OXFORD

RESEARCH ARTICLE

Two Chronic Stress Models Based on Movement Restriction in Rats Respond Selectively to Antidepressant Drugs: Aldolase C As a Potential Biomarker

Estibaliz Ampuero, PhD; Alejandro Luarte, BSc; Marcos Santibañez, PhD; Manuel Varas-Godoy, PhD; Jorge Toledo, BSc; Gabriela Diaz-Veliz, PhD; Gabriel Cavada, PhD; F. Javier Rubio, PhD; Ursula Wyneken, PhD

Facultad de Medicina, Universidad de los Andes, Chile (Dr Ampuero, Mr Luarte, Dr Santibañez, Mr Toledo, Drs Varas-Godoy, Cavada, Rubio, and Wyneken); Laboratorio de Neurobiologia, Centro de Investigaciones Biomedicas, Universidad Andres Bello, Santiago, Chile (Dr Ampuero); Facultad de Medicina, Universidad de Chile (Dr Diaz-Veliz); Laboratory of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden (Dr Varas-Godoy).

Correspondence: Ursula Wyneken, Laboratorio de Neurociencias, Facultad de Medicina, Universidad de los Andes; Mons. Alvaro del Portillo 12.455, Las Condes; 7550000 Santiago, Chile; phone: 056 2 6181353; fax: 056 2 2141752; (e-mail: uwyneken@uandes.cl).

Abstract

Background: Clinically depressed individuals respond to different types of antidepressants, suggesting that different neurobiological mechanisms may be responsible for their depression. However, animal models to characterize this are not yet available.

Methods: We induced depressive-like behaviors in rats using 2 different chronic stress models: restraint in small cages or immobilization in adaptable plastic cones. Both models increased anxiety responses evaluated by novelty-suppressed feeding and the elevated plus-maze; increased learned helplessness evaluated by the tail suspension and forced swimming tests; and increased anhedonia evaluated by the sucrose preference test.

Results: We assessed the ability of 2 different types of antidepressants to ameliorate depressive-like behaviors. We administered the serotonin reuptake inhibitor fluoxetine or the noradrenaline reuptake inhibitor reboxetine once daily for 28 days to rats that received either chronic restraint or immobilization stress, or no stress. Behavioral analysis revealed that fluoxetine ameliorated depressive-like behaviors when induced by chronic restraint stress, whereas reboxetine ameliorated these behaviors when induced by chronic immobilization stress. To further test biological differences between both models, we evaluated the levels of Aldolase C, an enzyme expressed by forebrain astrocytes that is regulated by antidepressant treatment, in the cerebrospinal fluid: chronic restraint stress, but not immobilization stress, increased the levels of Aldolase C. Moreover, the presence of astrocyte-derived Aldolase C-GFP in the cerebrospinal fluid indicates its central origin.

Conclusions: Two stress paradigms induced depressive-like behaviors that were sensitive to different antidepressant treatments. Biomarkers such as Aldolase C could help determine optimal antidepressant treatments for clinically depressed patients.

Keywords: Fluoxetine, reboxetine, restraint, immobilization, chronic stress

Introduction

Antidepressant compounds act by selectively inhibiting the uptake of 5-hydroxytryptamine (selective serotonin reuptake inhibitors [SSRIs]), such as fluoxetine, or noradrenaline (selective noradrenaline reuptake inhibitors [NRIs]), such as reboxetine from the synaptic cleft. Additional classes of antidepressants are monoamine oxidase inhibitors, dual serotonin and noradrenaline reuptake inhibitors, serotonin and dopamine reuptake inhibitors, or atypical antidepressants, all of which require chronic administration to be therapeutically effective (Papakostas and Fava, 2005). However, only less than one-third of patients respond to the first drug prescribed. Furthermore, the therapeutic effects are observable only after several weeks of treatment. Thus, patients who fail to respond to the first drug of choice may respond if they are switched to a different drug or if augmentation is prescribed, leading to prolonged treatment periods associated with significant costs for the health systems (Papakostas et al., 2008a, 2008b; Bradley and Lenox-Smith, 2013). Therefore, correctly selecting the first antidepressant drug, an election until now incompletely supported by scientific evidence, would allow shorter treatment periods. In that line, the availability of biomarkers able to predict drug responsiveness is currently an unmet need (Leuchter et al., 2010; Toups and Trivedi, 2012).

Animal models of stress constitute a widely used approximation to understand the impact of stress on the brain and cognition and on its relationship with psychiatric disorders. Exposure to stress induces epigenetic changes leading to structural and functional remodeling of neuronal networks (Lupien et al., 2009; Vialou et al., 2013). In rodents, a popular neurobiological tool to induce stress is the exposure to movement restriction, which, although constituting a physical interference, causes psychological stress when applied in a chronic manner and leads to the expression of depressive-like behaviors such as anhedonia, learned helplessness, and anxiety. In such a way, movement restriction can be attained by restraint in small wire mesh cages or by immobilization in adaptable plastic rodent immobilization bags (Magarinos and McEwen, 1995; Vyas et al., 2002). In the literature, both procedures are considered as equivalent with the exception of the intensity factor, which is supposed to be higher in the case of complete immobilization (Buynitsky and Mostofsky, 2009). Thus, immobilization would lead to increased depressive-like behaviors, higher effective antidepressant drug dosages, or increased levels of biomarkers when compared with restraint.

In the present study, we exposed adult male rats to repeated restraint or immobilization stress. We compared depressive-like behaviors as well as the responsiveness with treatment with an SSRI and an NRI. In the cerebrospinal fluid (CSF), we quantified the levels of Aldolase C as a potential biomarker, as this enzyme of the glycolytic/gluconeogenic pathway was previously shown to be regulated by fluoxetine (Sandoval et al., 2013). Based on the results, this study opens a promising avenue to advance in the detection of neurobiological differences among depressive-like states to guide future psychiatric treatments.

Methods

Animal Treatments

Adult male Sprague-Dawley rats of 250 to 280 g at the beginning of the experiments were used. All procedures involving animals were approved by the Universidad de los Andes Bioethical Committee and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering. The antidepressant drugs fluoxetine at doses of 0.7 or 3.5 mg/kg (Ely-Lilly Co., Indianapolis, IN), reboxetine at doses of 0.13 or 0.65 mg/kg (Pfizer, Pharmacia and Upjohn), or saline (vehicle) were administered by intraperitoneal injection once daily at 9:00 to 10:00 AM for 28 days. In each case, the lower drug dose had been shown by us to lead to clinically relevant plasma levels, induce brain-derived neurotrophic factor signaling at excitatory synapses, excitatory synaptic remodeling, and affect learning and memory (Wyneken et al., 2006; Ampuero et al., 2010, 2013; Rubio et al., 2013).

Antibodies

For Western blots, anti-GFP from AbCam (Ab6673) and 2 anti-Aldolase C antibodies were used with the same results: 1) SC 12065, (1:500) from Santa Cruz Biotechnology and 2) a monoclonal antibody (1:1000) kindly provided by Dr. Richard Hawkes (University of Calgary, Alberta, Canada).

