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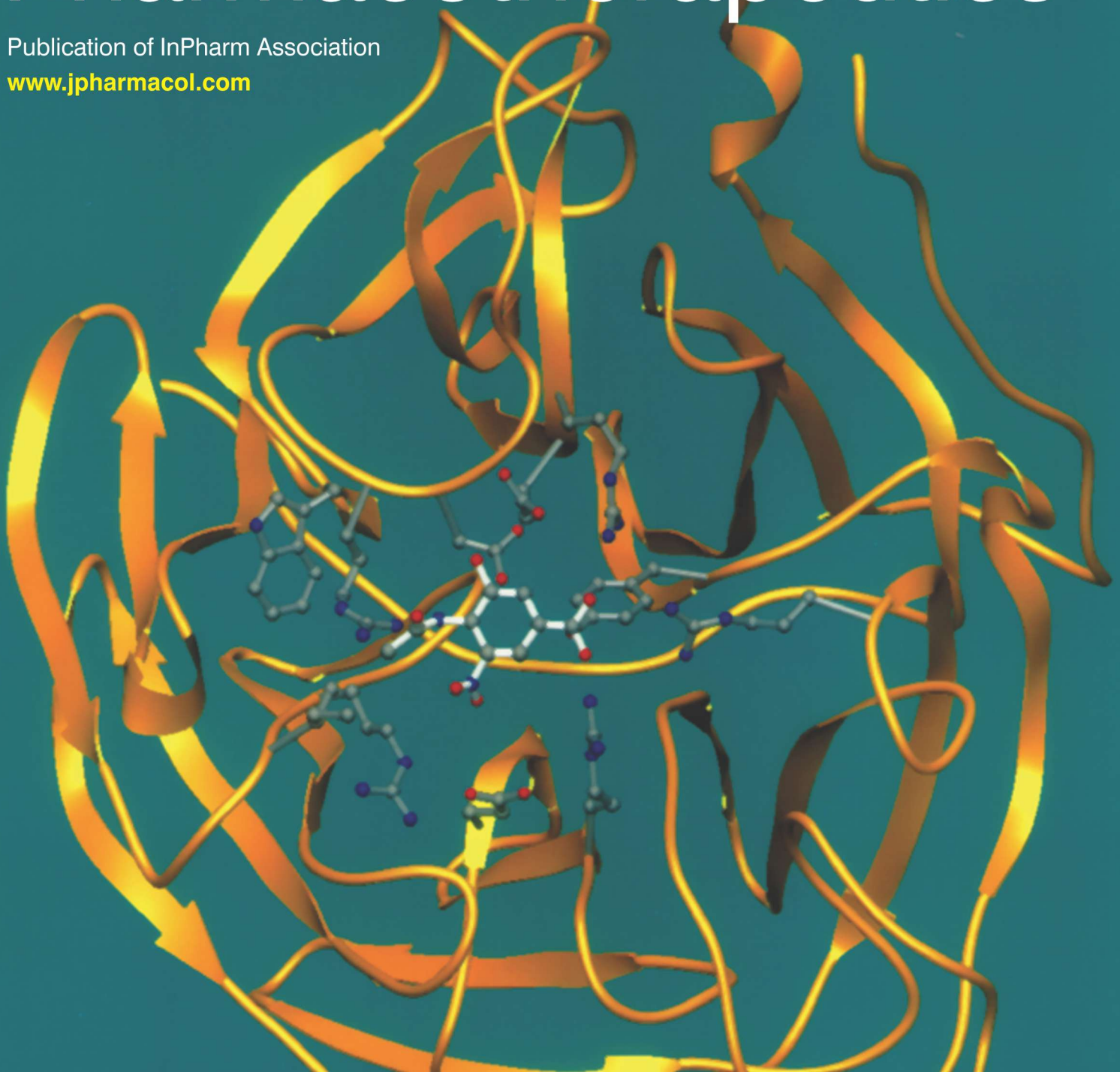

Research Paper

Effect of rolipram, a phosphodiesterase enzyme type–4 inhibitor, on γ-amino butyric acid content of the frontal cortex in mice exposed to chronic mild stress

