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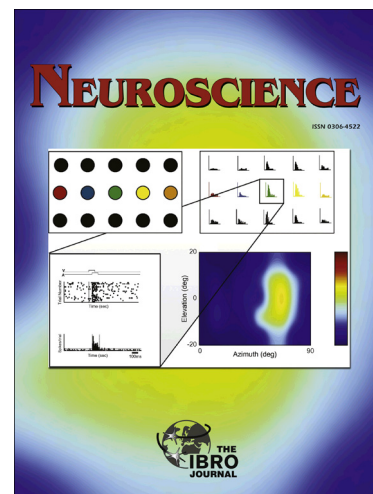
Impramine, fluoxetine and clozapine differently affected reactivity to positive and negative stimuli in a model of motivational anhedonia in rats

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Abbreviations

ANOVA, analysis of variance; BP, breaking point; DARPP-32, dopamine and cAMP-regulated phosphoprotein of Mr 32,000; FR, fixed-ratio; NAcS, nucleus accumbens shell; PR, progressive ratio; SDS, sodium dodecyl sulfate; Thr, threonine.

Abstract

Anhedonia is a relevant symptom in depression and schizophrenia. Chronic stress exposure induces in rats escape deficit, disrupts the dopaminergic response to palatable food and the competence to acquire sucrose self-administration (SA), thus configuring a possible model of motivational anhedonia. Repeated lithium administration reverts stress effects and brings back to control values the breaking point (BP) score, a measure of reward motivation. In this study, we tested on this model two antidepressants, imipramine and fluoxetine, and two antipsychotics, haloperidol and clozapine. The dopaminergic response to sucrose consumption was studied in non food-deprived rats in terms of dopamine D_1 receptor signaling in the nucleus accumbens shell (NAcS). More specifically, we studied the modifications in cAMP-regulated phosphoprotein Mr 32,000 (DARPP-32) phosphorylation pattern following sucrose consumption. Fluoxetine reverted the escape deficit and showed no effects on dopaminergic response and sucrose SA. Imipramine reverted sucrose SA and dopamine response deficit in half of the rats and the escape deficit in all animals. Haloperidol did not affect stress-induced deficits. Clozapine-treated rats recovered the dopaminergic response to sucrose consumption and the competence to acquire sucrose SA, although they still showed the escape deficit, thus confirming that motivation toward reward may be dissociated from that to punishment escape. These results indicate that imipramine or fluoxetine are not endowed with a rapid onset antianhedonic effect. On the other hand, clozapine treatment showed a motivational antianhedonic activity similar to that observed after lithium treatment.

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Anhedonia is considered a core symptom of depression and schizophrenia, although it is a symptom as difficult to define as to treat (Treadway and Zald, 2011). The DSM-IV-TR and the DSM-V (DSM-IV-TR®, 2000; DSM-V-TR®, 2014) refer to anhedonia as diminished interest or pleasure in response to stimuli perceived as rewarding during a premorbid state. Thus, clinical diagnosis does not discriminate between a decrease in motivation and a reduction in experienced pleasure, although the neurobiological mechanisms underpinning the consummatory ("liking") and preparatory ("wanting") behaviors controlled by positive stimuli clearly distinguish pleasure from motivation (Treadway and Zald, 2011). In rodents, responses to palatable food are a validated index of hedonic responsiveness (Willner et al, 1987) and, although palatability is independent of dopaminergic transmission (Berridge and Robinson, 1998), palatable food consumption induces a phasic increase in extraneuronal dopamine levels in the mesolimbic areas that confers to it incentive salience (Berridge, 2007). Non food-deprived rats can be trained to self-administer sucrose and the breaking point (BP) score can be recorded. BP measures the effort animals are willing to exert in order to obtain the reinforcing stimulus (Salamone et al, 2012) and is considered an index of animal motivation.

The ingestion of a food of unexpected palatability induces in non food-deprived rats a consistent dopaminergic response in the shell portion of the nucleus accumbens (NAcS) in terms of increased extraneuronal dopamine concentration and dopamine D_1 receptor-dependent signaling (Bassareo and Di Chiara, 1999; Gambarana et al, 2003; Rauggi et al, 2005). In particular, an increase in PKA-dependent phosphorylation of cAMP-regulated phosphoprotein Mr 32,000 (DARPP-32) in the Thr34 residue is observed and increases in both extraneuronal dopamine and phospho-Thr34-DARPP-32 levels are reduced after a second consumption of the same food, indicating that the actual hedonic value is dependent on novelty besides palatability (Danielli et al, 2010). Repeated exposure to unavoidable stress induces two distinct behavioral modifications in rats: reduced reactivity to aversive stimuli and reduced motivation to earn palatable food (Gambarana et al, 2001; Marchese et al, 2013). Moreover, unavoidable stress exposure disrupts the dopaminergic responses to palatable food consumption, and repeated lithium treatment reverts all these effects (Marchese et al, 2013). Thus, we proposed an experimental model that conforms to face validity for decreased appetitive motivation and is responsive to lithium treatment (Marchese et al, 2013), although clinical studies on lithium efficacy did not specifically address this issue. Modifications in signaling after palatable food consumption seem to match the modifications observed in extraneuronal dopamine levels (Danielli et al, 2010). Thus, we first verified whether modifications in dopamine D_1-dependent signaling represented a useful index of the NAcS dopaminergic response to the consumption of a natural reward. To this end, we studied whether these modifications following sucrose ingestion matched extraneuronal dopamine modifications in the NAcS of non food-deprived control rats and rats expressing chronic stress-induced decrease in appetitive motivation, treated or not with lithium. A pattern of changes consistent with the previously reported extraneuronal dopamine increase (Marchese et al, 2013) was observed after sucrose consumption in lithium-treated rats, exposed or not to chronic stress, confirming the efficacy of lithium to restore the dopaminergic response to palatable food in chronically stressed rats, as well as the validity of the proposed index. On these premises, using the same experimental protocol utilized with lithium (Marchese et al, 2013), we then studied the possible activity of some antidepressant and antipsychotic drugs in reinstating appetitive motivation in non food-deprived rats.

2. Experimental procedures

2.1 Animals

Experiments were carried out on male Sprague-Dawley rats (Charles River, Calco, Italy), weighing 200-225 g when the experimental procedures began, allowing 10 days of habituation to the animal colony. Animals were housed 4 - 5 per cage (bedding Lignocel® 3/4S, Harlan Laboratories, San Pietro al Natisone, Italy) in an environment maintained at a constant temperature and humidity with free access to food (4RF21, Mucedola, Settimo Milanese, Italy) and water. A 12 h reverse light/dark cycle (7:00 a. m. lights off, 7:00 p. m. lights on) was used. Experiments were carried out from 9:00 a. m. to 5:00 p. m. under a red light and controlled noise conditions. In all the experiments, body weight did not significantly differ between groups at the beginning and at the end of experimental procedures. The procedures used were in accordance with the European legislation on the use and care of laboratory animals (EU Directive 2010/63) and they were approved by the University of Siena Ethics Committee. All efforts were made to minimize the number of animals used and their suffering.

2.2 Immunoblotting

Rats were killed and the NAcS was excised using the rapid head-freeze dissection technique previously described (Danielli et al, 2010). Tissues were solubilized in boiling 1% sodium dodecyl sulfate (SDS) and 50 mM NaF. Small aliquots of the homogenate were used for protein determination by a modified Lowry protein assay method (DC protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed as previously described (Danielli et al, 2010). Briefly, proteins (30 μg) were loaded into 10% SDS–PAGE gels (Invitrogen, Carlsbad, CA, USA), transferred onto nitrocellulose membranes, and incubated with antibodies against phospho-Thr34 DARPP-32, phospho-Thr75 DARPP-32 and total DARPP-32 (Cell Signaling Technology, Beverly, MA, USA). Blots were developed using a chemiluminescence detection system (Pierce Biotechnology Inc., Rockford, IL, USA) and quantified with the Versa Doc 1000 Imaging System (Bio-Rad Laboratories). Samples containing the same amount of total proteins from rats in each experimental group were run on the same immunoblots and then analyzed together. To control for equal loading, blots were reprobed with the non-phosphorylation-state-specific antibody; when a greater than 10% difference in the levels of total DARPP-32 was detected, protein concentrations were determined again and a new immunoblotting experiment was performed. Thus, although levels of phosphorylated proteins were not normalized to the respective total protein levels, only the data obtained with equal protein loading were utilized. Stress exposure and lithium, imipramine, fluoxetine, haloperidol or clozapine 10-day administration *per sè* did not modify baseline expression levels of DARPP-32 and its Thr34 and Thr75 phosphorylated forms.

2.3 Chronic stress protocol

The experimental procedure, previously described (Gambarana et al, 2001), consisted in the induction of an escape deficit and its maintenance by exposure to minor unavoidable stressors. Briefly, rats were immobilized with a flexible wire-net and administered about 80 tail shocks (1 mA x 5 s, 1 every 30 s). Twenty-four h later, rats were exposed to a shock-escape test. Rats were then exposed on alternate days to unavoidable stressors, beginning 48 h after the escape test. Rats were exposed to stress sessions in the afternoon, 3 - 4 h after the end of SA sessions. Control rats were manipulated daily by experimenters. Since rats exposed to chronic stress show scarce interest in

sucrose pellets and a variable latency to approach and consume them, in order to study the dopaminergic response to sucrose consumption (immunoblotting experiments), rats were habituated for a week to be handled and 30 min before sacrifice the sucrose solution (10%) was administered orally.