Experimental Design and Stress Procedures

One week before starting the protocol, the animals were housed in groups of 4 to 5 animals per cage in a 12-h- (8:00 AM–8:00 PM) light/dark cycle at 22±1°C with standard rodent pellet food and water available ad libitum. During the experimental days, rats were maintained in their home cages except during exposure to stress for 2 hours daily. Nonstressed rats were handled for 2 minutes daily and then returned to their home cage. Two procedures of movement restriction were used: restraint in wire mesh cages or immobilization in plastic bags (Magarinos and McEwen, 1995; Vyas et al., 2002). The restraint cages of 18×6×6 cm allowed limited movements of the rats. In contrast, the cone-shaped plastic bags (18 cm of length) were adapted to prevent any movement of the animal while a large hole in the nose and mouth zone allowed breathing. Thus, the main experimental groups were no stress, restraint, and immobilization. The stress effects were evaluated at the behavioral level in 2 groups of rats: the first was exposed to 10 days of stress/no stress and tested for depressive-like behaviors on day 11 (n = 11–14) (Figure 1A). Body weight was controlled daily while the adrenal gland weight was determined on day 12. The second group was treated after 10 stress/no stress days for 28 days with fluoxetine, reboxetine, or vehicle administered by intra-peritoneal injection for 30 minutes before the beginning of the stress session (in total, 38 days of stress/no stress) to perform behavioral tests on days 39, according to the design of Czeh et al. (2007). This generated 15 experimental groups (n = 10–16 per experimental group) (Figure 2A).

Behavioral Tests

A battery of behavioral tests was used as described previously (Ampuero et al., 2010). Briefly, each animal was evaluated once for each test 24 hours after the last stress session (day 11 or day 39) following this sequence: novelty-suppressed feeding (NSF), spontaneous motor activity, elevated plus maze (EPM), tail suspension test (TST), and forced swim test (FST). Rats received no drugs on test days. A trained assistant, blind to the experimental condition, recorded the behavioral parameters. In the NSF test (to assess anxiety), rats were deprived of food 24 hours before behavioral testing. Then, animals were placed in a corner of
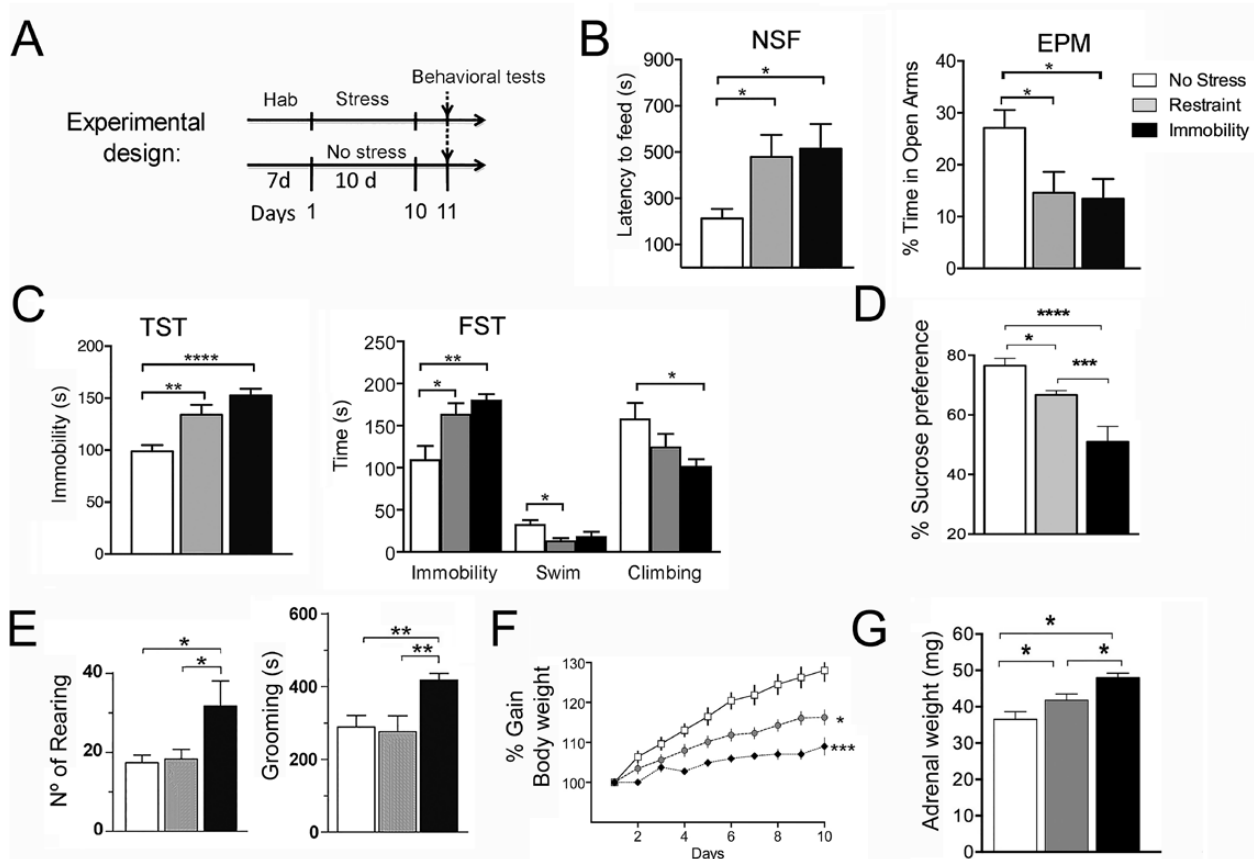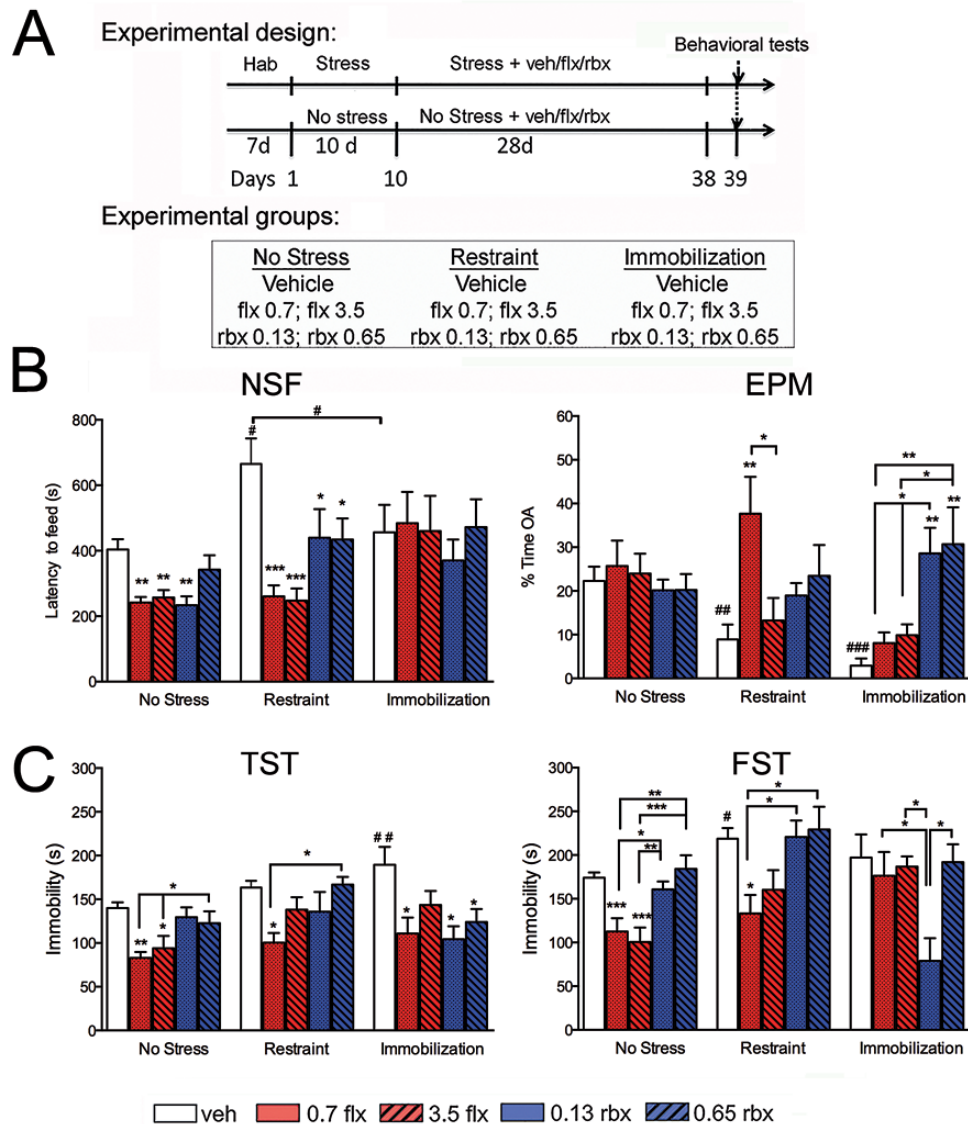
Figure 1. Depressive-like behaviors were induced after 10 days of stress by restraint or immobilization. (A) Experimental design: rats were habituated in their home cages for 7 days. Then, stress by restraint (grey) or immobilization (black) was applied for 10 days. The control groups (white) were nonstressed animals. Depressive-like behaviors were tested on day 11 and in the case of the forced swimming test, on day 12. (B) To evaluate anxiety, the latency in seconds to feed in the novelty suppressed feeding test (NSF) and the percentage of total time spent in the open arm in the elevated plus maze (EPM) were plotted. (C) To evaluate learned helplessness, the time in seconds spent in immobility during the tail suspension test (TST) and the forced swim test (FST) were measured. In addition, the swimming and climbing behaviors were quantified in the FST. (D) The sucrose preference test (SPT) was performed to evaluate anhedonia after 10 days of restraint or immobilization. (E) Spontaneous motor activity was recorded during 30 minutes. Left: rearing, the number of times that the rat rises both feet, was measured during 30 minutes. Right: grooming, total time (seconds) spent in grooming behavior. (F) The body weight gain was plotted along the first 10 days of stress exposure. (G) The adrenal gland weight was plotted after 10 days of stress exposure. The values correspond to the mean ± SEM. We performed ANOVA followed by a posthoc Newman-Keuls test to detect statistical significance. n = 11 to 14 animals per group. *$P<.05$; **$P<.01$, ***$P<.001$, and ****$P<.0001$.