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ABSTRACT

Objectives: To investigate the alterations in GABA levels by rolipram in the model of depression. **Materials and Methods:** The alteration of GABA content by rolipram as a phosphodiesterase enzyme type-4 inhibitor in the frontal cortex (FCx; as a brain region crucial for the control of emotion and cognition) obtained from male mice exposed to chronic mild stress (CMS)-induced anhedonia (the loss of pleasure or lack of sensitivity to pleasure stimuli) was recorded. **Results:** The results demonstrated the reversal of CMS-induced anhedonia after 3 weeks per os of rolipram in a dose of 0.1 mg/kg/day dissolved in distilled water. Furthermore, rolipram showed a significant reduction in duration of immobility in long-term behavioral changes recorded by the FST. Additionally, there was a significant increase in the GABA content of the FCx of rolipram-treated mice exposed to CMS-induced anhedonia. **Conclusions:** The present study suggested that GABA levels may be decreased in an animal model of depression and its reversal together with the behaviour improvement by rolipram could support the hypothesis that modification in GABAergic activity has a role in mood disorders. These effects may complement the antidepressant effect of rolipram that is originally mediated via inhibition of phosphodiesterase enzyme type-4 [PDE4] that increases cyclic adenosine monophosphate signalling the pharmacotherapy of depression.

Key words: Chronic mild stress, depression, forced swimming test, γ-amino butyric acid, rolipram

INTRODUCTION

Impairments of signal transduction that regulate neuroplasticity and cell survival are thought to be important mechanisms contributing to major depressive disorders.[1] In particular, cyclic adenosine monophosphate (cAMP) - mediated signalling appears to have a key role in the pathophysiology and pharmacotherapy of depression.[2] Elevating intracellular cAMP, either via inhibition of type-4 phosphodiesterase (PDE4), which specifically catalyzes the hydrolysis of cAMP, or stimulation of β- adrenergic receptors, produces antidepressant-like effects in animal models.[3-6]

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Cellular cAMP concentrations are determined by the relative activities of adenyl cyclases, which catalyze cAMP synthesis, and cAMP PDEs, which catalyze its hydrolysis.[7,8] To date, mammalian PDEs have been divided into 11 families.[9] Of these PDEs, PDE4 is particularly important for controlling intracellular cAMP concentrations and is considered to be

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a prime target for therapeutic intervention for a range of disorders such as depression, impaired cognition, asthma and inflammation.[10,11] Notably, PDE4 is the predominant mediator of hydrolysis of cAMP formed by stimulation of β-adrenergic receptors, which are involved in the mediation of the effects of antidepressant drugs.[12,13] Consistent with this, inhibition of PDE4 by rolipram produces antidepressant-like and memory-enhancing effects in animals.[5,14,15]

γ-amino-butyric acid (GABA) is the major inhibitory neurotransmitter in the brain and diminishes the activity of its target neurons. It is a major inhibitory neurotransmitter in the central nervous system and modulates the activity of several neurotransmitters including dopamine, serotonin, and norepinephrine (NE). GABA is synthesized in a single step from its precursor glutamate by glutamic acid decarboxylase. GABA is metabolized by successive transamination and oxidation to yield succinic semialdehyde and succinic acid, respectively. As a part of the transamination reaction, a recycling system is formed in which α-ketoglutaric acid is converted to the GABA precursor glutamate by GABA-glutamic acid transaminase.[16]

Petty *et al*.[17] reported that plasma GABA levels are relatively reduced in depressed patients. This study demonstrated that there is a well-proven tendency for depressed and bipolar patients to have lower levels of GABA in their blood plasma. These low plasma levels are thought to reflect lower brain levels.

The current theory of GABA and depression is that low plasma levels of GABA may identify an inheritable tendency for mood disorders such as depression or bipolar disease.[18]

Hence, the role of GABA is demonstrated in mood disorders so the possible effect of rolipram, as PDE4 inhibitor, needs to be investigated on this neurotransmitter. The present study investigated the alterations of GABA content by 3-week treatment of rolipram in the frontal cortex (FCx) [as a brain region crucial for the control of emotion and cognition] obtained from mice exposed to chronic mild stress (CMS)-induced anhedonia. The behavioral changes of the CMS without and with this antidepressant treatment was also tested using the forced swimming test (FST).

MATERIALS AND METHODS

Drug and chemicals

Rolipram (Sigma Chemicals Co.,St Louis, MO,USA) was dissolved in distilled water in a volume of 20 ml/ kg. GABA and norvaline standards (Sigma Chemicals Co), ethanol, [HPLC grade , MERCK], triethylamine [TEA, MERCK], phenylisothiocyanate [PITC, Sigma Chemicals Co.], hydrochloric acid (32%, MERCK), acetonitrile [MERCK], glacial acetic acid (Sigma Chemicals Co), sodium acetate anhydrous [MERCK].