2.4 Self-administration (SA) procedure

Experiments were conducted in operant chambers (MED Associates Inc., St. Albans, VT, USA) as previously described (Marchese et al, 2013). Chambers were enclosed in ventilated, sound-attenuating boxes and they contained two response levers; during SA testing, a lever-press response at the active lever delivered a sucrose pellet (Bio-Serv, Frenchtown, NJ, USA) into the food receptacle and produced no programmed result at the inactive lever. A cue light was located above the active lever. The house light was turned on at the start of each session. Experimental events and data collection were scheduled using MED Associates software (MED Associates Inc.). Rats were given daily 30-min sessions between 9:00 and 12:00 a.m. and had free access to standard food in the home cage before and after each session. Rats were exposed to a fixed-ratio (FR) 1 schedule until a criterion of 50 or more lever presses was reached for 2 consecutive days by the control group, then they were switched to an FR5 schedule. When a criterion of 50 or more responses was reached by the control group, rats were switched to a PR schedule, in which the number of responses required to receive a sucrose pellet was progressively increased in each session with a step size of 3. The schedule continued until 5 min had elapsed without a response (BP). BP was defined as the number of lever presses in the final completed ratio. The condition of deficit in appetitive motivation was induced by exposure to the chronic unavoidable stress protocol (see above) and evaluated considering the reinforcement efficacy of a hedonic stimulus (sucrose) in SA experiments (Marchese et al, 2013). The criterion for appetitive motivation deficit was a lever pressing rate in FR1 and FR5 schedules lower than 60% of the control group rate (Table 1).

2.5 Drugs

Imipramine and fluoxetine were dissolved in deionized/distilled water. Clozapine was dissolved in 1N HCl, then diluted to 0.1N HCl with distilled water and pH-adjusted to about 6 with NaOH. Haloperidol was dissolved in a combination of 5% lactic acid, NaOH and distilled water to obtain a pH of about 6 - 7. All drugs were administered i. p. at a volume of 1 ml/kg rat body weight at doses chosen on the basis of published results, twice a day, with the exception of fluoxetine, which was administered once daily (Salamone et al, 1996; Gambarana et al, 2001). Rats in the control groups received the same volume of saline or the appropriate vehicle. All drugs and chemicals were purchased from commercial sources. In order to reduce acute drug effects on behavioral performances, all drugs treatments were administered after SA training and about 18 h before the escape test.

2.6. Statistical analyses

Statistical analyses were performed on commercially available software (GraphPad Prism statistical package, GraphPad, San Diego, CA, USA). Data on DARPP-32 phosphorylation levels in response to sucrose consumption was subjected to two-way analysis of variance (ANOVA) with group as the between subject factor and time as the within subject factor. Data from SA (FR1 and FR5) experiments were analyzed with two-way repeated measures ANOVA with group as the between subject factor and session as the within subject factor. Data from the PR

schedule of the SA experiment (BP results) and on the escape numbers were analyzed using one-way ANOVA. Post-hoc analyses were performed by the Bonferroni's test when $p < 0.05$. To further analyze the results obtained after imipramine treatment, two-step cluster analysis using the Schwarz Bayesian criterion was employed (SPSS version 20). The advantage to the two-step clustering method is that it provides a measure of the most appropriate number of clusters using the Schwarz Bayesian Information Criterion. In order to demonstrate the stability of the cluster solution yielded from the two-step method, a K-Means cluster analysis was also employed using the number of clusters yielded from the two-step approach.

3. Results

3.1 *Experiment 1 - Effect of repeated lithium administration on DARPP-32 phosphorylation levels in response to palatable food consumption in control rats and rats showing a stress-induced deficit in appetitive motivation.*

In order to study whether repeated lithium administration could restore the response to a natural reward in rats exposed to chronic stress, the phosphorylation pattern of DARPP-32 in the NAcS was examined by immunoblotting at baseline and after the consumption of palatable food (sucrose pellets). Rats were divided into a control and a Chronic Stress group ($n = 20$ in each group). Rats in the Chronic Stress group were exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol for 3 weeks. Then, from day 21 half of the control and Chronic Stress rats received saline 1 ml/kg (Saline, $n = 10$, and Chronic Stress + Saline, $n = 10$), and half received lithium 0.8 mEq/kg (Li, $n = 10$, and Chronic Stress + Li, $n = 10$). Treatments were administered i. p. twice daily. After 8 days of treatment, animals in each group were sacrificed at baseline or 30 min after sucrose consumption. Analysis by two-way ANOVA of modifications in the phosphorylation levels of Thr34 and Thr75 DARPP-32 in response to sucrose consumption, expressed as the percentage of the respective time 0 levels, showed a significant effect of group (phospho-Thr34: $F_{3, 16} = 6.17$, $P < 0.01$; phospho-Thr75: $F_{3, 16} = 6.83$, $P < 0.01$), time (phospho-Thr34: $F_{1, 16} = 35.15$, $P < 0.001$; phospho-Thr75: $F_{1, 16} = 7.76$, $P < 0.05$) and their interaction (phospho-Thr34: $F_{3, 16} = 6.17$, $P < 0.01$; phospho-Thr75: $F_{3, 16} = 6.83$, $P < 0.01$). Post hoc analysis of phospho-Thr34 DARPP-32 levels showed that the response to sucrose consumption in the Stress group was blunted and differed from that in the Saline group ($P < 0.001$), while the response in the Li and Stress + Li groups was similar to that of the Saline rats and differed from that in the Stress group ($P < 0.001$; Fig.1a). Moreover, post hoc analysis showed that modifications in phospho-Thr75 DARPP-32 levels in the Stress and Li groups differed from those in the Saline group ($P < 0.001$, both comparisons), and in the Stress group they also differed from those in the Stress + Li group ($P < 0.05$) (Fig. 1b). These results indicate that lithium treatment restored the dopaminergic response to a natural reward in rats exposed to chronic stress and complement previous data that demonstrated a restored dopaminergic response in the NacS in terms of dopamine output by microdialysis (Marchese et al, 2013). Moreover, they confirm that modifications in the pattern of DARPP-32 phosphorylation represent a useful index of the NAcS dopaminergic response to a natural reward (Scheggi et al, 2013) and were studied in the following experiments.

3.2.1 *Experiment 2a - Effect of repeated imipramine administration on DARPP-32 phosphorylation levels in response to palatable food consumption in control rats and rats showing a stress-induced deficit in appetitive motivation.*

In order to study whether imipramine treatment was able to restore the response to a natural reward in a model of stress-induced deficit in appetitive motivation, the phosphorylation pattern of DARPP-32 in the NAcS was examined by immunoblotting at baseline and after the consumption of palatable food. Rats were divided into a control (n = 26) and a Chronic Stress group (n = 28). Rats in the Chronic Stress group were exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol for 3 weeks. Then, beginning on day 21, 12 rats of the control group and 12 Chronic Stress rats received saline 1 ml/kg (Saline and Chronic Stress + Saline), while 14 rats of the control group and 16 Chronic Stress rats received imipramine 5 mg/kg (IMI and Chronic Stress + IMI). Treatments were administered i. p. twice daily. After 8 days of treatment, animals in each treatment group were sacrificed at baseline or 30 min after sucrose consumption. Analysis by two-way ANOVA of modifications in the phosphorylation levels of Thr34 DARPP-32 in response to sucrose consumption, expressed as the percentage of the respective time 0 levels, showed a significant effect of group ($F_{3, 21}$ = 6.94, $P < 0.01$), time ($F_{1, 21}$ = 14.25, $P < 0.01$) and their interaction ($F_{3, 21}$ = 6.94, $P < 0.01$). In particular, post hoc analysis of phospho-Thr34 DARPP-32 levels showed a blunted response to sucrose consumption in the Stress, IMI and Stress + IMI groups ($P < 0.001$ versus the Saline group; Fig. 2a). Interestingly, the response to palatable food in terms of levels of phospho-Thr34 DARPP-32 in the Stress + IMI rats also differed from that in the Stress group ($P < 0.05$), suggesting that imipramine treatment reverted to some extent the effects induced by stress exposure on hedonic response. To clarify whether imipramine treatment induced a partial effect in the whole treated population or a full effect in part of the treated population, a two-step cluster analysis using the Schwarz Bayesian criterion was performed with phospho-Thr34 DARPP-32 levels as dependent variables to categorize rats into *non responders* and *responders*. The two-step cluster analysis showed that it was possible to divide data from all the experimental groups into two separate clusters (Fig. 2c). The first cluster grouped all the "time 0" rats and the Stress + Saline at 30 min group (*non responders*) and the second cluster grouped all Saline at 30 min rats (*responders*). In the group of 7 IMI at 30 min rats, 4 rats were grouped into the *non responders* cluster and 3 were grouped into the *responders* cluster. Moreover, in the group of 8 Stress + IMI at 30 min rats, 4 rats were grouped into the *non responders* cluster and 4 rats were grouped into the *responders* cluster. Analysis of modifications in the phosphorylation levels of Thr75 DARPP-32 in response to sucrose, expressed as the percentage of the respective time 0 levels, showed a significant effect of group ($F_{3, 18}$ = 3.63, $P < 0.05$), time ($F_{1, 18}$ = 5.65, $P < 0.05$) and their interaction ($F_{3, 18}$ = 3.63, $P < 0.05$). In particular, post hoc analysis showed that phospho-Thr75 DARPP-32 levels decreased only in the Saline group and differed from those in the Stress + Saline, IMI, and Stress + IMI groups ($P < 0.05$, $P < 0.001$ and $P < 0.01$, respectively; Fig. 2b). Thus, cluster analysis of Thr75 DARPP-32 phosphorylation levels was not performed. These results indicated that under imipramine treatment only about half of the rats showed an intense dopaminergic response to a natural reward and that imipramine treatment restored the response in 50% of the rats exposed to a chronic stress protocol.