a plastic cage (80 × 70 × 40 cm) with its floor covered with 2 cm of wooden bedding. A food pellet was placed on a round filter paper (10 cm diameter) in the center of the box. We recorded the latency in seconds to begin feeding in a maximum time span of 15 minutes. The amount of food consumed in 5 minutes in the home cage was controlled to discard a general disturbance in food intake. For spontaneous motor activity measurements, rats were individually placed in cages of (30 × 30 × 35 cm), located inside a soundproof chamber, and total activity in counts, number of rearings, and time spent in grooming behavior were evaluated during a period of 30 minutes. In the EPM (to assess anxiety), we used an apparatus elevated 83 cm above the ground consisting of 2 opposed open arms (50 × 10 cm) and 2 opposed closed arms, both of 50 × 10 cm, connected by a central platform (10 × 10 cm). Each animal was placed on the central platform facing one of the open arms. During a 5-minute interval, the number of open and closed arms entries, plus the time spent in open and enclosed arms, was measured in dim light. We calculated the percentage of time in open arms and the ratio between the time in the open arms and total time in arms. In the TST (to assess learned helplessness), rats were individually suspended by the tail to a horizontal bar elevated 40 cm from the

table. During the test session of 6 minutes, the time in seconds spent in a completely immobile posture was measured. In the FST (to assess learned helplessness), we used an acrylic cylinder of 50 cm (height) × 20 cm (diameter) filled with warm water (height of 30 cm). Rats were exposed for 15 minutes to the swimming pool on the pretest day (11 or 39). On the test day (12 or 40), the total time of the following behaviors was measured in 5 minutes: escape or climbing behavior, that is, upward-directed movements of the forepaws along the cylinder walls; swimming behavior; and immobility. To assess anhedonia with the sucrose preference test (SPT), a different groups of rats was used following the protocols of Figures 1A and 2A (Willner et al., 1987). Rats were habituated to choose between drinking 1% sucrose or tap water during 3 hours daily for 5 days after the beginning of the stress protocol. After completion of the 10-day or 38-day protocol, rats were water deprived for 12 hours to measure the individual sucrose consumption during 1 hour in their home cage.

CSF Collection

CSF of 3 rats was collected from the cisterna magna and pooled in one sample as reported (Sandoval et al., 2013). In Western

Figure 2. Fluoxetine (flx) and reboxetine (rbx) selectively reverted depressive-like behaviors in rats exposed to restraint or immobilization. (A) Experimental design: stress or no stress was applied for 28 days concomitant with one of the following treatments: vehicle (veh), fluoxetine (flx) at 0.7 or 3.5 mg/kg, or reboxetine (rbx) at 0.13 or 0.65 mg/kg. Behavioral tests were performed on day 39. This design generated 15 experimental groups, 5 groups per condition (no stress, restraint, or immobilization). (B) The anxiety measures obtained in the novelty suppressed feeding test (NSF) (latency to feed) and elevated plus maze (EPM) (percent of time in open arms) are shown in the left and right panels, respectively. (C) The learned helplessness expressed as immobility in seconds in the tail suspension test (TST) and forced swim test (FST) are shown in the left and right panels, respectively. Number of animals: no stress: veh = 12, 0.7 flx = 10, 3.5 flx = 11, 0.13 rbx = 11, 0.65 rbx = 14; restraint: veh = 14, 0.7 flx = 9, 3.5 flx = 11, 0.13 rbx = 11, 0.65 rbx = 11; immobilization: veh = 10, 0.7 flx = 10, 3.5 flx = 12, 0.13 rbx = 10, 0.65 rbx = 12. The values correspond to mean ± SEM. We performed ANOVA followed by a posthoc Newman-Keuls test to detect significance within groups (drug-treated animals exposed to no stress, restraint, or immobilization compared with the respective vehicle-treated group). *$P < .05$, **$P < .01$, ***$P < .001$; #$P < .05$, ##$P < .001$, ###$P < .001$ denote statistical differences between the stress, vehicle-treated condition and the no-stress, vehicle-treated value observed in each test (white bars).

blots (n = 3–8 independent CSF pools per experimental condition), equal amounts of CSF protein were loaded per lane (30 μg).