Animals

Thirty-six male albino mice, weighing 20-25 g, were used all over experimental procedures. They were randomly allocated into three groups, number of animals in each group was 12. Mice were allowed 1 week to acclimatize to the surroundings before beginning any experimentation. Animals were housed in individual plastic cages. Food and tap water were available *ad libitum* for the duration of the experiments unless otherwise noted. Sucrose solution (2%) was available *ad libitum* for one week preceding the experimental procedures to allow adaptation to the taste of sucrose. The temperature was maintained at 22±2°C. The light-dark cycle (LD) was on a 12 h LD cycle with lights on at 06:00 a.m. and off at 06:00 p.m., unless otherwise noted during the stress procedure (6 weeks). All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocol

Mice were weighed and each one was placed in an individual cage. To introduce the mouse to sucrose solution and to obtain baseline data on sucrose consumption, mice were given a bottle of 2% sucrose. Twenty-four hours later, the bottles were removed and weighed to measure liquid intake. The water bottles were then replaced. Sucrose intake was measured again for a 1-h period. On the basis of body weight and sucrose intake (during the 24- and 1-h period), mice were assigned to either experimental or control groups (n=12 in each group). Body weight, in addition to sucrose consumption, was used to separate animals in an effort to minimize future changes in sucrose intake caused by differences in body size. Experimental animals were exposed to 6 weeks of chronic mild stress. Antidepressant-treated animals received a daily dose *per os* [po] of rolipram starting from the beginning of the 3rd week up to the end of the 6th week of CMS. The control animals were left undisturbed during the 6 week-period, except for scheduled daily po administration of distilled water in the last 3 weeks simulating the test group of treated animals, in addition to cleaning, feeding and weighing procedures.

During the stress period, control and experimental animals were weighed weekly. A 1-h sucrose test was given to all animals once a week. At the end of the experiment, a forced-swimming test (FST) was done to assess long-term behavioral changes of the chronic stress protocol according to Solberg *et al*.[19]

Drug administration and forced swimming test

Where indicated, mice were given *per os* with a once daily dose of either distilled water (control group), rolipram (0.1

mg/kg/day) dissolved in distilled water in the last 3 weeks of exposure to CMS. The injected volume did not exceed 20 ml/kg body weight. This dose was selected by a pilot study that was done before the start of the experimental study and denoted the presence of changes by its administration.

The FST is used to test the behavioral despair in rodents.[20] It can be seen as a way to measure "fighting spirit" of mice. In the first 2 min., the animal was allowed to adjust to the new conditions, then, the immobility time that alternated with conditions of enhanced motor activity was measured. Immobility time was measured with a stopwatch for the next 4 minutes.[21] Mice were removed from their cages and placed in individual glass cylinders (diameter 15 ml) containing water at 22-24°C at a depth of 14-16 cm so that they could not escape and could not touch the bottom. The animals were placed in the cylinders for observation in a 6-min test swim. Two swimming sessions were conducted: An initial 15-min pretest followed 24 h later by a 6-min test). The duration of immobility was measured for a 6-min period. The duration of immobility during the last 4 min of the 6 min test was measured by two trained experimenters. The mouse was considered as immobile when it stopped struggling and moved only to remain floating in the water, keeping its head above water. Shorter immobility time is an indicator of the stronger antidepressant effect of the tested substance.[21]

Chronic stress procedure

The chronic stress procedure was adopted from Willner *et al*.[22] Solberg *et al*.[19] The protocol consisted of the following stressors:

- 16-h water deprivation (water bottles were removed from cage during this time)
- 5 min-tail suspension (animals were held upside down by their tail with metal tongs)
- 1-to2-h restraint (animals were placed in a 50 ml conical tube with breathing holes)
- 30-45 min paired housing (the mouse was placed in the cage of another mouse of the stress group, each week the home cage mouse alternated)
- Soiled cage (100 ml 16-18°C water was poured into the cage)
- 5-min forced swim in cold water (16-18°C)

Each week, the stressors were presented in a different order and given at different times of the day.

