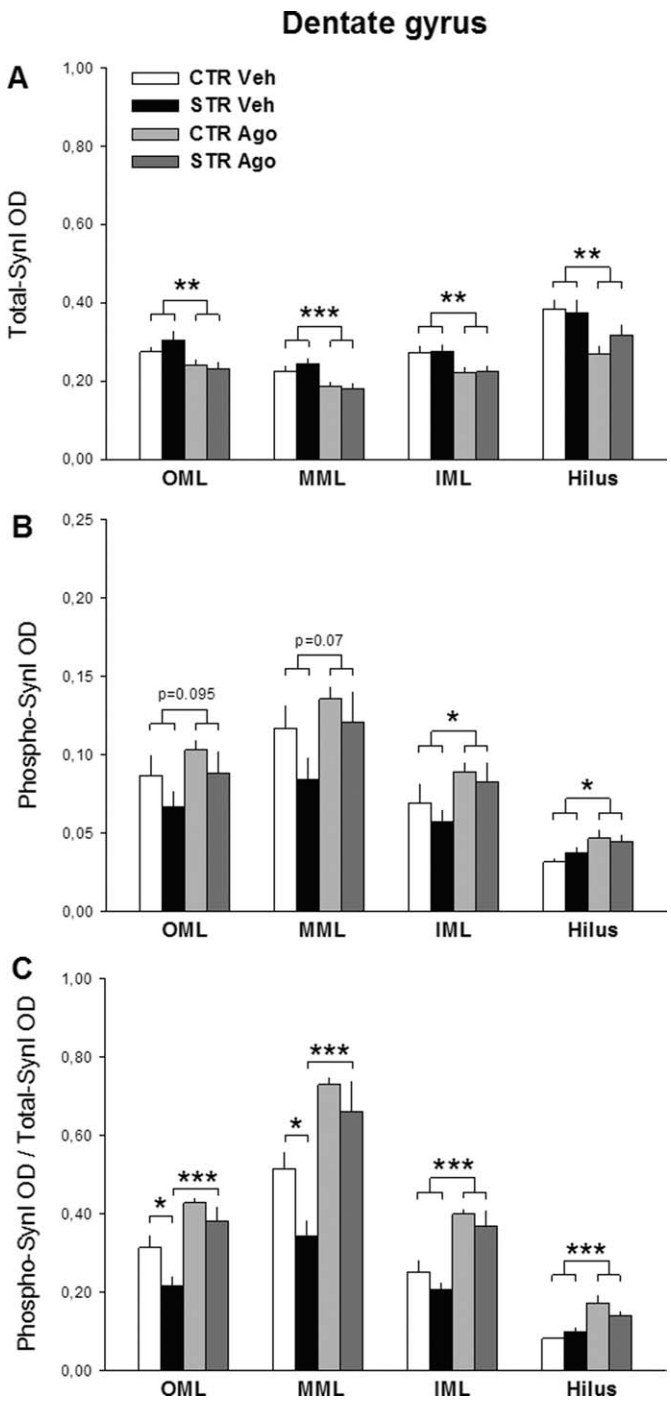

Fig. 4. Effects of chronic stress and agomelatine treatment on total and phosphorylated SynI expression in different layers of the hippocampal dentate gyrus. Although stress had no significant effect on total SynI-ir, agomelatine decreased it in all molecular layers of the DG and in the hilus (**A**). Moreover, agomelatine treatment significantly increased phospho-SynI-ir in the IML and hilus and showed a trend toward increase in the OML and MML (**B**). The ratio between phosphorylated and total SynI revealed a significant stress-induced decrease in the fraction of phospho-SynI in the OML and MML, which was abolished by agomelatine (**C**). DG, dentate gyrus; OML, outer molecular layer; MML, middle molecular layer; IML, inner molecular layer. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DG also showed a drug effect (Fig. 4B). Agomelatine treatment significantly increased phospho-SynI-ir in the IML and hilus of both control and stressed rats ($F_{1,28} = 5.27$, $P = 0.03$; $F_{1,28} = 7.50$, $P = 0.01$, respectively) and showed a trend toward an increase in the OML and MML ($F_{1,28} = 2.98$, $P = 0.095$; $F_{1,28} = 3.58$, $P = 0.069$, respectively). Although stress seemed to decrease phospho-SynI-ir in the molecular layers of the DG, these effects did not reach statistical significance. Effects on SynI phosphorylation in the DG became clearer when the ratio between phosphorylated and total SynI was calculated (Fig. 4C). Significant stress and drug effects were found in the OML ($F_{1,28} = 7.92$, $P = 0.009$; $F_{1,28} = 25.87$, $P = 0.00002$, respectively) and in the MML ($F_{1,28} = 7.57$, $P = 0.01$; $F_{1,28} = 28.06$, $P = 0.00001$, respectively), whereas only a significant drug effect was observed in the IML ($F_{1,28} = 33.55$, $P = 0.000003$) and in the hilus ($F_{1,28} = 23.72$, $P = 0.00004$). Post hoc analysis revealed a significant stress-induced decrease in the ratio of SynI phosphorylation in the OML and MML (CTR Veh vs. STR Veh: $P = 0.02$ and $P = 0.02$, respectively), which was abolished by concomitant agomelatine treatment (STR Veh vs. STR Ago: $P = 0.0004$ and $P = 0.0002$, respectively).