Plasmids

A system of piggyBac transposon donor and helper plasmids was used in this study. The piggybac donor plasmid to drive the expression of enhanced green fluorescent protein (pBCAG-eGFP) was previously described (Chen and LoTurco, 2012). To make the plasmid express Aldolase C fused to GFP (pBCAG-AldoC-GFP), rat Aldolase C was polymerase chain reaction amplified from Aldolase C rat cDNA (Origene plasmid RR200828) and inserted into *Eco*RI and *Age*I sites of pBCAG-eGFP under the control of

CAG promoter. The piggybac helper plasmid pPBCAG-PBase, kindly provided by Joseph Lo Turco (University of Connecticut), was modified replacing CAG promotor by glial fibrillary acid protein (GFAP) promotor, generating the pPBGFAP-PBase plasmid.

In Utero Electroporation

We used the methodology of Rosen et al. (2007). To target astrocytes, electroporation was performed at E18-19 (gliogenic stage) and the somatosensory (barrel) cortex was targeted. Pregnant rats were anesthetized by intra-peritoneal injection with ketamine (50 mg/kg) and xylazine (5 mg/kg), the uterine horns exposed, and 1 to 2 μL of a plasmid mixture of pPBGFAP-PBase

(1 µg/µL) and pBCAG-AldoC-GFP (1 µg/µL) mixed with Fast Green (Sigma, 1 mg/mL) was injected with pulled glass capillaries (P97, Sutter Instruments) into the lateral ventricles by a pressure picopump (PV830, World Precision Instruments). Then, a 60- to 70-V electric pulse delivered by a 500-µF capacitor (previously charged with a power supply) was used for electroporation. The voltage pulse discharge occurred across a pair of silver-plated oval electrodes (1×0.5 cm) placed on the lateral surface of one of the cerebral hemispheres. After birth, rats were allowed to grow until adulthood and male rats were exposed to stress by restraint for 10 days or to no stress. Alternatively, pure astrocyte cell culture was performed on day P1 using the electroporated region as starting material following a well-established protocol (Ramirez et al., 2005). After 15 days in culture, microglial cells were discarded by shaking the culture while astrocytes were obtained after trypsinization of the attached cells and replated at low density to allow proliferation to reach confluence. Astrocytes presented the typical polygonal, flat morphology and expressed the astrocyte marker GFAP.

Statistical Analysis

Behavioral data were collected as depicted in Figures 1 and 2. The GraphPad PRISM 6.0 software was used to perform 1-way ANOVA followed by a Newman-Keuls posthoc to test the statistical significance of behavioral parameters. Two-way ANOVA followed by Newman-Keuls was used for the SPT and data obtained in CSF Western blots. For all tests, $P<.05$ was considered significant.

Results

Repeated Stress for 10-Day Induced Depressive-Like Behaviors in Rats

After 10 days of stress by restraint or immobilization (Figure 1), anxiety levels (Figure 1B) increased compared with the no-stress condition both in the NSF, as reflected by augmented latency to feed ($F_{(2,32)}=4.23$, $P<.05$), and in the EPM, as reflected by less time spent in the open arms ($F_{(2,32)}=4.113$, $P<.05$). In the NSF, the amount of food consumed in the home cage during the 30 minutes after completion of the test period was measured as a control of feeding behavior. No differences were detected among groups (no stress: $1.21±0.1$ g; restraint: $0.93±0.17$ g; immobilization: $0.86±0.15$ g; $F_{(2,23)}=1.927$, $P=.16$). In turn, learned helplessness evaluated in the TST and FST (Figure 1C, left and right panels, respectively) indicated that immobility increased by both stress procedures (TST: $F_{(2,27)}=14.39$, $P<.0001$ and FST: $F_{(2,28)}=6.94$; $P=.004$). Thus, no differences could be detected among both stress groups when assessing anxiety or learned helplessness in the TST and FST. However, active behaviors decreased differentially in the FST: restraint reduced swimming, while immobilization reduced climbing (swimming: $F_{(2,28)}=3.47$, $P=.044$ and climbing: $F_{(2,28)}=4.13$, $P=.027$).

In addition, in the SPT (Figure 1D), animals exposed to immobilization consumed less sucrose than animals exposed to restraint ($F_{(2,37)}=17.39$, $P<.0001$), revealing increased anhedonia after immobilization. Importantly, the spontaneous motor activity measurements, that is, number of crossings in the open field, revealed no global motor deficiencies ($F_{(2,34)}=1.47$, $P=.24$) (not shown), while in the motor tests (Figure 1E), immobilization increased both rearing ($F_{(2,30)}=5.47$, $P<.01$) and grooming ($F_{(2,28)}=4.83$, $P<.05$), 2 behaviors used as anxiety-like measures (Kalueff and Tuohimaa, 2005). Restraint had no effect on these

behaviors. Similarly, immobilization induced larger changes than restraint on the body weight gain and adrenal weights during the first 10 days of the experimental design (Figure 1F-G) (body weight gain: $F_{(2,28)}=8.47$, $P<.001$ and adrenal weight $F_{(2,25)}=16.06$, $P<.0001$). Taken together, the behavioral data after 10 days of stress indicate that anxiety and learned helplessness are affected similarly by both procedures, while the intensity factor caused by increased immobilization revealed larger effects on anhedonia, anxiety-like motor behaviors, and physiological variables.