3.2.2 *Experiment 2b - Effects of repeated imipramine administration on performance in the SA paradigm of control rats and rats showing a stress-induced deficit in appetitive motivation.*

Preliminary experiments showed that imipramine administration did not impair the ability to acquire sucrose SA in control rats (Table 2). Thus, this experiment investigated whether imipramine treatment would reverse an

established stress-induced deficit in appetitive motivation. Rats were divided into two groups: control rats (Ctr, $n =$ 18) and rats exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol (Stress, $n = 37$). Rats began SA training as described in the Experimental procedures section and illustrated in Fig. 3. When the condition of deficit in appetitive motivation was established according to the criteria described above in section 2.4 (Table 1), treatments began (day 21). The 18 control rats received saline (1 ml/kg, Saline) and the 37 rats exposed to the stress protocol were divided into two groups: 16 rats received saline (1 ml/kg, Chronic stress + Saline) and 21 rats received imipramine (5 mg/kg, Chronic stress + IMI), while continuing stress exposure. Treatments were administered i. p. twice daily. After 7 days of treatment (day 28), SA training with an FR5 schedule was resumed (Fig. 3). Analysis of lever presses by two-way ANOVA revealed a significant effect of group ($F_{2, 52} = 11.56$, $P < 0.001$), session ($F_{6, 312} = 6.71$, $P < 0.001$), but not their interaction. Post hoc analysis demonstrated that acquisition of the operant behavior was reduced in the Chronic stress + Saline ($P < 0.05$, session 2; $P < 0.001$, all other sessions, versus the Saline group), and a similar performance was observed in the Chronic stress + IMI group ($P < 0.01$, session 1; $P < 0.05$, all other sessions, versus the Saline group) (Fig. 4a). Analysis of the BP by one-way ANOVA showed a significant difference between groups ($F_{2, 52} = 11.84$, $P < 0.001$). Post hoc analysis confirmed that chronic stress exposure reduced the motivation to lever pressing for sucrose pellets (Chronic stress + Saline versus Saline group: $P < 0.001$) and imipramine treatment only partially restored the incentive motivation in stressed rats, as the BP value in the Chronic stress + IMI group was different from the BP values in the Chronic stress + Saline and Saline groups ($P < 0.05$, both comparisons) (Fig. 4b). Response to antidepressants shows high interindividual variability in both clinical practice (Joffe et al, 1996; Geschwind et al, 2011) and animal studies (Vaugeois et al, 1997; Jama et al, 2008) and in the latter case it is often possible to divide an outbred population into experimental substrains based on sensitivity to pharmacological treatment. Thus, to further evaluate the response to the antidepressant, a two-step cluster analysis using the Schwarz Bayesian criterion was performed in order to determine whether rats with appetitive motivation deficit showed different sensitivity to treatment, with the BP values as dependent variables to categorize rats into *non responders* and *responders*. Two-step cluster analysis revealed the existence of three discrete clusters (Fig. 4c). The first cluster grouped rats with very high motivation, represented by 4 Saline rats and 4 Stress + IMI rats (*high responders*); the second cluster grouped rats with a intermediate degree of motivation, represented by the remaining 14 Saline rats and 8 Stress + IMI rats (*responders*); the third cluster categorized rats with low levels of motivated behavior, represented by the 16 Chronic stress + Saline and 9 Stress + IMI rats (*non responders*). Thus, cluster analysis categorized the Stress + IMI rats into separate clusters on the basis of their antidepressant treatment response and indicated that imipramine treatment was efficacious in reverting the condition of stress-induced appetitive motivation deficit in 57% of the rats. Twenty-four h after the last SA session, rats were exposed to the escape test to verify their reactivity to avoidable aversive stimuli. The number of escapes was different between groups (one-way ANOVA, $F_{2, 52} = 50.12$, $P < 0.001$); post hoc analysis demonstrated a clear-cut escape deficit in the Chronic stress + Saline group ($P < 0.001$ versus the Saline group), while the performance of Chronic stress + IMI was not different from that of the Saline group (Table 3). This experiment indicate that in rats exposed to a chronic stress protocol repeated imipramine treatment had antianhedonic effects only in a subgroup of treated rats, while it restored the capability to escape avoidable noxious stimuli in the whole group.

3.3.1 *Experiment 3a - Effect of repeated fluoxetine administration on DARPP-32 phosphorylation levels in response to palatable food consumption in control rats and rats showing a stress-induced deficit in appetitive motivation.*

In order to study whether fluoxetine treatment was able to restore the response to a natural reward in a model of stress-induced deficit in appetitive motivation, the phosphorylation pattern of DARPP-32 in the NAcS was examined by immunoblotting at baseline and after the consumption of sucrose pellets. Rats were divided into a control ($n = 20$) and a Chronic Stress group ($n = 20$). Rats in the Chronic Stress group were exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol for 3 weeks. Then, beginning on day 21, 10 rats in the control group and 10 Chronic Stress rats received saline 1 ml/kg (Saline and Chronic Stress + Saline), while 10 rats of the control group and 10 Chronic Stress rats received fluoxetine 5 mg/kg (FLX and Chronic Stress + FLX). Treatments were administered i. p. once daily. After 8 days of treatment, animals in each treatment group were sacrificed at baseline or 30 min after sucrose consumption. Analysis by two-way ANOVA of modifications in the phosphorylation levels of Thr34 and Thr75 DARPP-32 in response to sucrose consumption, expressed as the percentage of the respective time 0 levels, showed a significant effect of group (phospho-Thr34: $F_{3, 16} = 7.58$, $P < 0.01$; phospho-Thr75: $F_{3, 16} = 3.76$, $P < 0.05$), time (phospho-Thr34: $F_{1, 16} = 10.78$, $P < 0.001$; phospho-Thr75: $F_{1, 16} = 22.04$, $P < 0.001$) and their interaction (phospho-Thr34: $F_{3, 16} = 7.58$, $P < 0.01$; phospho-Thr75: $F_{3, 16} = 3.76$, $P < 0.05$). Post hoc analysis of phospho-Thr34 DARPP-32 levels showed that the response to sucrose consumption in the Stress, FLX and Stress + FLX groups was blunted ($P < 0.001$ versus the Saline group) (Table 4). Post hoc analysis showed that, compared to the Saline group, the modifications in phospho-Thr75 DARPP-32 levels differed in the Stress group ($P < 0.001$) and in the FLX and Stress + FLX groups ($P < 0.01$, both comparisons) (Table 4). These results indicate that fluoxetine treatment blunted the dopaminergic response to a natural reward and did not restore this response in rats exposed to the chronic stress protocol.

3.3.2 *Experiment 3b - Effects of repeated fluoxetine administration on performance in the SA paradigm of control rats and rats showing a stress-induced deficit in appetitive motivation.*

Preliminary experiments showed that fluoxetine administration did not impair the ability to acquire sucrose SA in control rats (Table 2). Thus, this experiment investigated whether fluoxetine treatment would reverse an established stress-induced deficit in appetitive motivation. Rats were divided into two groups: control rats (Ctr, $n = 8$) and rats exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol (Stress, $n = 16$). Rats began SA training as described in the Experimental procedures section and illustrated in Fig. 3. When the motivational deficit was established (Table 1), treatments began (day 21). The 8 control rats received saline (1 ml/kg, Saline) and the 16 rats exposed to the stress protocol were divided into two groups: 8 rats received saline (1 ml/kg, Chronic stress + Saline) and 8 rats received fluoxetine (5 mg/kg, Chronic stress + FLX), while continuing stress exposure. Treatments were administered i. p. once daily. After 7 days of treatment (day 28), SA training with an FR5 schedule was resumed (Fig. 3). Analysis of lever presses by two-way ANOVA revealed a significant effect of group ($F_{2, 21} = 10.54$, $P < 0.001$), session ($F_{6, 126} = 12.09$, $P < 0.001$), but not their interaction. Post hoc analysis demonstrated that acquisition of the operant behavior was reduced, compared to the Saline group, in the Chronic stress + Saline ($P < 0.01$, sessions 1 and 5; $P < 0.01$, session 6; $P < 0.001$, session 7) and in the Chronic stress +

FLX group ($P < 0.05$, session 2; $P < 0.01$, sessions 3, 4, 5, 6 and 7; $P < 0.001$, session 1) (Fig. 5a). Analysis of the BP by one-way ANOVA showed a significant difference between groups ($F_{2, 21} = 9.26$, $P < 0.001$). Post hoc analysis confirmed that chronic stress exposure reduced motivation to lever pressing for sucrose pellets (Chronic stress + Saline versus Saline group: $P < 0.01$) and that fluoxetine treatment was not able to restore the incentive motivation in stressed rats ($P < 0.05$ versus the Saline group) (Fig. 5b). Twenty-four h after the last SA session, rats were exposed to the escape test to verify their reactivity to avoidable aversive stimuli. The number of escapes was different between groups (one-way ANOVA, $F_{2, 21} = 29.06$, $P < 0.001$); post hoc analysis demonstrated a clear-cut escape deficit in the Chronic stress + Saline, while the Chronic stress + FLX group and Saline group had similar performances (Table 3). This experiment indicate that repeated fluoxetine treatment did not counteract the condition of stress-induced motivational anhedonia, while it restored the capability to escape avoidable noxious stimuli in the whole group.