CA3 area. Two-way ANOVA revealed a significant drug effect on total SynI-ir in the CA3 area of the hippocampus (Fig. 5A). Stress had no significant effect on total SynI expression, but agomelatine treatment decreased total SynI-ir in all layers of the CA3 area of both control and stressed rats (SO: $F_{1,28} = 10.98$, $P = 0.003$; SL: $F_{1,28} = 10.20$, $P = 0.004$; SR: $F_{1,28} = 13.56$, $P = 0.001$). Phospho-SynI expression in the CA3 area was low (Fig. 5B). Agomelatine treatment significantly increased phospho-SynI-ir in the SO ($F_{1,28} = 9.02$, $P = 0.006$). A significant drug effect was found on the ratio between phosphorylated and total SynI in all layers of the CA3 area (Fig. 5C; SO: $F_{1,28} = 19.46$, $P = 0.0001$; SL: $F_{1,28} = 6.88$, $P = 0.01$; SR: $F_{1,28} = 6.35$, $P = 0.02$).

CA1 area. Two-way ANOVA revealed a significant drug effect on total SynI-ir in the CA1 area of the hippocampus (Fig. 6A). Stress in itself did not affect total SynI expression. Agomelatine decreased total SynI-ir in all layers of the CA1 area of both control and stressed rats (SO: $F_{1,28} = 18.85$, $P = 0.0002$; SR: $F_{1,28} = 21.23$, $P = 0.00008$; SLM: $F_{1,28} = 14.05$, $P = 0.0008$). Phospho-SynI expression in the CA1 area was low, except in the SLM (Fig. 6B). Agomelatine treatment significantly increased phospho-SynI-ir in the SO and SR ($F_{1,28} = 10.82$, $P = 0.003$; $F_{1,28} = 7.47$, $P = 0.01$, respectively). When the ratio between phosphorylated and total SynI was calculated (Fig. 6C), a significant drug effect was found in all layers of the CA1 area (SO: $F_{1,28} = 29.67$, $P = 0.000008$; SR: $F_{1,28} = 21.61$, $P = 0.00007$; SLM: $F_{1,28} = 8.61$, $P = 0.007$).

Lateral posterior thalamic nucleus

SynI expression in the lateral posterior thalamus was measured for control purposes, because this nucleus