Depressive-Like Behaviors Induced by Restraint or Immobilization Are Selectively Reverted by Fluoxetine and Reboxetine

We then tested the effectiveness of 28 day-treatment with fluoxetine (a SSRI), or reboxetine (an NRI) on rats stressed for 10 days with both procedures (Figure 2A). The respective higher drug dose, representing 5-fold of the low dose, was used to test whether augmentation might play a role in antidepressant effectiveness. After 38 days, vehicle-treated animals displayed anxiety and learned helplessness measures (Figure 2B-C, white bars). Interestingly, anxiety measures in the NSF were not observed after immobilization, revealing a possible adaptation of this behavior to chronic immobilization. In turn, the learned helplessness dimension increased by restraint in the FST but by immobilization in the TST, suggesting that both tests may rely on differential, yet-unknown neurobiological substrates. This reflects the advantages of using several paradigms to assess depressive-like behaviors. In nonstressed animals (Figure 2, first group of bars of each plot), fluoxetine decreased anxiety in the NSF and learned helplessness in the TST and FST, while reboxetine had an effect only on anxiety in the NSF. The middle and right groups of bars in turn show the effect of drugs on animals that display increased depressive-like behaviors. Anxiety values assessed in the NSF and EPM revealed that in rats exposed to restraint, both fluoxetine doses effectively reduced latency to feed (restraint: $F_{(4,33)}=6.61$, $P=.005$). In contrast, in rats exposed to immobilization, no effect was observed in the NSF after antidepressant drug treatments, and this was due to the fact that the latency to feed in immobilized vehicle-treated animals did not differ from nonstressed animals; in this dimension, immobilized animals adapted to the stressor. In the EPM, only the low fluoxetine dose reverted anxiety in restrained animals, while both reboxetine doses, but not fluoxetine, effectively increased the time in open arms in immobilized animals (restraint: $F_{(4,35)}=4.016$, $P=.009$; immobilization: $F_{(4,37)}=6.52$, $P=.0004$). In turn, Figure 2C shows that in rats exposed to restraint, 0.7 mg/kg fluoxetine effectively reduced immobility in the TST ($F_{(4,36)}=2.95$, $P=.033$) and FST ($F_{(4,30)}=4.03$, $P=0.011$), while in rats exposed to immobilization, both reboxetine doses were effective in the TST ($F_{(4,38)}=3.82$, $P=.011$); however, only the low dose of reboxetine was effective in the FST ($F_{(4,30)}=3.83$, $P=.016$). These data suggested that low fluoxetine was effective in reverting depressive-like behaviors after restraint, while reboxetine was effective after immobilization with 2 exceptions: in the NSF, reboxetine also decreased anxiety in restrained animals and in the TST, fluoxetine was also effective in immobilized animals. It should be emphasized that the behavioral measures after effective drug treatments are reverted to values very similar to those obtained in nonstressed and noninjected rats (Figure 1). For example, in the TST, nonstressed and noninjected rats presented $98.9±5.9$ seconds of immobility. This increases to $140±6.6$ seconds in nonstressed but vehicle-injected animals

(Figure 2). After immobilization, immobility in the TST increases further to 189.5±20.4 seconds, and this increase is reverted by 0.13 mg/kg reboxetine to 100.4±11 seconds. In the case of fluoxetine, for example, in the NSF, 0.7 mg/kg fluoxetine decreases latency to 241±17 (no stress) or 260.6±33.5 (restraint) seconds, not different from the value in nonstressed and noninjected animals (212±41). Based on the results indicating that the lower drug doses were effective, these were used in the SPT (Figure 3). Anhedonia was induced after 38 days of restraint, and fluoxetine but not reboxetine reverted it. After 38 days of immobilization, no anhedonia could be observed, suggesting that an adaptive response occurred in a proportion of animals and reboxetine had no effect. Thus, the anhedonia data after immobilization could be separated into 2 groups: nonanhedonic group (with a sucrose consumption of 90±1.9 mL) and an anhedonic group with a mean consumption of 57.6±2 mL (P=.001). However, this categorization needs further investigation. Overall, the pharmacological results show a differential functional effect of antidepressant drugs on behaviors induced either by restraint or immobilization, thus suggesting that different neurobiological substrates are responsible for behavioral alterations.

Aldolase C in the CSF Was Upregulated after Restraint but Not after Immobilization

The incorporation of biomarkers would be extremely helpful to predict antidepressant effectiveness. We recently reported that an astrocyte-derived metabolic enzyme, Aldolase C, is upregulated by fluoxetine and is present in exosome-like microvesicles isolated from astrocyte cell culture media (Sandoval et al., 2013). In that line, we found that reboxetine did not regulate Aldolase C levels (unpublished results, Marcos Santibañez). We thus quantified relative Aldolase C levels in the CSF of 9 experimental groups, including no stress, restraint, and immobilization groups treated with the low, behaviorally effective fluoxetine and reboxetine doses (Figure 4A). Interestingly, restraint led to a 9.2±0.6-fold upregulation of Aldolase C, and this was not observed in the CSF of immobilized rats, confirming our hypothesis that different neurobiological mechanisms are activated in both stress models. Specifically, astrocyte function (the cell type that releases Aldolase C in the forebrain) might be differentially

affected. As already reported, fluoxetine, but not reboxetine, let to a 4±0.7-fold change in Aldolase C in nonstressed animals. The significance of this finding is further discussed in the corresponding section. To confirm that brain astrocytes can be a probable source of Aldolase C in the CSF, we performed in utero electroporation introducing a plasmid for expression of Aldolase C-GFP into forebrain astrocytes. Adult male rats were stressed by restraint or not stressed and the CSF was collected. As shown in Figure 4B (left panels), pure astrocyte cultures obtained from the electroporated region expressed the recombinant protein as revealed by Western blots using either anti-GFP or anti-Aldolase C antibodies. Instead, astrocyte cultures using the nonelectroporated hemisphere as starting material did not express Aldolase C-GFP. When the CSF was collected from adult electroporated rats, we found that Aldolase C-GFP detected by an antibody against GFP or Aldolase C could be detected in the CSF (right panels). Taken together, the main conclusion is that in pharmacologically untreated animals, Aldolase C levels in the CSF can be considered as a marker to distinguish restraint from immobilization, while both a behaviorally effective treatment (fluoxetine) as well as a behaviorally ineffective treatment (reboxetine) reduced Aldolase C in restrained animals, suggesting that the presence of the enzyme is not related to antidepressant activity but to the "disease" state. Importantly, Aldolase C of brain origin reaches the CSF, and thus it could serve as a predictive tool for the choice of a therapeutically effective antidepressant drug family able to reverse anhedonia, learned helplessness, and anxiety.

Discussion

Our study highlights the advantage of identifying subtypes of stress-induced diseases to develop distinctive molecular pathway-directed therapies in the future. We show that depressive-like behaviors induced by 2 stress protocols based on movement restriction are selectively responsive to treatment with a SSRI or a NRI. Specifically, depressive-like behaviors induced by restraint were effectively reverted by fluoxetine, but not by reboxetine (with exception of the anxiety measure in the NSF). A further neurobiological distinction is revealed by the differential presence of Aldolase C in the CSF. Although further studies are necessary to consider Aldolase C as a key candidate for translational research, the molecular analysis of the exosome fraction containing this enzyme in body fluids opens the hope that focusing research on quantitative identification of their content (ie, transcriptomic and/or proteomic analysis) in clinically categorized subtypes of major depressive disorders, anxiety disorders or stress phenotypes could represent a major advance in the search of biomarker patterns for the implementation of "personalized treatments."