3.4.1 *Experiment 4a - Effect of repeated haloperidol administration on DARPP-32 phosphorylation levels in response to palatable food consumption in control rats.*

In order to study whether haloperidol administration affected the response to a natural reward, the phosphorylation pattern of DARPP-32 in the NAcS was examined by immunoblotting at baseline and after the consumption of sucrose pellets. Rats were divided into two groups: 10 rats received vehicle (1 ml/kg, Vehicle) and 10 rats received haloperidol (0.1 mg/kg, HAL). Treatments were administered i. p. twice daily. After 8 days of treatment, animals in each group were sacrificed at baseline or 30 min after sucrose consumption. Analysis by two-way ANOVA of modifications in the phosphorylation levels of Thr34 and Thr75 DARPP-32 in response to sucrose consumption, expressed as the percentage of the respective time 0 levels, showed a significant effect of treatment (phospho-Thr34: $F_{1, 16} = 4.94$, $P < 0.05$; phospho-Thr75: $F_{1, 16} = 4.63$, $P < 0.05$), time (phospho-Thr34: $F_{1, 16} = 14.22$, $P < 0.01$; phospho-Thr75: $F_{1, 16} = 27.51$, $P < 0.001$) and their interaction (phospho-Thr34: $F_{1, 16} = 4.94$, $P < 0.05$; phospho-Thr75: $F_{1, 16} = 4.63$, $P < 0.05$). Post hoc analysis of phospho-Thr34 and phospho-Thr75 DARPP-32 levels demonstrated that the response to sucrose consumption in the HAL group was blunted (phospho-Thr34: 126 ± 17.3 % versus 193 ± 26.4 % in the Vehicle group, $P < 0.05$; and phospho-Thr75: 79.1 ± 9.9 % versus 50.1 ± 6.7 % in the Vehicle group, $P < 0.05$).

3.4.2 *Experiment 4b - Effects of repeated haloperidol administration on performance in the SA paradigm of control rats and rats showing a stress-induced deficit in appetitive motivation.*

Preliminary experiments showed that haloperidol administration did not impair the ability to acquire sucrose SA in control, as haloperidol-treated rats attained a BP score not different from the score of vehicle treated rats (Table 2). Thus, this experiment investigated the effect of haloperidol treatment on an established stress-induced deficit in appetitive motivation. Rats were divided into two groups: control rats (Ctr, $n = 6$) and rats exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol (Stress, $n = 12$). Rats began SA training as described in the Experimental procedures section and illustrated in Fig. 3. When the condition of appetitive motivation deficit was established (Table1), treatments began (day 21). The 6 control rats received vehicle (1 ml/kg, Vehicle) and the 12 rats exposed to the stress protocol were divided into two groups: 6 rats received saline (1 ml/kg, Chronic stress + Saline) and 6 rats received haloperidol (0.1 mg/kg, Chronic stress +

HAL), while continuing stress exposure. Treatments were administered i. p. twice daily. After 7 days of treatment (day 28), SA training with an FR5 schedule was resumed (Fig. 3). Analysis of lever presses by two-way ANOVA revealed a significant effect of group ($F_{2, 15} = 5.36$, $P < 0.05$), session ($F_{5, 75} = 5.68$, $P < 0.001$) and their interaction ($F_{10, 75} = 4.73$, $P < 0.001$). Post hoc analysis demonstrated that, compared to the Saline group, acquisition of the operant behavior was reduced in the Chronic stress + Vehicle group ($P < 0.05$, session 5; $P < 0.01$, session 3 and 4; $P < 0.001$, session 6) and in the Chronic stress + HAL group ($P < 0.05$, session 5; $P < 0.01$, session 6) (Fig. 6a). Analysis of the BP by one-way ANOVA showed a significant difference between groups ($F_{2, 15} = 13.72$, $P < 0.001$). Post hoc analysis confirmed that chronic stress exposure reduced motivation to lever pressing for sucrose pellets (Chronic stress + Vehicle versus Vehicle group: $P < 0.01$) and haloperidol treatment did not restore the incentive motivation in stressed rats (Chronic stress + HAL versus the Vehicle group: $P < 0.001$) (Fig. 6b). Twenty-four h after the last SA session, rats in each group were exposed to the escape test to verify their reactivity to avoidable aversive stimuli. The number of escapes was different between groups (one-way ANOVA, $F_{2, 15} = 25.99$, $P < 0.001$); post hoc analysis demonstrated a clear-cut escape deficit in the Chronic stress + Vehicle and Chronic stress + HAL groups ($P < 0.001$ versus the Vehicle group, both comparisons), showing that haloperidol did not restore the ability to escape avoidable noxious stimuli (Table 3). These results indicate that while repeated haloperidol treatment did not impair the rat's competence to operate for sucrose, it did not counteract the effects of chronic stress exposure on the responses to positive and negative stimuli.

3.5.1 *Experiment 5a - Effect of repeated clozapine administration on DARPP-32 phosphorylation levels in response to palatable food consumption in control rats and rats showing a stress-induced deficit in appetitive motivation.*

In order to study the effect of clozapine administration on the response to a natural reward in control and anhedonic rats, the phosphorylation pattern of DARPP-32 in the NAcS was examined by immunoblotting at baseline and after the consumption of sucrose pellets. Rats were divided into a control ($n = 22$) and a Chronic Stress group ($n = 24$). Rats in the Chronic Stress group were exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol for 3 weeks. Then, beginning on day 21, 12 rats of the control group and 12 Chronic Stress rats received vehicle 1 ml/kg (Vehicle and Chronic Stress + Vehicle), while 10 rats of the control group and 12 Chronic Stress rats received clozapine 5 mg/kg (CLZ and Chronic Stress + CLZ). Treatments were administered i. p. twice daily. After 8 days of treatment, animals in each treatment group were sacrificed at baseline or 30 min after sucrose consumption. Analysis by two-way ANOVA of modifications in the phosphorylation levels of Thr34 and Thr75 DARPP-32 in response to sucrose consumption, expressed as the percentage of the respective time 0 levels, showed a significant effect of group (phospho-Thr34: $F_{3, 38} = 3.30$, $P < 0.05$; phospho-Thr75: $F_{3, 36} = 5.47$, $P < 0.01$), time (phospho-Thr34: $F_{1, 38} = 21.52$, $P < 0.001$; phospho-Thr75: $F_{1, 36} = 28.27$, $P < 0.001$) and their interaction (phospho-Thr34: $F_{3, 38} = 3.30$, $P < 0.05$; phospho-Thr75: $F_{3, 36} = 5.47$, $P < 0.01$). Post hoc analysis of modifications in phospho-Thr34 DARPP-32 levels showed that the response to sucrose consumption in the Stress group was blunted ($P < 0.001$ versus the Saline group), while the response in the CLZ and Stress + CLZ groups was similar to that of the Vehicle rats and differed from that in the Stress group ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 7a). Moreover, post hoc analysis showed that modifications in phospho-Thr75 DARPP-32 levels in the Stress,

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CLZ and Stress + CLZ groups differed from those in the Vehicle group ($P < 0.001$ for the Stress and Stress + CLZ groups, $P < 0.01$ for the CLZ group), and in the CLZ group they also differed from those in the Stress group ($P < 0.05$) (Fig. 7b). These results indicate that repeated clozapine treatment restored the dopaminergic response to a natural reward in rats exposed to chronic stress.