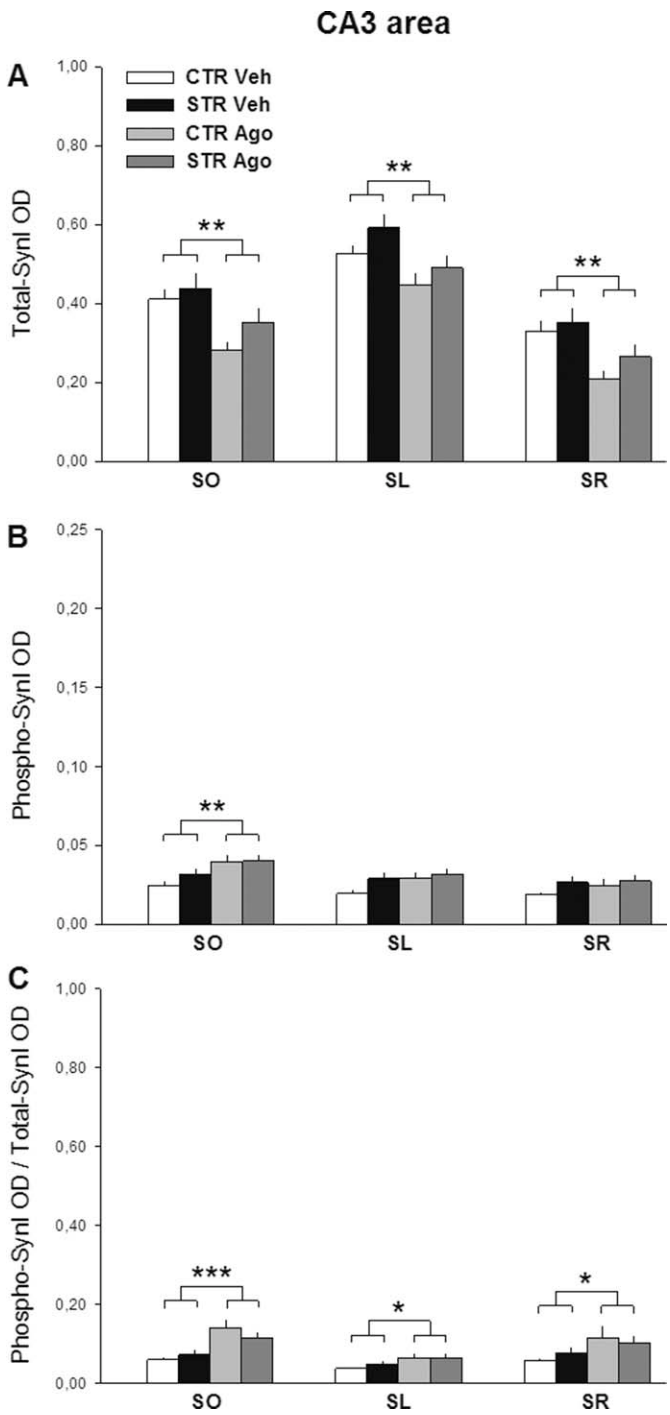

Fig. 5. Effects of chronic stress and agomelatine treatment on total and phosphorylated SynI expression in different layers of the hippocampal CA3 area. Although stress had no significant effect on total SynI-ir, agomelatine decreased it in all layers of the CA3 area (**A**). Moreover, agomelatine treatment significantly increased phospho-SynI-ir in the SO (**B**). The ratio between phosphorylated and total SynI revealed a significant agomelatine-associated increase in the fraction of phospho-SynI in all layers of the CA3 area (**C**). SO, stratum oriens; SL, stratum lucidum; SR, stratum radiatum. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