We used chronic movement restriction to induce depressive-like behaviors in animals (Buynitsky and Mostofsky, 2009). Even though theoretically improved animal models based on exposure to different types of chronic stressors have been implemented more recently, able to recapitulate more accurately features of major depressive disorder and thus approaching construct, face, and predictive validity (Nestler and Hyman, 2010), the molecular mechanisms compromised in chronic stress and major depressive disorder may differ fundamentally, for example (Andrus et al., 2012) indicating that the general validity of animal models for mood disorders is still limited (Belzung, 2014). Although the lack of physical activity is known to be associated with increasingly higher prevalence rates of chronic diseases, including psychiatric disorders (Booth et al., 2012), the stress-inducing
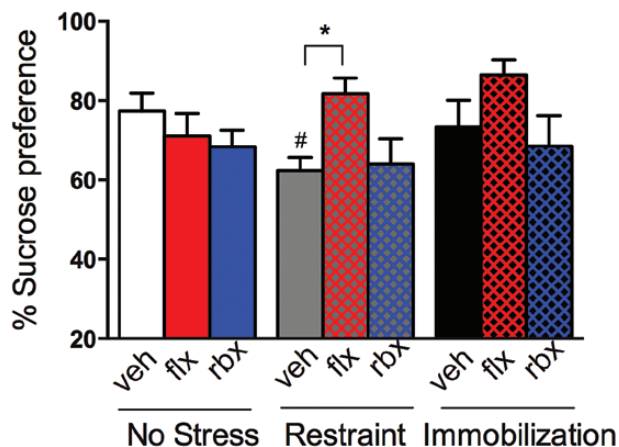

Figure 3. Effect of 0.7 mg/kg fluoxetine or 0.13 mg/kg reboxetine on anhedonia after the 38-day protocol. The sucrose preference test (SPT) was performed in 5 to 6 rats per experimental condition. The values correspond to the mean±SEM. Statistical significance was tested with a 2-way ANOVA followed by a posthoc Newman-Keuls test. *P<.05 compared with stressed, vehicle-treated rats; #P<.05 compared with nonstressed, vehicle-treated rats.
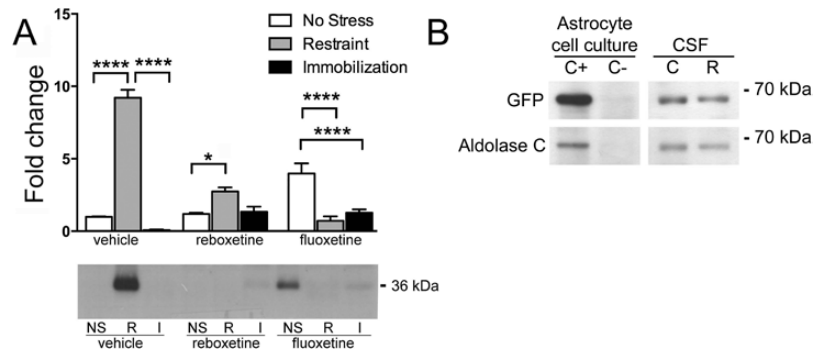
Figure 4. (A) Aldolase C level in the cerebrospinal fluid (CSF) is upregulated by restraint but not by immobilization. Upper panel: relative quantification of Aldolase C levels in Western blots of CSF obtained from nonstressed rats or from rats exposed to stress treated with 0.7 mg/kg fluoxetine or 0.13 mg/kg reboxetine. The values represent the mean ± SEM relative optic density of Aldolase C bands. Two-way ANOVA followed by a posthoc Bonferroni test was performed. Lower panel: representative Western blot of Aldolase C after loading of equal amounts of CSF protein obtained from rats in each experimental condition. N = 3–5 independent determinations per experimental group. I, immobilization; NS, no stress, R, restraint. ***$P < .001$, ****$P < .0001$. (B) Aldolase C-GFP expressed in forebrain astrocytes was detected in homogenates of pure astrocyte cultures derived from the electroporated hemisphere (C +) or as a negative control from the nonelectroporated hemisphere (C -) as well as in the CSF of no-stress (NS) animals or of animals exposed to restraint (R) for 10 days. The blots represent the results of the same membrane that was probed with anti-GFP antibody (upper panel) or with anti-Aldolase C antibody (lower panel). The blots are representative of 3 independent experiments.

procedures used by us might lack construct validity. However, they reliably induced anxiety and depressive-like features in animals (contributing to face validity), and these behaviors could be reversed by antidepressant treatment, meeting predictive or pharmacological validity.

The increased severity of immobilization compared with restraint should lead to augmented neurobiological abnormalities. This was the case in the measurements of body weight gain, adrenal weight, sucrose preference, and motor behaviors such as rearing and grooming when measured after 10 stress sessions. In turn, active behaviors (but not immobility time) in the FST were differentially affected; while restraint decreased swimming, immobilization affected primarily climbing. This differential effect of both stress procedures on active behaviors could also be used as a predictor for drug sensitivity: fluoxetine treatment (effective in restraint) increases swimming, while reboxetine (effective in immobilization) increases climbing (Cryan et al., 2002). An additional difference between both stress models is that 38 days of immobilization suppressed anhedonia and the increased latency to feed in the NSF, both behaviors influenced by a motivational component, suggesting that adaptive mechanisms are operating. It is known that repeated homotypic stressor exposure can cause habituation in subjects able to successfully cope with stress (Feder et al., 2009), and this was indeed the case in about 50% of the rats that adapted to the repetitive immobilization sessions and thus can be considered as resilient. However, the remaining 50% did not adapt. Further experiments are necessary to clarify this incongruity. In that line, it was reported that repetitive immobilization on boards, a stressor that resembles in severity immobilization in plastic bags, causes only a transient reduction in saccharin intake (Pastor-Ciurana et al., 2014), that is, anhedonia "adapted" to the stressor. In any case, while reboxetine had no effect, fluoxetine decreased the dispersion of data. Anhedonia is regulated by the mesolimbic system involved in motivated behaviors. It is conceivable that immobilization-induced changes specific to this circuit are not sensitive to reboxetine, while other brain circuits, for example those involving the amygdala and thus anxiety behaviors, are sensitive to reboxetine, explaining the fact that this NRI reverses immobilization-induced anxiety and learned helplessness but not anhedonia or latency to feed in the NSF. Regarding the differences between stress by immobilization

or restraint, we conclude that more than the severity of the stressor, different neurobiological processes are recruited in both cases.