3.5.2 *Experiment 5b - Effects of repeated clozapine administration on performance in the SA paradigm of control rats and rats showing a stress-induced deficit in appetitive motivation.*

Preliminary experiments showed that clozapine administration did not impair the ability to acquire sucrose SA in control rats (Table 2). Thus, this experiment investigated whether clozapine treatment would reverse an established stress-induced deficit in appetitive motivation. Rats were divided into two groups: control rats (Ctr, $n = 5$) and rats exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol (Stress, $n = 14$). Rats began SA training as described in the Experimental procedures section and illustrated in Fig. 3. When the condition of appetitive motivation deficit was established (Table1), treatments began (day 21). The control rats received vehicle (1 ml/kg, Vehicle) and the 16 rats exposed to the stress protocol were divided into two groups: 6 rats received saline (1 ml/kg, Chronic stress + Vehicle) and 8 rats received clozapine (5 mg/kg, Chronic stress + CLZ), while continuing stress exposure. Treatments were administered i. p. twice daily. After 7 days of treatment (day 28), SA training with an FR5 schedule was resumed (Fig. 3). Analysis of lever presses by two-way ANOVA revealed a significant effect of group ($F_{2, 16} = 4.44$, $P < 0.05$) and session ($F_{5, 80} = 7.38$, $P < 0.001$), but not their interaction. Post hoc analysis demonstrated that acquisition of the operant behavior was reduced in the Chronic stress + Vehicle compared to the Vehicle group ($P < 0.05$, session 2, 3, 4 and 6; $P < 0.01$, session 5) and clozapine treatment did not completely counteract the effect of stress exposure since the performance of the Chronic stress + CLZ group was not different from that of the Chronic stress + Vehicle or the Vehicle group (Fig. 6c). Analysis of the BP by one-way ANOVA showed a significant difference between groups ($F_{2, 16} = 7.41$, $P < 0.01$). Post hoc analysis confirmed that chronic stress exposure reduced motivation to lever pressing for sucrose pellets (Chronic stress + Vehicle versus Vehicle group: $P < 0.01$), while clozapine treatment restored the incentive motivation in stressed rats (Chronic stress + CLZ versus the Chronic stress + Vehicle group: p < 0.05, Fig. 6d).

Twenty-four h after the last SA session, rats in each group were exposed to the escape test to verify their reactivity to avoidable aversive stimuli. The number of escapes was different between groups (one-way ANOVA, $F_{2, 16} = 16.96$, $P < 0.001$); post hoc analysis demonstrated a clear-cut escape deficit in the Chronic stress + Vehicle and Chronic stress + CLZ groups ($P < 0.001$ and $P < 0.01$ versus the Vehicle group, respectively), showing that clozapine did not restore the ability to escape avoidable noxious stimuli (Table 3). This experiment indicates that clozapine treatment restored the motivation to operate for a reward in rats exposed to chronic stress, but not the capability to escape avoidable noxious stimuli.

4. Discussion

The present results show that lithium reinstated the increase in phosphoThr34-DARPP-32 levels induced by sucrose consumption in the NAcS of rats exposed to chronic stress, thus confirming that dopamine D_1 receptor-dependent signaling matches the modifications observed in extraneuronal dopamine levels (Danielli et al, 2010). Anhedonia is a symptom common to different types of psychopathology and considered particularly relevant in depression and

schizophrenia (Pelizza and Ferrari, 2009). Thus, we focused our interest on drugs used to treat these two disorders and selected two antidepressants, imipramine and fluoxetine, and two antipsychotics, haloperidol and clozapine, to be tested on our model. After 8 days of treatment, about half of the imipramine-treated rats and all fluoxetine-treated rats showed a blunted dopaminergic response to palatable food, yet during a total 3 week-administration, these drugs did not modify the competence to acquire sucrose SA. A 3-week treatment with imipramine or fluoxetine completely reverts the repeated stress-induced escape deficit (Gambarana et al, 2001) and prevents the disrupting effect of chronic stress on the acquisition of an appetitive instrumental behavior (Ghiglieri et al, 1997). Accordingly, in the present study stressed rats treated with imipramine or fluoxetine, tested at the end of the SA protocol, showed an escape capacity similar to that of control animals. However, repeated imipramine treatment only reinstated the dopaminergic response to sucrose in about 50 % of the chronically stressed rats, and about the same percentage of animals acquired sucrose SA and reached a BP score similar to that of control animals. Stressed rats treated with fluoxetine did not show the dopaminergic response to sucrose, did not acquire sucrose SA, and they showed a BP score similar to that of stressed rats. Analogously, a study of the effects of a 2-week treatment with fluoxetine or desipramine on anhedonia induced by chronic social defeat demonstrated the efficacy of these antidepressant treatments in about 45% of the rats showing a stress-induced increase in intracranial self-stimulation threshold (Der-Avakian et al, 2014). The failure of fluoxetine and the efficacy of imipramine, limited to a subgroup of stressed rats, to revert the negative effects of stress exposure on sucrose SA does not necessarily imply that these compounds are devoid of antianhedonic activity. In a condition of reduced sucrose consumption induced by chronic mild stress, fluoxetine reverted this deficit after a 9-week treatment, while imipramine was effective after a 3-week treatment (Muscat et al, 1992; Monleon et al, 1995). Thus, a more prolonged treatment with imipramine or fluoxetine might also have resulted in a reinstated competence to acquire sucrose SA in rats exposed to the chronic stress protocol. What the present results suggest is that repeated imipramine or fluoxetine administration, at variance with lithium treatment (Marchese et al, 2013), is not endowed with a rapid onset effect on stress-induced appetitive deficit, while the effects on the reactivity to noxious stimuli showed a faster onset. An early antianhedonic activity may be clinically relevant, as clinical studies show that although antidepressant treatments affect both positive and negative emotion processing (Rawlings et al, 2010; Harmer et al, 2011), it is the early improvement in positive affect that best predicts treatment outcome (Wichers et al, 2009; Geschwind et al, 2011). In other words, the increase in positive affect discriminates between treatment responders and non-responders. While haloperidol and NAc dopamine depletion do not modify food palatability and consumption (Salamone et al, 1996; Berridge and Robinson, 1998), they may impair lever pressing for sucrose, depending on the ratio schedule requirement (Aberman and Salamone, 1999; Salamone et al, 2007). Repeated haloperidol administration, at the dose of 0.1 mg/kg twice a day, decreased the dopaminergic response to sucrose consumption, but it did not significantly affect the rat's competence to acquire sucrose SA. Since the basal aspects of food motivation are mainly sustained by palatability, as demonstrated in non food-deprived rats consuming sucrose or saccharin (Scheggi et al, 2013), and remain intact after interference with mesolimbic dopaminergic transmission (Salamone and Correa, 2002; Kelley et al, 2005), it was predictable that haloperidol would not impair animal's performance on a relatively non-demanding SA schedule. On the other hand, chronic stress exposure abolished the dopaminergic response to sucrose consumption and its disrupting effects on sucrose SA were already evident under the FR1

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schedule. That is, stress also interfered with the basal, dopamine-independent motivation for food. Repeated haloperidol treatment did not revert the stress-induced behavioral deficits.

In the present study we observed that a repeated treatment with clozapine or haloperidol did not modify the pattern of DARPP-32 phosphorylation, compared with saline-treated rats (data not shown), at variance with results obtained after acute administrations *in vivo* or *ex vivo* (Pozzi et al, 2003; Bateup et al, 2008). Moreover, repeated clozapine treatment did not interfere with the dopamine D_1 receptor-mediated modifications in DARPP-32 phosphorylation induced by sucrose consumption and it did not modify the rat's competence to acquire sucrose SA. Clozapine-treated stressed rats showed a reduced performance in the FR5 schedule experiments compared to control rats, while a complete reversal of stress-induced effect was observed in the BP score. These results do not inherently conflict with the decreased lever pressing for sucrose previously observed in rats receiving similar doses of clozapine, since this effect was interpreted as likely due to sedative activity to which tolerance developed within 14 days of treatment (Salamone et al, 1996). In the present study, rats completed the SA protocol at 21-22 days of drug administration. Moreover, while in the study by Salamone et al. (1996) rats were tested 30 min after clozapine administration, in this study SA sessions were performed about 15-16 h after the evening dose, to avoid acute effects. Thus, repeated clozapine treatment reinstated the basal, dopamine-independent, motivational appetitive competence disrupted by chronic stress exposure. Moreover, since it restored the dopaminergic response to sucrose consumption, likely it also reinstated the dopamine-dependent motivational responsiveness.

In the search for more refined definitions of anhedonia the terms "consummatory anhedonia", which refers to deficits in the hedonic response to rewards (Salamone et al, 2007), and "motivational anhedonia", which refers to a diminished motivation to pursue them, were proposed (Treadway and Zald, 2011). These terms are intended to roughly correspond to the reward-processing components of "liking" and "wanting" proposed in the preclinical literature (Berridge and Robinson, 1998; Berridge, 2009). However, other authors restrict the term anhedonia to the emotional reaction to reward and consider confounding the extension of the definition to the pursuit of rewards (Markou et al, 2013). In the present model, the motivation to pursue a hedonic stimulus, sucrose, that rats perceive as palatable, is measured. Thus, the disrupting effect of unavoidable stress exposure on sucrose SA acquisition - so far termed appetitive deficit or decreased appetitive motivation - delineates a model that, according to Treadway and Zald (2011), can be defined as "motivational anhedonia".

4.1 Conclusions

This study shows that the antidepressant drugs tested differently affected the reactivity to positive and negative stimuli, as fluoxetine had no antianhedonic activity and imipramine was active only in a subgroup of rats, while both drugs reinstated the reactivity to aversive stimuli in all rats. Of the two antipsycotic drugs tested, clozapine showed an antianhedonic activity, but did not revert the hyporeactivity towards aversive stimuli, while haloperidol did not modify the effects of chronic stress exposure. The administration of the four drugs, haloperidol included, to non-stressed rats did not impair the competence to acquire sucrose SA. The results obtained with haloperidol strengthen the tenet that the baseline drive to operate for a natural reward, such as a palatable food, is dopamine-independent (Salamone et al, 1996). Moreover, the treatments that showed antianhedonic activity (lithium, clozapine, and imipramine in the "responder" rats) also restored the acute dopaminergic response to palatable food. Thus, the study

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suggests that the dopamine-dependent component of the motivation to operate for a palatable food is also restored by effective "antianhedonic treatments".