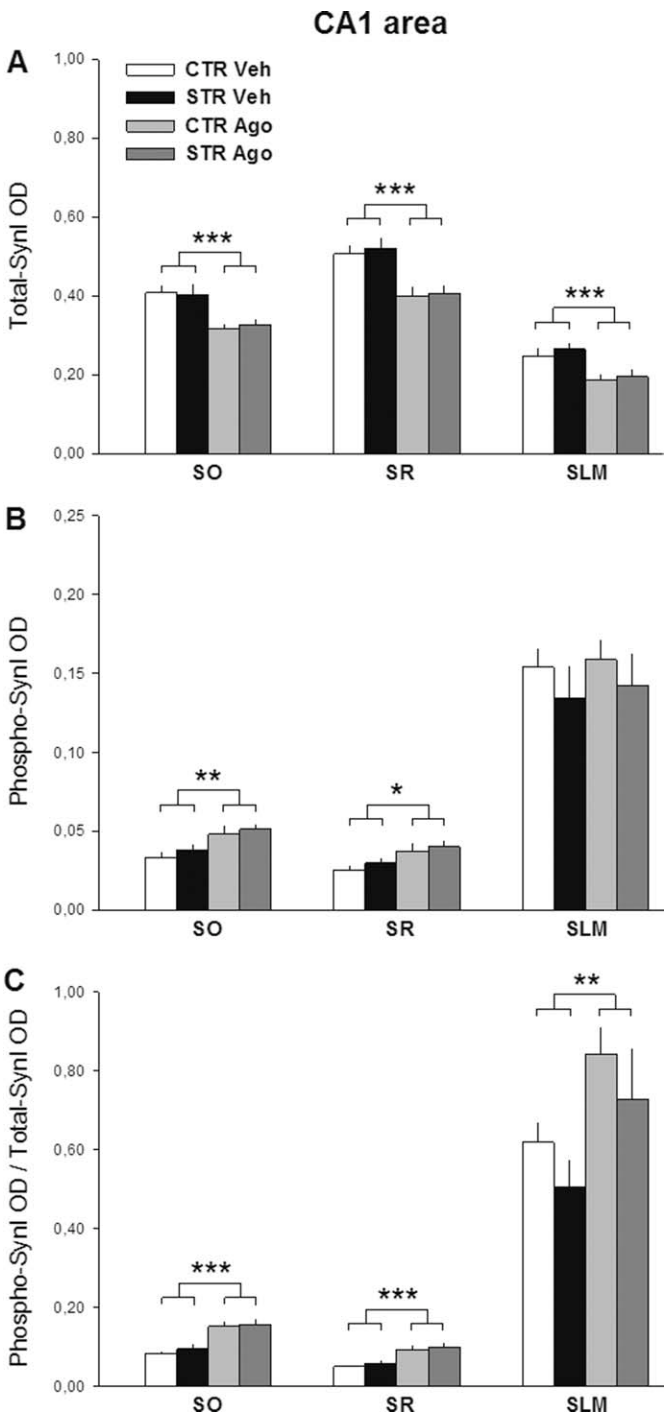

Fig. 6. Effects of chronic stress and agomelatine treatment on total and phosphorylated SynI expression in different layers of the hippocampal CA1 area. Although stress had no significant effect on total SynI-ir, agomelatine decreased it in all layers of the CA1 area (**A**). Moreover, agomelatine treatment significantly increased phospho-SynI-ir in the SO and SR (**B**). The ratio between phosphorylated and total SynI revealed a significant agomelatine-associated increase in the fraction of phospho-SynI in all layers of the CA1 area (**C**). SO, stratum oriens; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

TABLE I. Optical Densities of Total and Phosphorylated SynI-ir and Their Ratios in Mediorostral and Laterorostral Parts of the Lateral Posterior Thalamic Nucleus (LPMR and LPLR)*

	Experimental group			
Brain region	CTR Veh	STR Veh	CTR Ago	STR Ago
Total SynI				
LPMR	0.30 ± 0.03	0.29 ± 0.04	0.24 ± 0.03	0.24 ± 0.03
LPLR	0.26 ± 0.04	0.23 ± 0.03	0.19 ± 0.02	0.21 ± 0.03
Phospho-SynI				
LPMR	0.18 ± 0.02	0.16 ± 0.02	0.19 ± 0.01	0.17 ± 0.03
LPLR	0.18 ± 0.02	0.15 ± 0.02	0.19 ± 0.01	0.16 ± 0.03
Phospho-SynI/total SynI ratio				
LPMR	0.61 ± 0.10	0.56 ± 0.09	0.80 ± 0.11	0.73 ± 0.18
LPLR	0.71 ± 0.12	0.65 ± 0.10	0.97 ± 0.12	0.74 ± 0.18

*There were no significant changes between the groups.

is not directly involved in processes associated with chronic stress and antidepressant treatment. Table I depicts COD values of total SynI-ir and SynI phosphorylation in mediorostral and laterorostral parts of the lateral posterior thalamic nucleus (LPMR and LPLR). No significant changes were found.

DISCUSSION

Chronic stress and agomelatine treatment induced region-specific changes in the immunoreactivity of total and phosphorylated SynI protein (see Table II). In the mPFC, chronic stress increased total SynI expression in all cortical layers. Although agomelatine did not affect total SynI-ir in control animals, it partially abolished the stress-induced effects. To examine changes in the degree of SynI phosphorylation, the ratio between the phosphorylated and total forms (also referred to as the fraction of phosphorylated SynI) was calculated. Chronic stress robustly decreased the fraction of phosphorylated SynI in all layers of the mPFC; the effects of agomelatine were nonsignificant. In the hippocampus, chronic stress did not influence total SynI-ir, whereas agomelatine significantly reduced it in both control and stressed animals. However, chronic stress selectively decreased the fraction of phosphorylated SynI in the outer and middle molecular layers (OML and MML) of the dentate gyrus (DG). Agomelatine completely abolished this stress-induced effect and increased the fraction of phosphorylated SynI in all layers of the DG and CA3 and CA1 areas. As

