Biomedicine & Pharmacotherapy 128 (2020) 110249

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

The mood stabilizing properties of AF3581, a novel potent GSK-3β inhibitor

Valeria Capurro[a,1], Massimiliano Lanfranco[a,1], Maria Summa[a], Pier Francesca Porceddu[a], Mariasole Ciampoli[a], Natasha Margaroli[a], Lucia Durando[b], Beatrice Garrone[b], Rosella Ombrato[b], Serena Tongiani[b], Angelo Reggiani[a,*]

[a] D3 Validation Research Line, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genova, Italy
[b] Angelini Pharma S.p.A., Viale Amelia, 70–00181, Rome, Italy

ARTICLE INFO

Keywords:
Bipolar disorders
Depression
GSK3beta
Drug Discovery
Neuropharmacology

ABSTRACT

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype of a novel class of ATP-competitive GSK-3β inhibitors having the common N-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzyme and highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype of a novel class of ATP-competitive GSK-3β inhibitors having the common N-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzyme and highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype of a novel class of ATP-competitive GSK-3β inhibitors having the common *N*-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzymeand highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of

* Corresponding author.
E-mail address: angelo.reggiani@iit.it (V. Capurro).
[1] Equal contribution.

https://doi.org/10.1016/j.biopha.2020.110249
Received 22 January 2020; Received in revised form 29 April 2020; Accepted 10 May 2020

depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype

of a novel class of ATP-competitive GSK-3β inhibitors having the common *N*-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzyme and highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype of a novel class of ATP-competitive GSK-3β inhibitors having the common *N*-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzyme and highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype of a novel class of ATP-competitive GSK-3β inhibitors having the common *N*-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzyme and highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine protein kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK-3β has been linked to several disease conditions. There is now large evidence on the role of GSK-3β in the pathophysiology of mood disturbances with special regard to bipolar disorders. In the present study we further investigated the role of GSK-3β in bipolar disorders by studying AF3581, the prototype of a novel class of ATP-competitive GSK-3β inhibitors having the common *N*-[(1- alkylpiperidin-4-yl) methyl]-1H-indazole-3-carboxamide scaffold. Based on previous studies, AF3581 inhibits GSK-3β in the nanomolar range on purified human enzyme and highly selective with respect to other kinases. Current study demonstrates that the compound has efficacy both in the chronic mild stress paradigm of depression (mimicking the down phase of bipolar disorder) and on mice aggressiveness in the resident intruder model (mimicking the up phase). These findings underline the importance of aberrant GSK-3β activity in the development/ maintenance of mood oscillation in this peculiar pathological condition. Moreover, the present work also suggests a therapeutic potential for selective GSK-3 β inhibitors in the management of bipolar disorders patients.

1. Introduction

Lithium has been used for over half a century as the mood stabilizer of choice, but its use in the treatment of bipolar disorder (BD) has some important limitations may not be always favorable to bipolar patients, because of the intrinsic risk of precipitation of the manic phase. Moreover, lithium has narrow safety margins due to its rather unspecific pharmacological profile. This liability can significantly reduce drug tolerability and possibly patient compliance [1].

Since the discovery of lithium psychotropic properties, its mechanism of action has been the subject of intense investigation. An intriguing feature is that lithium inhibits GSK-3β activity and that the same inhibitory effect seems to be shared by other mood stabilizers such as valproate, raising the hypothesis of a role of GSK-3β in disease

development and/or maintenance [2–4].

GSK-3β is a widespread signaling molecule regulating many cellular processes associated to cell structure, gene expression and apoptosis and GSK-3β hyperactivity has been linked to several disease conditions such as inflammation, neurodegeneration, alteration of circadian clock and psychiatric disorders [3].

There is now evidence both in animals and in humans that hyperactivity of GSK-3β can be associated to bipolar disorders

For example, induction of depressive states in animals increases GSK-3β activity, while heterozygous GSK-3β mice (+/−) have reduced depression-like immobility in the Forced Swimming Test [5], suggesting that, following reduction of GSK-3β activity, a kind of natural antidepressant behavior occurs. Moreover, lithium and GSK-3β inhibitors have shown efficacy in mood disorders animal models [6–9].