5. Acknowledgements

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Figure legends

Fig. 1. Modifications in phospho-Thr34 (a) and phospho-Thr75 (b) DARPP-32 levels in response to sucrose consumption in control and chronically stressed rats treated or not with lithium. Rats in the Chronic Stress group were exposed to the sequence of unavoidable stress-escape test (day 1 and 2) and then to the stress protocol for 3 weeks. Then, beginning on day 21, rats in the control group and Chronic Stress rats received saline 1 ml/kg (Saline and Chronic Stress + Saline), or lithium 0.8 mEq/kg (Li and Chronic Stress + Li), twice a day i. p. After 8 days of treatment, animals in each group were sacrificed at baseline or 30 min after sucrose consumption. Data are presented as mean ± S.E.M. of percentage modification in phospho-DARPP-32 levels compared to levels in the baseline group. *** $P < 0.001$ versus the Saline group; ### $P < 0.001$, # $P < 0.05$ versus the Chronic Stress + Saline group; §§ $P < 0.01$ versus the Li group (Bonferroni's test).

Fig. 2. Modifications in DARPP-32 phosphorylation levels in response to sucrose consumption in control and chronically stressed rats treated or not with imipramine. Rats in the Chronic Stress group were exposed to the stress protocol for 3 weeks as described in the legend to Fig. 1. Then, beginning on day 21, 12 rats of the control group and 12 Chronic Stress rats received saline (1 ml/kg, Saline and Chronic Stress + Saline), while 14 rats of the control group and 16 Chronic Stress rats received imipramine (5 mg/kg, IMI and Chronic Stress + IMI), twice a day i. p. After 8 days of treatment, animals in each group were sacrificed at baseline or 30 min after sucrose consumption. Data are presented as mean ± S.E.M. of percentage modification in phospho-DARPP-32 levels compared to levels in the baseline group. (a) phospho-Thr34 and (b) phospho-Thr75 DARPP-32 levels. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus the Saline group; # $P < 0.05$ versus the Chronic Stress + Saline group (Bonferroni's test). (c) Two-step cluster analysis of phospho-Thr34 DARPP-32 levels as dependent variables revealed the existence of two discrete clusters. The cluster under the dotted line grouped all rats at baseline, all Chronic Stress + Saline rats and about half rats in the IMI and Chronic Stress + IMI groups after sucrose consumption. The cluster above the dotted line grouped all rats in the Saline group and half of the rats in the IMI and Chronic Stress + IMI groups after sucrose consumption.

Fig. 3. Outline of experimental protocol for the self administration (SA) protocol. Rats were exposed to the unavoidable stress session on day 1 and were tested for escape on day 2. On day 3 they began the chronic stress protocol (Stress). A group of rats were not exposed to the stress protocol (Control). After 7 days of stress exposure or non stressful manipulation, the two groups of rats were trained to press a lever for sucrose pellets under FR1 and FR5 schedules of reinforcement. When the Stress rats attained the criterion for appetitive motivation deficit, Control and Stress rats were divided into two groups that received saline/vehicle or drug for 7 days; then they resumed the FR5 and PR schedules, while treatments continued concomitant with exposure to the chronic stress protocol. At the end of SA protocol, rats were tested for the escape.

Fig. 4. Fixed Ratio 1 (FR1), Fixed Ratio 5 (FR5) responses and Progressive Ratio (PR) schedules for sucrose pellets in control and chronically stressed rats treated or not with imipramine. Rats were exposed to chronic stress protocol as described in the legend to Fig. 3 and when the condition of appetitive motivation deficit was established in the stress group, they began treatment with saline (1 ml/kg, Chronic Stress + Saline) or imipramine (5 mg/kg, Chronic Stress + IMI) twice a day i. p. for 7 days; then they resumed the FR5 (a) and PR schedules (b), while treatments continued concomitant with exposure to the chronic stress protocol. Data are presented as mean ± S.E.M. of the number of correct responses (a) or BP values (b). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the Saline group; [#] $P < 0.05$ versus the Chronic Stress + Saline group (Bonferroni's test). (c) Two-step cluster analysis of BP value as dependent variables revealed the existence of three discrete clusters. The clusters of responders (*high responders* and r*esponders*) grouped all the Saline rats and about half of the Chronic Stress + IMI rats; the cluster of *non responders* grouped the Chronic Stress + Saline rats and about half of the Chronic Stress + IMI rats.

Fig. 5. Fixed Ratio 1 (FR1), Fixed Ratio 5 (FR5) responses and Progressive Ratio (PR) schedules for sucrose pellets in control and chronically stressed rats treated or not with fluoxetine. Rats were exposed to chronic stress protocol as described in the legend to Fig. 3 and when the condition of appetitive motivation deficit was established in the stress group, they began treatment with saline (1 ml/kg, Chronic Stress + Saline) or fluoxetine (5 mg/kg, Chronic Stress + FLX), once a day i. p. for 7 days; then they resumed the FR5 (a) and PR schedules (b), while treatments continued concomitant with exposure to the chronic stress protocol. Data are presented as mean ± S.E.M. of the number of correct responses (a) or BP values (b). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus the Saline group (Bonferroni's test).

Fig. 6. Fixed Ratio 1 (FR1), Fixed Ratio 5 (FR5) responses and Progressive Ratio (PR) schedules for sucrose pellets in control and chronically stressed rats treated or not with haloperidol (a, b) or with clozapine (c, d). Rats were exposed to chronic stress protocol as described in the legend to Fig. 3 and when the condition of appetitive motivation deficit was established in the stress group, they began treatment with vehicle (1 ml/kg, Chronic Stress + Vehicle), haloperidol (0.1 mg/kg, Chronic Stress + HAL) or clozapine (5 mg/kg, Chronic Stress + CLZ), twice a day i. p. for 7 days; then they resumed the FR5 (a, c) and PR schedules (b, d), while treatments continued concomitant with exposure to the chronic stress protocol. Data are presented as mean ± S.E.M. of the number of correct responses (a, c) or BP values (b, d). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the Vehicle group; [#] $P < 0.05$ versus the Chronic Stress + Vehicle (Bonferroni's test).

Fig. 7. Modifications in phospho-Thr34 (a) and phospho-Thr75 (b) DARPP-32 levels in response to sucrose consumption in control and chronically stressed rats treated or not with clozapine. Rats in the Chronic Stress group were exposed to the stress protocol for 3 weeks as described in Fig. 1. Then, beginning on day 21, Control and Chronic Stress rats received vehicle (1 ml/kg, Chronic Stress + Vehicle) or clozapine (5 mg/kg, Chronic Stress

+ CLZ), twice a day i. p. Two groups of rats not exposed to the stress protocol received vehicle (1 ml/kg, Vehicle) or clozapine (5 mg/kg, CLZ), twice a day i. p. After 8 days of treatment, animals in each group were sacrificed at baseline or 30 min after sucrose consumption. Data are presented as mean ± S.E.M. of percentage modification in phospho-DARPP-32 levels compared to levels in the baseline group. *** $P < 0.001$, ** $P < 0.01$ versus the Vehicle group; ## $P < 0.01$, # $P < 0.05$ versus the Chronic Stress + Vehicle group (Bonferroni's test).

Tables

Table 1. Responding for sucrose pellets under fixed-ratio (FR)1 and FR5 in control rats and in rats exposed to chronic stress protocol.

Groups	FR1 (5th session)	FR5 (3rd session)
Experiment 2b	Number of correct responses	
CTR	83.66 ± 7.4 ***	55.61 ± 4.7 ***
Chronic Stress	34.32 ± 4.0	28.10 ± 3.2
Experiment 3b		
CTR	82.62 ± 6.9 ***	59.25 ± 7.5 ***
Chronic Stress	30.87 ± 5.1	22.87 ± 4.3
Experiment 4b		
CTR	119.66 ± 22.9 ***	55.61 ± 4.7 **
Chronic Stress	27.83 ± 5.4	27.16 ± 5.9
Experiment 5b		
CTR	99.40 ± 17.1 ***	68.60 ± 11.6 **
Chronic Stress	33.07 ± 5.5	27.88 ± 6.6

Rats were exposed to the unavoidable stress session on day 1 and were tested for escape on day 2. On day 3 they began the chronic stress protocol (Chronic Stress). A group of rats were not exposed to the stress protocol (control group, CTR). After a 7-day stress exposure, the two groups of rats were trained to press a lever for sucrose pellets under FR1 and FR5 schedules of reinforcement. Data are presented as mean ± S.E.M. of the number of correct responses in the 5th session in FR1 schedule and the 3rd session in FR5 schedule. *** $P < 0.001$, ** $P < 0.01$ versus the Chronic Stress group.

Table 2. Responding for sucrose pellets under progressive ratio in control rats and in rats treated with imipramine, fluoxetine, haloperidol or clozapine.