TABLE II. Effects of Chronic Footshock Stress and Agomelatine Treatment on Total and Phosphorylated SynI (Total SynI and p-SynI, Respectively) in the mPFC and Hippocampal Subregions (Data Presented in Figures 2, 4–6)*

	Main stress effect (compared with CTR)			Main agomelatine effect (compared with Veh)			STR Ago vs. STR Veh		
Brain region	Total SynI	p-SynI	p-SynI/total SynI	Total SynI	p-SynI	p-SynI/total SynI	Total SynI	p-SynI	p-SynI/total SynI
mPFC									
Layer I	↑↑	n.s.	↓↓	n.s.	n.s.	n.s.	↓↓	n.s.	n.s.
Layer II	↑↑	n.s.	↓↓	n.s.	n.s.	n.s.	↓	n.s.	n.s.
Layer III	↑↑	n.s.	↓↓	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Layer V	↑↑	n.s.	↓↓	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Layer VI	↑↑	n.s.	↓↓	n.s.	n.s.	n.s.	↓	n.s.	n.s.
DG									
OML	n.s.	n.s.	↓↓	↓↓	↑	↑↑	n.s.	n.s.	↑↑
MML	n.s.	n.s.	↓↓	↓↓	↑	↑↑	n.s.	n.s.	↑↑
IML	n.s.	n.s.	n.s.	↓↓	↑↑	↑↑	n.s.	n.s.	n.s.
Hilus	n.s.	n.s.	n.s.	↓↓	↑↑	↑↑	n.s.	n.s.	n.s.
CA3									
SO	n.s.	n.s.	n.s.	↓↓	↑↑	↑↑	n.s.	n.s.	n.s.
SL	n.s.	n.s.	n.s.	↓↓	n.s.	↑↑	n.s.	n.s.	n.s.
SR	n.s.	n.s.	n.s.	↓↓	n.s.	↑↑	n.s.	n.s.	n.s.
CA1									
SO	n.s.	n.s.	n.s.	↓↓	↑↑	↑↑	n.s.	n.s.	n.s.
SR	n.s.	n.s.	n.s.	↓↓	↑↑	↑↑	n.s.	n.s.	n.s.
SLM	n.s.	n.s.	n.s.	↓↓	n.s.	↑↑	n.s.	n.s.	n.s.

*CTR, control group; Veh, vehicle; STR, stress group; Ago, agomelatine; mPFC, medial prefrontal cortex; DG, dentate gyrus; OML, outer molecular layer; MML, middle molecular layer; IML, inner molecular layer; SO, stratum oriens; SL, stratum lucidum; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. ↑↑, Significant increase; ↑, trend to increase; ↓↓, significant decrease; ↓, trend to decrease; n.s., no statistically significant change.

expected, neither chronic stress nor agomelatine treatment changed SynI-ir in the lateral posterior thalamic nucleus.

SynI appears to be a stress- and antidepressant-responsive gene product, regulated in a region-dependent manner. To interpret the changes observed in our study, it is necessary to consider the function of this presynaptic protein. SynI acts as a phosphorylation-state-dependent regulator of synaptic vesicle mobilization and hence neurotransmitter release (Greengard et al., 1993; Chi et al., 2001; Cesca et al., 2010). SynI tethers synaptic vesicles to the actin filaments, thereby forming a reserve pool of vesicles away from the docking site. Phosphorylation of SynI reduces its binding to synaptic vesicles and cytoskeleton, which allows vesicles to enter the readily releasable pool and to be released during a presynaptic depolarization. Thus, it is thought that the dephosphorylated form of SynI provides an inhibitory constraint for synaptic vesicle exocytosis, which is relieved upon SynI phosphorylation (Greengard et al., 1993). Regulation of the relative number of vesicles in the releasable and reserve pools represents an important mechanism by which neurons regulate neurotransmitter release and, therefore, the efficiency and strength of synaptic signaling (Hilfiker et al., 1999).