In the 38-day experimental group, fluoxetine treatment improved depressive-like measures in vehicle-injected nonstressed animals in the NSF, TST, and FST. This might be due to the stress induced by the injection procedure during 28 days. Synaptic and molecular as well as behavioral effects in nonstessed rats have already been reported in previous studies after fluoxetine injection (Rantamaki et al., 2007; Ampuero et al., 2010; Rubio et al., 2013; Guirado et al., 2014), although chronic fluoxetine delivered in the drinking water to nonstressed rats also induced antidepressant-like effects (Shishkina et al., 2007) or affected synaptic plasticity (Dringenberg et al., 2014). Thus, it is not possible to distinguish whether the injection by itself, the treatment period, or the drug is responsible of the observed effects. In any case, the stress procedures increased depressive-like behaviors compared with nonstressed vehicle-treated rats and more importantly, the behavioral measures improved to values very similar to those obtained previous to the beginning of the 28-day treatment, suggesting that these are the best behavioral measures that can be reached in the reported experimental conditions.

Heterogeneity of major depressive and/or anxiety disorders has firmly been proposed, although a large overlap at the genetic as well as symptomatic levels may exist. However, distinct mechanistic pathways represented by completely different gene subsets or their variants might be affected in genetically homogeneous subcategories (Flint and Kendler, 2014). At the symptomatic level, atypical depression, with an early age of onset, is characterized by mood reactivity, anxiety, fatigue, hypersomnia, and increased appetite, whereas melancholic depression is characterized by nonreactivity of mood, anhedonia, psychomotor disturbances, insomnia, and weight loss. Although still controversial, atypical depression, associated with HPA hypo-reactivity, can effectively be treated with SSRIs, whereas melancholic depression, associated with HPA hyperreactivity, responds mainly to tricyclic antidepressants as well as to MAO inhibitors and dual reuptake inhibitors (Peselow et al., 1992; Perry, 1996; Stewart et al., 1998; Tzanakaki et al., 2000; Joyce et al., 2002; Brown, 2007). In that line, meta-analysis suggests that patients resistant to SSRIs are benefited by switching

to an NRI over switching to a different SSRI (Papakostas et al., 2008a), while the overall efficacy of fluoxetine and reboxetine in treating major depressive disorder is similar (Papakostas et al., 2008b).

Risking a gross oversimplification, in the animal models described here, restraint induces more atypical-like features such as HPA axis adaptation and less intense symptoms in anhedonia and anxiety-like measures, while immobilization induced more melancholic-like features such as HPA hyper-reactivity, intense anhedonia, and a pronounced weight loss.

In our study, animals with similar genetic susceptibility reproduced depressive-like behaviors that might ultimately represent 2 different mechanistic pathways leading to the expression of depressive-like symptoms. In such a way, microRNAs, thought to play a pivotal role in the pathophysiology underlying major depressive disorder, are differentially regulated by restraint or immobilization (Dwivedi, 2014). For example, miR-709 was found to be regulated in the hippocampus using restraint in plastic tubes (Rinaldi et al., 2010; Babenko et al., 2012) but not after immobilization (Meerson et al., 2010), thus suggesting that these molecules could be used as biomarkers of subtypes, an issue that to our knowledge has not yet been addressed. Interestingly, Aldolase C, an enzyme with several nonglycolitic functions, including RNA-binding properties (Canete-Soler et al., 2005), is differentially present in the CSF after restraint or immobilization. It is possible that Aldolase C interacts with different RNA species besides the mRNA for the light neurofilament chain, thus explaining the surprising fact that the enzyme is regulated both by fluoxetine and restraint, although to differential levels. If the accompanying RNA strand (mRNA or microRNA) is different, the "physiological" state of the astrocyte could be reflected by the Aldolase C binding partner and not by the sole levels of the protein. Consistent with previous results, our data indicate that forebrain astrocytes release Aldolase C (Sandoval et al., 2013) and that this process is activated after restraint, but not after immobilization. This is supported by the fact that Aldolase C-GFP, when expressed in astrocytes, can be detected in the CSF. One may consider several possibilities to explain why the levels of the recombinant protein were not regulated by restraint: (1) only a subpopulation of astrocytes contributes to Aldolase C in the CSF. This is an interesting possibility because, for example, astrocytes in the prefrontal cortex might contribute in a different degree than somatosensory or motor cortices, especially under stress conditions. (2) Regulation is lost in astrocytes that overexpress the protein; or (3) the variability of the number or volume of electroporated astrocytes per rat, thus impeding visualization of regulation.

The main contribution of the present work is the finding that stress-induced behavioral abnormalities using 2 different procedures based on movement restriction responded selectively to antidepressant drugs and that a biomarker for this differential sensitivity could be detected. This is the first time that 2 different depressive-like states were generated in animals and compared at various levels of analysis.

Acknowledgements

Statement of Interest

None.

References

Ampuero E, Rubio FJ, Falcon R, Sandoval M, Diaz-Veliz G, Gonzalez RE, Earle N, Dagnino-Subiabre A, Aboitiz F, Orrego F, Wyneken U (2010) Chronic fluoxetine treatment induces structural plasticity and selective changes in glutamate receptor subunits in the rat cerebral cortex. Neuroscience 169:98–108.

Ampuero E, Stehberg J, Gonzalez D, Besser N, Ferrero M, Diaz-Veliz G, Wyneken U, Rubio FJ (2013) Repetitive fluoxetine treatment affects long-term memories but not learning. Behav Brain Res 247:92–100.

Andrus BM, Blizinsky K, Vedell PT, Dennis K, Shukla PK, Schaffer DJ, Radulovic J, Churchill GA, Redei EE (2012) Gene expression patterns in the hippocampus and amygdala of endogenous depression and chronic stress models. Mol Psychiatry 17:49–61.

Babenko O, Golubov A, Ilnytskyy Y, Kovalchuk I, Metz GA (2012) Genomic and epigenomic responses to chronic stress involve miRNA-mediated programming. PLoS One 7:e29441.

Belzung C (2014) Innovative drugs to treat depression: did animal models fail to be predictive or did clinical trials fail to detect effects? Neuropsychopharmacology 39:1041–1051.

Booth FW, Roberts CK, Laye MJ (2012) Lack of exercise is a major cause of chronic diseases. Comprehens Physiol 2:1143–1211.

Bradley AJ, Lenox-Smith AJ (2013) Does adding noradrenaline reuptake inhibition to selective serotonin reuptake inhibition improve efficacy in patients with depression? A systematic review of meta-analyses and large randomised pragmatic trials. J Psychopharmacol 27:740–758.

Brown WA (2007) Treatment response in melancholia. Acta psychiatrica Scandinavica Supplementum:125–129.

Buynitsky T, Mostofsky DI (2009) Restraint stress in biobehavioral research: Recent developments. Neurosci Biobehav Rev 33:1089–1098.

Canete-Soler R, Reddy KS, Tolan DR, Zhai J (2005) Aldolases a and C are ribonucleolytic components of a neuronal complex that regulates the stability of the light-neurofilament mRNA. J Neurosci 25:4353–4364.

Chen F, LoTurco J (2012) A method for stable transgenesis of radial glia lineage in rat neocortex by piggyBac mediated transposition. J Neurosci Methods 207:172–180.

Cryan JF, Markou A, Lucki I (2002) Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci 23:238–245.