Groups	BP (Last session completed)
Experiment 2b	
CTR	35.80 ± 2.9
IMI	28.50 ± 4.1
Experiment 3b	
CTR	37.80 ± 9.0
FLX	36.17 ± 5.5
Experiment 4b	
CTR	35.67 ± 6.8
HAL	25.33 ± 4.9
Experiment 5b	
CTR	34.88 ± 5.5
CLZ	28.50 ± 3.8

Rats were administered imipramine, fluoxetine, haloperidol or clozapine for 8 days; when stable responses under FR1 and FR5 reinforcement schedules were established, rats were switched to a progressive ratio (PR) schedule with a step size of 3. Data are presented as mean ± S.E.M. of the breaking point (BP) values.

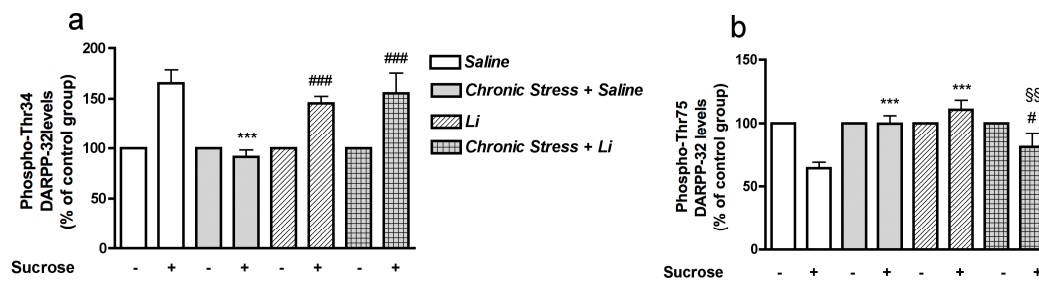**Table 3. Number of escapes scored by rats at the end of SA training**

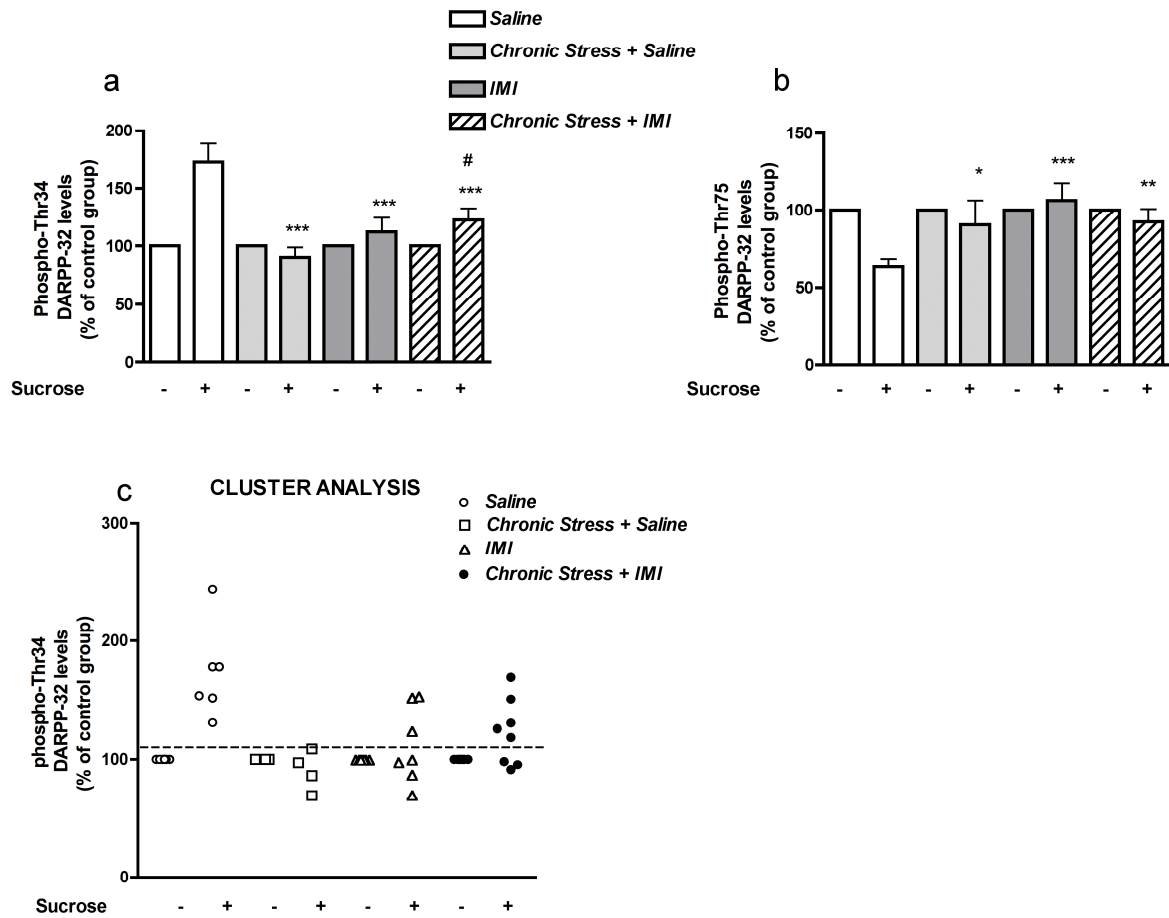| Groups	Number of escapes
Experiment 2b	
Saline	21.6 ± 1.2
Chronic Stress + Saline	6.6 ± 1.4 ***
Chronic Stress + IMI	18.0 ± 0.7 [###]
Experiment 3b	
Saline	21.1 ± 2.0
Chronic Stress + Saline	5.1 ± 1.2 ***
Chronic Stress + FLX	15.9 ± 1.0 [###]
Experiment 4b	
Vehicle	19.5 ± 2.2
Chronic Stress + Vehicle	4.8 ± 1.6 ***
Chronic Stress + HAL	3.7 ± 21.2 ***
Experiment 5b	
Vehicle	21.4 ± 1.6
Chronic Stress + Vehicle	2.2 ± 0.8 ***
Chronic Stress + CLZ	9.1 ± 2.7 **