Our data reveal that chronic footshock stress selectively decreased the fraction of phosphorylated SynI in the mPFC and in the OML and MML of the DG. Interestingly, total SynI-ir was significantly increased after chronic stress in the mPFC, but not in any of the hippocampal subregions. Becausee phosphorylated SynI allows vesicle release, it is tempting to speculate that the decrease in the ratio of SynI phosphorylation resulting from prolonged stress may lead to a lower vesicle release. Chronic stress in our study selectively affected the degree of SynI phosphorylation in the OML and MML of the DG, the layers that receive input from the entorhinal cortex (Witter et al., 2000). This finding thus indicates that chronic stress may compromise synaptic transmission from the entorhinal–hippocampal projections. This notion is substantiated by an observation that chronic stress reduces the ability to induce long-term potentiation at this pathway (Pavlides et al., 2002; Alfarez et al., 2003). Vesicle release, however, not only depends on SynI but is regulated by multiple other synaptic proteins. Our results suggest that, despite the effect on SynI, chronic footshock did not alter the expression of another presynaptic marker, GAP-43 (data not shown). Elucidation of stress-associated changes in this complex synaptic machinery, although undoubtedly important, was beyond the scope of this study, which used SynI primarily as a marker of chronic stress and agomelatine treatment-induced effects on the brain.

In our experiment, chronic footshock did not change SynI expression in other synapses of the hippocampal circuitry. These findings are in agreement with a report showing that chronic restraint stress did not influence SynI-ir in the inner molecular layer (IML) or hilus of the DG or in the CA3 and CA1 areas, although it changed the expression of other synaptic markers, synaptobrevin and synaptophysin (Gao et al., 2006). However, altered expression patterns of SynI protein and mRNA were observed throughout the hippocampal subregions after chronic mild stress (Wu et al., 2007; Silva et al., 2008), suggesting that distinct stress paradigms may have different effects on synaptic marker regulation. Moreover, stress effects on SynI may depend on the duration of the stress experience and on the animal strain (Alfonso et al., 2006; Iwata et al., 2006).

The effects of chronic footshock on SynI-ir were region specific. Our data indicate that this form of stress altered SynI expression in the entire mPFC; however, its effects in the hippocampus were less severe and were restricted to the OML and MML of the DG. Such region-dependent changes suggest that chronic footshock stress distinctly affect different cell populations. In line with this notion, region-specific regulatory effects of stress and glucocorticoids have been observed previously (Joels, 2006; McEwen, 2007; Lei and Tejani-Butt, 2010) also on SynI expression (Bessa et al., 2009; Muller et al., 2011). In accordance with our findings, a recent report showed a chronic restraint stress-induced increase in prefrontocortical SynI expression without change in the hippocampus (Muller et al., 2011). Interestingly, in our study, chronic footshock stress increased total SynI-ir in the mPFC but decreased the degree of its phosphorylation. Given that the dephosphorylated form of SynI provides an inhibitory constraint for vesicle release, this result might indicate a chronic stress-related decrease in prefrontocortical neurotransmission. Experimental studies assessing stress effects on SynI expression, especially in the mPFC, are scarce, and reports on hippocampal changes are rather inconsistent, requiring future research on this topic.

Nevertheless, the literature suggests that the PFC is more sensitive to stress at the level of synaptic transmission compared with the hippocampus (Moghaddam, 1993; Muller et al., 2011). The latter brain region, however, has been the focus of many recent studies on stress because of its deleterious effects on hippocampal neurogenesis. Our experiments using the chronic footshock stress model have also revealed stress-associated reductions in newborn cell proliferation and neuronal maturation in the hippocampus, which were abolished by agomelatine treatment (Dagyte et al., 2009, 2010). It is not clear whether changes in hippocampal neurogenesis precede or follow alterations in synaptic machinery or whether they are functionally related. A recent report on early-life stress suggested that reduced neurogenesis and altered dendritic complexity do not necessarily affect the ability to elicit synaptic plasticity (Oomen et al., 2010). However, microarray studies in mice heterozygous for the synaptic vesicle protein vesicular glutamate transporter 1, proposed as a genetic model of depression, revealed regulation of genes involved in neurogenesis and synaptic transmission (Tordera et al., 2011).

Importantly, agomelatine treatment completely abolished chronic stress-induced decrease in the fraction

of phosphorylated SynI in the OML and MML and may thereby rescue stress-associated synaptic changes in the entorhinal–hippocampal projections. Moreover, agomelatine increased the ratio of SynI phosphorylation in all hippocampal subregions of both control and stressed animals, which may boost synaptic transmission and strengthen neuronal communication in the entire hippocampus.