Sucrose test [once/week]

Preliminary data have shown that mice prefer a 2% sucrose solution over regular unsweetened water (pilot study). Once each week, each mouse was given a bottle of 2% sucrose for a 1-h period, this occurs 6 hours after lights out. After 1-hour, the bottle was removed and total sucrose consumption was calculated.

Determination of GABA content in homogenates of frontal cortices of mice

The GABA level in the tissue homogenates of the FCx was determined according to Gunawan *et al*.[23] and Rossetti and Lombard.[24]

The high performance liquid chromatography (HPLC) method with precolumn phenyl-iso-thio-cyanate (PITC) derivatization was used for the determination of GABA levels in the homogenate of the FCx of the brain of mice different in groups. The measurement scale of the data was in nmol/mg tissue protein.

The FCx obtained from each mouse was homogenized, then samples were centrifuged in a cooling (4°C) centrifuge at 15,000 rpm for 10 minutes. The supernatant was aspirated and transferred to an Eppendorff tube, while the pellet was kept at -70°C until assayed for its total protein content . Each sample was derivatized via drying 100 µl of the aspirated supernatant in the centrivap, under vacuum. The residue was dissolved in 20 µl of ethanol-water-triethylamine (2:2:1) and evaporated to dryness under vacuum. A 30 µl of ethanol-water-triethylamine-phenylisothiocyanate [PITC] (7:1:1:1) was added to the residue and allowed to react for 20 min. at room temperature to form the PITC-derivatives of the amino acids. Excess reagent was then evaporated under vacuum. The mobile phase of HPLC consisted of solvents A and B: Solvent A: 0.1 M sodium acetate buffer (pH = 5.8), solvent B: Acetonitrile: Water (60:40, v:v). A mixture of 80% solvent A and 20% solvent B was adjusted for the "isocratic" HPLC separations. Flow rate was set at 0.6 ml/min. The injected sample was 20 µl. The peaks were detected at 254-nm wave length. Standard curves for GABA and norvaline were plotted using norvaline 2 nmol/20 µl as an internal standard. The ratio of the peak area of each concentration of each standard to the peak area of the internal standard was determined and entered against the concentration of the standard, in a simple regression procedure.

Quantification of the total tissue protein

This was done according to Bradford.[25] The aim is to relate the GABA concentration to the total tissue protein.

Analysis of the data

The data obtained are presented as means ± SEM of mean and evaluated using one-way ANOVA, followed by Bonferroni's post hoc determination, using GraphPad Prism version 3.00 for Windows 97 (Graph Pad Software, San Diego, CA, USA).

RESULTS

Effect of 3-weeks rolipram administration on CMS-induced anhedonia in mice

Figure 1 demonstrates the reversal of anhedonia after 3 weeks *po* administration of rolipram 0.1 mg/kg/day to male

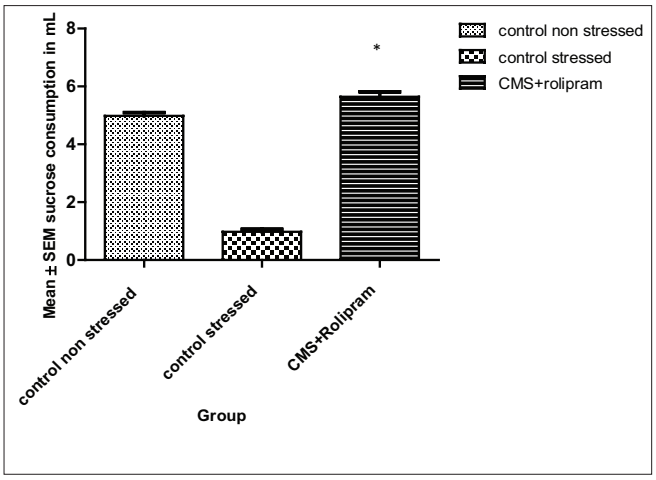

Figure 1: Sucrose consumption in mL in tested groups of mice; Demonstrated the sucrose consumption in mL in tested male albino mice of the different groups; control saline-treated, chronic stress -with and without antidepressant-treatment. Data are means ± SEM from 12 animals per group. *P<0.05 = a significant reduction versus saline control group. **P<0.05 = a significant increase in the CMS- treated groups versus the stressed nontreated group [groups 2 and 3]