Czeh B, Muller-Keuker JI, Rygula R, Abumaria N, Hiemke C, Domenici E, Fuchs E (2007) Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment. Neuropsychopharmacology 32:1490–1503.

Dringenberg HC, Branfield Day LR, Choi DH (2014) Chronic fluoxetine treatment suppresses plasticity (long-term potentiation) in the mature rodent primary auditory cortex in vivo. Neur Plasticity 2014:571285.

Dwivedi Y (2014) Emerging role of microRNAs in major depressive disorder: diagnosis and therapeutic implications. Dialogues Clin Neurosci 16:43–61.

Feder A, Nestler EJ, Charney DS (2009) Psychobiology and molecular genetics of resilience. Nat Rev Neurosci 10:446–457.

Flint J, Kendler KS (2014) The genetics of major depression. Neuron 81:484–503.

Guirado R, Perez-Rando M, Sanchez-Matarredona D, Castren E, Nacher J (2014) Chronic fluoxetine treatment alters the structure, connectivity and plasticity of cortical interneurons. Int J Neuropsychopharmacol 17:1635–1646.

Joyce PR, Mulder RT, Luty SE, Sullivan PF, McKenzie JM, Abbott RM, Stevens IF (2002) Patterns and predictors of remission, response and recovery in major depression treated with fluoxetine or nortriptyline. Aus New Zeal J Psychiatry 36:384–391.

Kalueff AV, Tuohimaa P (2005) Mouse grooming microstructure is a reliable anxiety marker bidirectionally sensitive to GABAergic drugs. Eur J Pharmacol 508:147–153.

Leuchter AF, Cook IA, Hamilton SP, Narr KL, Toga A, Hunter AM, Faull K, Whitelegge J, Andrews AM, Loo J, Way B, Nelson SF, Horvath S, Lebowitz BD (2010) Biomarkers to predict antidepressant response. Curr Psychiatry Rep 12:553–562.

Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434–445.

Magarinos AM, McEwen BS (1995) Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors. Neuroscience 69:83–88.

Meerson A, Cacheaux L, Goosens KA, Sapolsky RM, Soreq H, Kaufer D (2010) Changes in brain MicroRNAs contribute to cholinergic stress reactions. J Molec Neurosci 40:47–55.

Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nat Neurosci 13:1161–1169.

Papakostas GI, Fava M (2005) Monoamine-based pharmacotherapy. In: Biology of depression: from novel insights to therapeutic strategies. 1st ed. (M LJW, ed), pp 87–140. Weinheim, Germany:.

Papakostas GI, Fava M, Thase ME (2008a) Treatment of SSRI-resistant depression: a meta-analysis comparing within- versus across-class switches. Biol Psychiatry 63:699–704.

Papakostas GI, Nelson JC, Kasper S, Moller HJ (2008b) A meta-analysis of clinical trials comparing reboxetine, a norepinephrine reuptake inhibitor, with selective serotonin reuptake inhibitors for the treatment of major depressive disorder. Eur Neuropsychopharmacol 18:122–127.

Pastor-Ciurana J, Rabasa C, Ortega-Sanchez JA, Sanchis-Olle M, Gabriel-Salazar M, Ginesta M, Belda X, Daviu N, Nadal R, Armario A (2014) Prior exposure to repeated immobilization or chronic unpredictable stress protects from some negative sequels of an acute immobilization. Behav Brain Res 265:155–162.

Perry PJ (1996) Pharmacotherapy for major depression with melancholic features: relative efficacy of tricyclic versus selective serotonin reuptake inhibitor antidepressants. J Affect Disord 39:1–6.

Peselow ED, Sanfilipo MP, Difiglia C, Fieve RR (1992) Melancholic/endogenous depression and response to somatic treatment and placebo. Am J Psychiatry 149:1324–1334.

Ramirez G, Toro R, Dobeli H, von Bernhardi R (2005) Protection of rat primary hippocampal cultures from A beta cytotoxicity by pro-inflammatory molecules is mediated by astrocytes. Neurobiol Dis 19:243–254.

Rantamaki T, Hendolin P, Kankaanpaa A, Mijatovic J, Piepponen P, Domenici E, Chao MV, Mannisto PT, Castren E (2007) Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cgamma signaling pathways in mouse brain. Neuropsychopharmacology 32:2152–2162.

Rinaldi A, Vincenti S, De Vito F, Bozzoni I, Oliverio A, Presutti C, Fragapane P, Mele A (2010) Stress induces region specific alterations in microRNAs expression in mice. Behav Brain Res 208:265–269.

Rosen GD, Bai J, Wang Y, Fiondella CG, Threlkeld SW, LoTurco JJ, Galaburda AM (2007) Disruption of neuronal migration by RNAi of Dyx1c1 results in neocortical and hippocampal malformations. Cereb Cortex 17:2562–2572.

Rubio FJ, Ampuero E, Sandoval R, Toledo J, Pancetti F, Wyneken U (2013) Long-term fluoxetine treatment induces input-specific LTP and LTD impairment and structural plasticity in the CA1 hippocampal subfield. Front Cell Neurosci 7:66.

Sandoval M, Luarte A, Herrera-Molina R, Varas-Godoy M, Santibanez M, Rubio FJ, Smit AB, Gundelfinger ED, Li KW, Smalla KH, Wyneken U (2013) The glycolytic enzyme aldolase C is up-regulated in rat forebrain microsomes and in the cerebrospinal fluid after repetitive fluoxetine treatment. Brain Res 1520:1–14.

Shishkina GT, Kalinina TS, Dygalo NN (2007) Up-regulation of tryptophan hydroxylase-2 mRNA in the rat brain by chronic fluoxetine treatment correlates with its antidepressant effect. Neuroscience 150:404–412.

Stewart JW, Garfinkel R, Nunes EV, Donovan S, Klein DF (1998) Atypical features and treatment response in the National Institute of Mental Health Treatment of Depression Collaborative Research Program. J Clin Psychopharmacol 18:429–434.

Toups M, Trivedi MH (2012) Biomarkers and the future of treatment for depression. Cerebrum: the Dana forum on brain science 2012:6.

Tzanakaki M, Guazzelli M, Nimatoudis I, Zissis NP, Smeraldi E, Rizzo F (2000) Increased remission rates with venlafaxine compared with fluoxetine in hospitalized patients with major depression and melancholia. Int Clin Psychopharmacol 15:29–34.

Vialou V, Feng J, Robison AJ, Nestler EJ (2013) Epigenetic mechanisms of depression and antidepressant action. Annu Review Pharmacol Toxicol 53:59–87.

Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci 22:6810–6818.

Willner P, Towell A, Sampson D, Sophokleous S, Muscat R (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology (Berl) 93:358–364.

Wyneken U, Sandoval M, Sandoval S, Jorquera F, Gonzalez I, Vargas F, Falcon R, Monari M, Orrego F (2006) Clinically relevant doses of fluoxetine and reboxetine induce changes in the TrkB content of central excitatory synapses. Neuropsychopharmacology 31:2415–2423.