Rats were exposed to the unavoidable stress session on day 1 and were tested for escape on day 2. On day 3 they began treatment with saline or vehicle (1 ml/kg) or imipramine (5 mg/kg), fluoxetine (5 mg/kg), haloperidol (0.1 mg/kg) or clozapine (5 mg/kg); treatments continued concomitant with exposure to the chronic stress protocol. A group of rats not exposed to the stress protocol received saline or vehicle. Treatments were administered i. p. twice a day with the exception of fluoxetine. After 8 days of treatment, all groups began the self administration (SA) training. Twenty-four h after the last SA session they were tested for escape. Scores are expressed as mean number of escapes ± S.E.M. in 30 consecutive trials. *** $P < 0.001$, ** $P < 0.01$ versus the Saline group; [###] $P < 0.001$ versus the Chronic Stress + Vehicle group (Bonferroni's test).
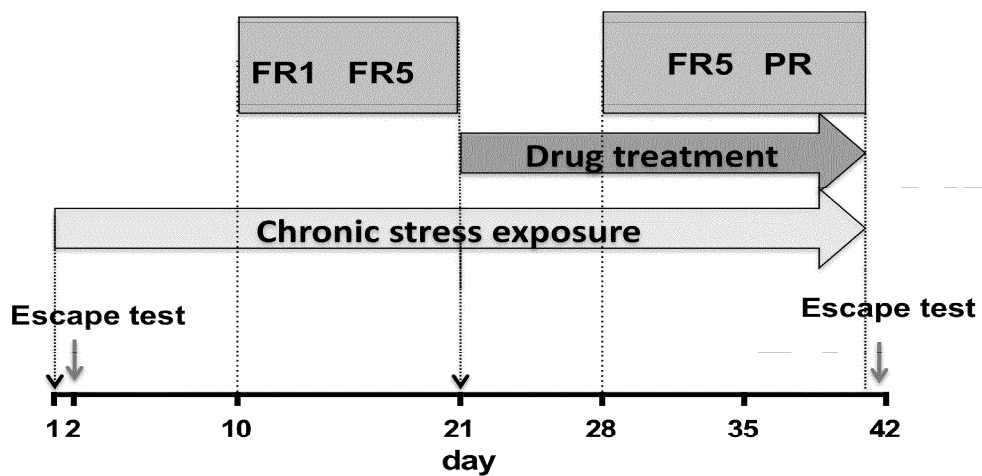
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Table 4. Modifications in DARPP-32 phosphorylation levels in response to sucrose consumption in control and chronically stressed rats treated or not with fluoxetine.
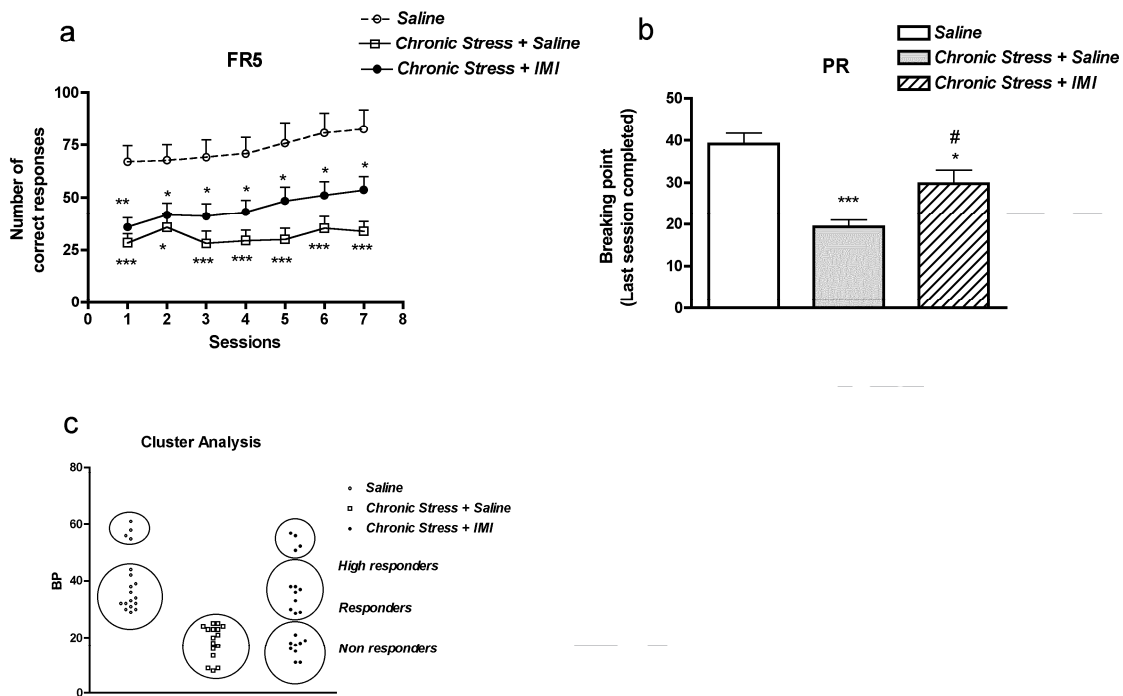
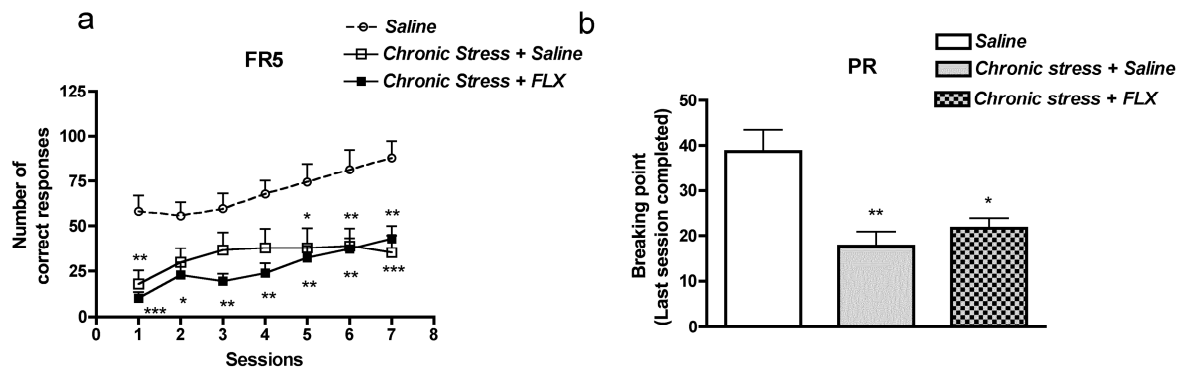| Groups	Baseline (0 min)	30 min after sucrose consumption
Experiment 3a	Phospho-Thr34 DARPP-32 phosphorylation levels	
Saline	100	165.2 ± 13.6 ***
Chronic Stress + Saline	100	91.4 ± 6.6
FLX	100	111.5 ± 16.3
Chronic Stress + FLX	100	108.2 ± 6.5
	Phospho-Thr75 DARPP-32 phosphorylation levels	
Saline	100	64.1 ± 5.1**
Chronic Stress + Saline	100	92.5 ± 10.7
FLX	100	88.9 ± 4.8
Chronic Stress + FLX	100	90.0 ± 4.9

Rats were exposed to the unavoidable stress session on day 1 and were tested for escape on day 2. On day 3 they were exposed to the chronic stress protocol. On day 21 they began treatment with saline (1 ml/kg, Chronic Stress+Saline) or fluoxetine (5 mg/kg, Chronic Stress+FLX). Treatments continued concomitant with exposure to the chronic stress protocol. Two groups of rats not exposed to the stress protocol received saline (1 ml/kg, Saline) or fluoxetine (5mg/kg, FLX). After 8 days of treatment, animals in each group were sacrificed at baseline or 30 min after sucrose consumption. Data are expressed as mean ± S.E.M. of percentage modification in phospho-DARPP-32 levels versus levels in the baseline (time 0) group. *** $P < 0.001$, ** $P < 0.01$ versus the response of Chronic Stress + Saline, FLX and Chronic Stress + FLX groups (Bonferroni's test).
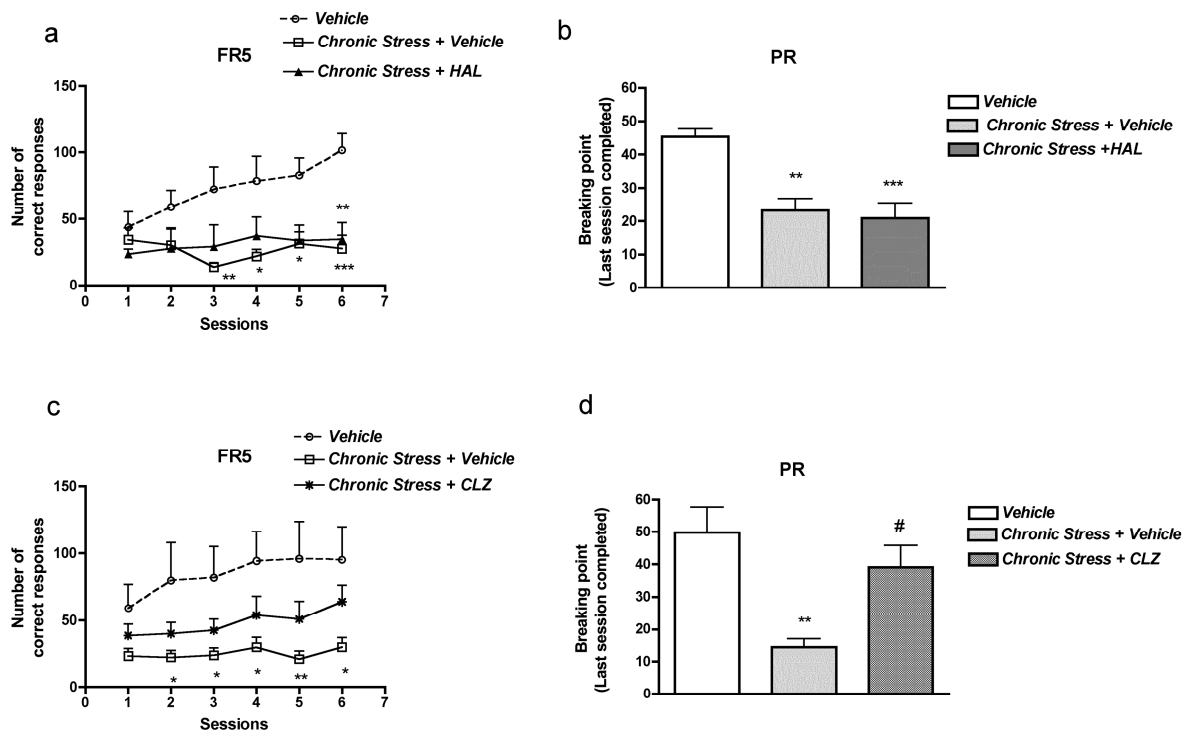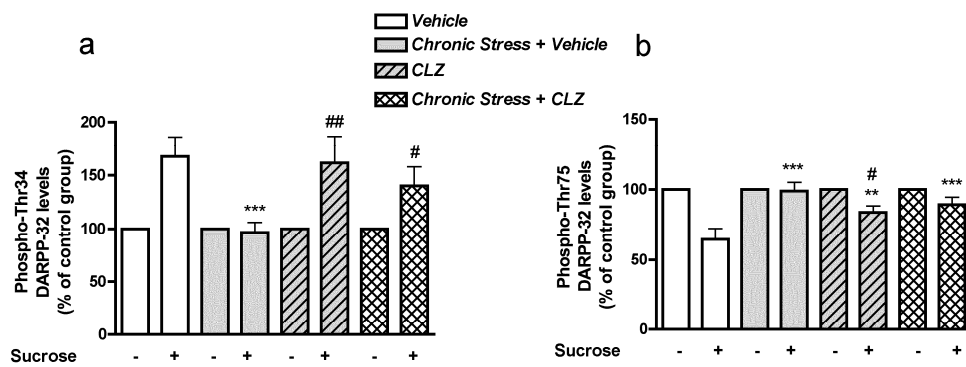
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a FR5

- Vehicle
- Chronic Stress + Vehicle
- Chronic Stress + HAL

b PR

- Vehicle
- Chronic Stress + Vehicle
- Chronic Stress +HAL

c FR5

- Vehicle
- Chronic Stress + Vehicle
- Chronic Stress + CLZ

d PR

- Vehicle
- Chronic Stress + Vehicle
- Chronic Stress + CLZ

Highlights

- Stress-induced disruption of sucrose SA models motivational anhedonia
- Antidepressants differently affected reactivity to positive and negative stimuli
- Clozapine reverted stress-induced motivational anhedonia