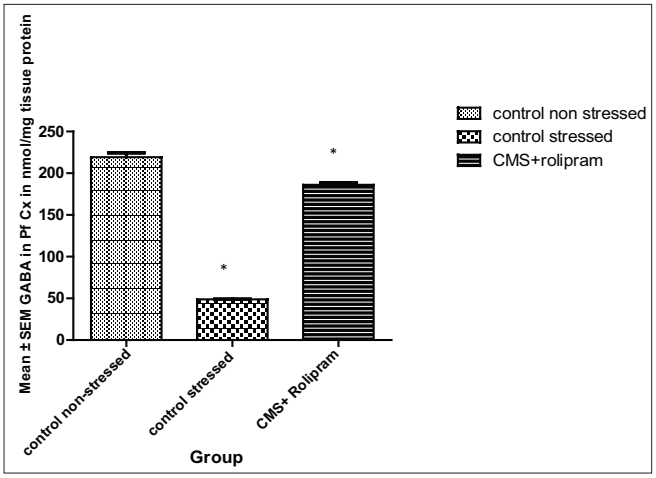

Figure 3: GABA contents in the frontal cortices homogenates in the tested mice; Demonstrated changes in the GABA content in the homogenates of frontal cortices isolated from male albino mice of the different groups; control saline-treated, chronic stress -with and without rolipram-treatment. Data are means± SEM from 12 animals per group. *P<0.05 = a significant decrease in GABA content versus saline control group. **P<0.05 = a significant increase in GABA content in the CMS-treated groups versus the stressed non-treated group [groups 2 and 3]

albino mice continuously exposed to CMS protocol. Sucrose consumption in mL of the different groups (control, CMS, CMS+rolipram) was calculated. In comparison to the control-saline administered group, the CMS group was associated with a (-80.32%) decrease in sucrose consumption [0. 98±0.09 versus the control value 4.98±0.11 mL as mean±SEM]. This decrease was reversed in the rolipram-treated groups to an increase of +13.25 % of the control group level [5.64±0.18 versus the control value of 4.98±0.11mL as mean±SEM].

Effect of 3-week administration of rolipram on the duration of immobilty in the forced swimming test (FST)

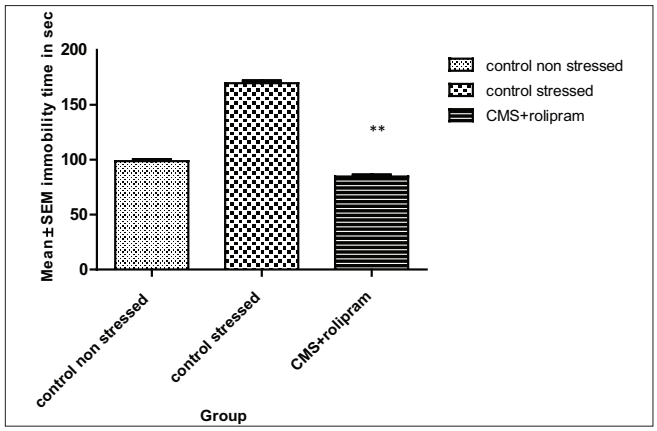

Figure 2: Duration of immobility in seconds in tested mice; Demonstrated changes in the duration of immobility in seconds in male albino mice of the different groups; control saline-treated, chronic stress -with and without rolipram-treatment. Data are means ± SEM from 12 animals per group. *P<0.05 = a significant increase in the immobility duration versus saline control group. **P<0.05 = a significant decrease in the immobility duration in the CMS- treated groups versus the stressed nontreated group [groups 2 and 3]

Figure 2 showed a significant [P< 0.05] reduction in immobility time measured in seconds (in the FST) after treatment of mice, exposed to CMS model, with rolipram compared to stressed nontreated group.

Effect of 3-week administration of rolipram on the GABA level in the FCx homogenates of tested mice

Figure 3 represents the changes in GABA concentration in the FCx of the control, CMS, CMS+rolipram treated mice.

CMS decreased significantly (P<0.05) the GABA concentration in the FCx. GABA concentration of CMS mice was increased significantly (P<0.05) by rolipram treatment compared to stressed nontreated group.