Our study shows that agomelatine treatment decreased the expression of total SynI in the hippocampus. These data are in line with the previously observed effects of various antidepressants on SynI. Similarly to our findings, decreases in total SynI in the hippocampal subregions were reported after treatment with a selective serotonin reuptake inhibitor (SSRI), fluvoxamine, a tricyclic antidepressant, imipramine, and a monoamine oxidase inhibitor, tranylcypromine (Rapp et al., 2004; Iwata et al., 2006). Also, a reduced SynI-ir in hippocampal synaptosomes and synaptic membranes was found after chronic administration of an SSRI, fluoxetine, and a noradrenaline reuptake inhibitor, reboxetine (Barbiero et al., 2007). Furthermore, a glucocorticoid receptor antagonist, mifepristone, and a mood stabilizer, lithium, were shown to reverse the chronic stress-induced alterations in hippocampal SynI expression (Wu et al., 2007; Silva et al., 2008). Thus, although endowed with a different primary mechanism, agomelatine exerts downstream effects on SynI similar to those of classical antidepressants.

Notably, agomelatine modulates SynI in a region-specific manner. Although it changes SynI expression in the hippocampus of both control and stressed animals, this novel antidepressant does not alter SynI-ir in the mPFC of control rats, yet it partially reverses the stress-induced effects. Such regional differences might depend on distinct expression of MT_1, MT_2, and 5-HT_{2C} receptors, implicated in agomelatine action. These receptors are highly expressed in brain areas involved in the pathophysiology of depression; however, whereas 5-HT_{2C} receptors are reported to show similar density in both the mPFC and the hippocampus, MT_1/MT_2 receptors are more densely expressed in the latter region (Weaver et al., 1989; Clemett et al., 2000). This could explain, at least partially, the stronger effects of agomelatine in the hippocampus. Lack of agomelatine-associated prefrontocortical changes in SynI-ir in control animals may also arise from its potentially distinct effects on afferents to the mPFC. MT_1, MT_2, and 5-HT_{2C} receptors are differentially expressed in multiple brain areas that send their input to the mPFC (Clemett et al., 2000; Pandi-Perumal et al., 2008). Thus, the effects of agomelatine may differ per brain region; however, SynI expression in the mPFC may not adequately reflect presynaptic changes in its afferents. Analysis of additional synaptic markers across the brain may, therefore, provide a more thorough understanding of the molecular correlates that underlie the action mechanism of this antidepressant.

The literature suggests that agomelatine enhances basal (pre)frontocortical noradrenergic and dopaminergic transmission as well as increasing basal noradrenaline release in the hippocampus (Millan et al., 2003, 2005). Unlike classical antidepressants, agomelatine does not influence serotonin release (Millan et al., 2003, 2005). Recent findings show that, in (pre)frontocortical synaptosomes, agomelatine abolishes the acute stress-induced increase in glutamate release but has no effect on depolarization-evoked glutamate release in nonstressed animals (Musazzi et al., 2010). The setup of our experiment does not allow determination of specific neurotransmitters released because of chronic stress and agomelatine treatment. Although the selective stress effect in the OML and MML of the DG hints at the involvement of glutamate, the agomelatine-induced uniform changes in SynI-ir across all layers of the hippocampal subregions argue against a sole contribution of glutamate. Multiple neurotransmitter systems have been suggested to play a role in stress, depression, and the action of antidepressant drugs (Joca et al., 2007; Millan, 2009; Dagyte et al., 2011b; McEwen et al., 2010). Thus, altered SynI-ir patterns in our experiment may reflect the net result of chronic stress and agomelatine effects on different neurotransmitters and neuromodulators. Altogether, it is plausible that agomelatine adjusts the balance of various neurotransmitters in stress-related brain regions and thereby exerts its beneficial effects on neuroplasticity.

The present study reveals the effects of chronic stress and the antidepressant agomelatine on the expression of total and phosphorylated SynI in several stress-related brain regions, the mPFC and the hippocampal DG, CA3, and CA1 areas. Both chronic stress and agomelatine treatment were associated with region-specific changes in SynI. Importantly, agomelatine prevented some of the stress effects in the mPFC and completely abolished stress-induced alterations in the hippocampal DG. The primary role of SynI is the regulation of vesicle turnover, so it is predicted that changes in its expression affect synaptic plasticity. Our data thus indicate that modulation of synaptic function underlies the action mechanism of agomelatine, which may include more efficient synaptic signaling in specific stress-related brain areas.

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