DISCUSSION

In the present study, 3-weeks single daily dose of rolipram induced an increase in the GABA content of the FCx of mice exposed to chronic mild stress-induced anhedonia compared to stressed non-treated group. The FCx, homogenates were analyzed in the present study for their GABA content. The selection of this area is based upon the fact that it plays a crucial role in processes involved in the control of mood, cognition and motor behavior, these functions that are compromised in depression.[26]

In the present study, CMS reduced the consumption of the sucrose solution, that was evident within 3 weeks after the beginning of stress. A 3-weeks treatment with rolipram increased the consumption to values higher than that reported

with the normal saline control levels. The development of an "anhedonia-like" condition has been also confirmed by the forced swimming test (FST). The duration of immobility was almost completely reversed by rolipram treatment of the CMS-exposed mice. The immobility displayed in rodents subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in turn may reflect depressive disorders in humans. Meanwhile, the FST is a sensitive test reflecting the state of immobility simulating behavioral despair in human.[27]

Brambilla *et al*.[16] reviewed the available literature on the preclinical and clinical studies involving GABAergic neurotransmission in mood disorders. Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter present almost exclusively in the central nervous system (CNS), distributed across almost all brain regions, and expressed in interneurons modulating local circuits. The role of GABAergic dysfunction in mood disorders was first proposed since long years ago.

Sanacora *et al*.[18] showed that there was a low concentration of GABA in plasma and cerebrospinal fluid (CSF) of individuals with major depression. In addition to that, low GABA concentration, measured by proton [(1)H] magnetic resonance spectroscopy (MRS) study, has also been found in the occipital cortex of depressed subjects and when these patients were treated with SSRI, the results revealed a normalization of the low GABA concentration, suggesting a role of GABA in the mechanism of antidepressant action.[28]

An experiemental study investigated the effects of rolipram, as an antidepressant drug, on excitatory and inhibitory amino acid neurotransmission systems in young and aged Wistar rat brains. The investigators used in vitro autoradiography with [3H]MK-801, [3H]glycine, D[3H]aspartate, and [3H]muscimol to label N-methyl-D-aspartate (NMDA) receptors, glycine modulatory sites, glutamate transport sites, and gamma-aminobutyric acid-A (GABA) receptors, respectively. Rolipram (0.01 or 0.1 mg/kg, *per os*) or its vehicle (distilled water) was administered once a day for 4 weeks. The highest binding of [3H]MK-801, [3H] glycine, and D-[3H]aspartate was seen in the hippocampus in vehicle-treated rats. No significant differences in these binding activities were seen between young and aged rat brains. [3H] Muscimol binding was the highest in the cerebellum, and decreased in many brain regions in aged rats. Rolipram treatment resulted in an increase in [3H]MK-801 binding in the dentate gyrus in both young and aged rats, remarkable reductions in D-[3H]aspartate binding in many regions of both young and aged rats, and no or minimal changes in [3H]glycine and [3H] muscimol binding. These results suggest that rolipram treatment modifies the excitatory amino acid neurotransmission system. Based upon these results, a recommendation was developed towards further studies of rolipram on inhibitory neuronal transmitters like GABA.[29]

Additionally, it was found that PDE4 inhibitors enhance antidepressant-induced increases in the expression of cAMP and brain-derived neurotrophic factor (BDNF) in the rat hippocampus.[30] These studies guided other authors to study the possible link between cAMP, BDNF and GABA.[31]

Juan-Fita *et al*.[32] reported that rolipram, enhances the inotropic effect of NE in the rat heart. The study presumably attributes this effect to the inhibition of PDE4 activity. The brain cAMP regulating system and its downstream elements in association of catecholamines as norepinephrine and dopamine play a pivotal role in the therapeutic effects of antidepressants. Itoh *et al*.,[33] demonstrated that repeated administration of imipramine (1.25–10 mg/kg, i.p.) or rolipram (1.25 mg/kg, i.p.) reduced the number of escape failures in learned helplessness rats. BDNF levels of the FCx and hippocampus were significantly increased by treatment with a combination of rolipram and imipramine compared to those in imipramine-treated rats. These results indicated that coadministration of phosphodiesterase type 4 inhibitors with antidepressants may be more effective for depression therapy and suggest that elevation of the cAMP signal transduction pathway is involved in the antidepressive effects. A possible relationship between cAMP and GABA was proposed to the antidepressant's effect of rolipram as a phosphodiesterase inhibitor; however, further investigations should be done in this field.

In conclusion, from a neurochemical and behavioral points of view, the present study pointed to a modulating role of rolipram on GABA content in the frontal cortex homogenates of the CMS-exposed mice that is a model of human depression with a high degree of validity.

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