In man, an elevated GSK-3β activity has been reported in post-mortem samples from ventral prefrontal cortex of patients with major depression disorder [10]. More recently, it has been found that an inherited mutation of the DISC1 gene that is clinically characterized by high frequency of mood disorders, is associated to GSK-3β gene dysregulation (increased activity) [11,12].

The goal of the present study was to verify the mood stabilizing properties of AF3581, a recently described potent ATP-competitive and selective GSK-3β inhibitor [13] to assess its potential as a treatment for bipolar disorders.

This compound belongs to a novel class of competitive GSK-3β inhibitors having the common *N*-[(1-alkylpiperidin-4-yl)methyl]-1H-indazole-3-carboxamide scaffold and it can be considered as a significant advancement compared to the existing, in view of its high plasma stability and overall drug-like properties.

Unfortunately, there are no reliable animal models featuring the BDs condition in human. Therefore, we studied AF3581 in distinct animal models for the down phase (anhedonia assessed with the chronic mild stress paradigm) and for the up phase (mania/aggressiveness with the resident intruder model). The assumption was that efficacy in both conditions could predict efficacy on the global bipolar disorder condition in humans.

In addition, to further confirm that the pharmacological effects induced by AF3581 are related to GSK-3β inhibition, the in vivo induction of pSer9-GSK-3β expression (inactive form of the enzyme) as qualitative index of GSK-3β engagement by AF3581 has been also determined.

2. Materials and methods

2.1. Animals and drug treatment

C57BL/6 J male mice of 8–10 weeks old (Charles River Laboratories Italia, Calco, Italy) were used in each study. They were housed in 6 per cages and allowed for two weeks to habituate in a climate-controlled animal facility (21 ± 2 °C) and maintained on a 12-h light/dark cycle (light on: 7 a.m. − 7 p.m.), before beginning experimentation.

Mice testing was conducted during the light phase and food was withdrawn the night before. Mice were weighted and monitored daily. All experiments were carried out in accordance with the guidelines established by the European Communities Council Directive (Directive 2010/63/EU of 22 September 2010) and approved by the National Council on Animal Care of the Italian Ministry of Health. All efforts were made to minimize animal suffering and to use the minimal number of animals required to produce reliable results.

Drugs: amphetamine, fluoxetine and lithium (Sigma-Aldrich, St. Louis, MO, USA); AF3581 (synthesized by Angelini SpA). AF3581, fluoxetine and lithium were always administered by oral gavage (5 mL/kg) unless otherwise specified and were dissolved in PEG 400/Tween 80/Saline solution at 10/10/80% in volume.

2.2. Locomotor activity with amphetamine treatment

10 C57BL/6 J male mice were used in each experimental group. They were food deprived the day before the experiment. At the day of the test, mice were allowed to explore the empty opaque open field arena (40 × 40 × 40 cm, Ugo Basile Srl, Gemonio, Italy) for 20 min (habituation). Then, mice were withdrawn from the apparatus, injected orally with AF3581 (with a range of doses between 0.3 and 30 mg/kg) and the placed back. After 15 min, amphetamine (2 mg/kg i.p.) was given and motility was recorded for the subsequent 90 min (test phase) to measure drug effect on amphetamine hyperactivity. All sessions were videotaped and the total distance travelled was analyzed with ANY-maze software (Stoelting Co.).

2.3. Chronic mild stress procedure (CMS)

A well-accepted model to mimic depressive-like state in mice is the Chronic Mild Stress (CMS) procedure.

Mice were randomly divided in two groups: one group of C57BL/6 J mice was exposed to 5–7 weeks of CMS (CMS mice). The other group, the non-stressed mice (NO CMS mice), was used as control.

CMS protocol was adapted from Elizalde et al. [14] and Nollet at al [15]. and was applied for 5–7 weeks consecutively. Briefly, CMS was induced by exposing mice to at least two of the following stressors daily:

- *restraint* - mice were placed in a 50 mL plastic tube (falcon) with openings in both sides for breathing for 1 h;
- *social stress* - mice were introduced in an empty cage previously occupied by another individual for 2 h;
- *without sawdust* - the sawdust is removed from the cage for 2 h;
- *damp sawdust* - the floor of each cage was covered with damp sawdust for 2 h;
- *"bath"* - the sawdust of each cage is removed and replaced by about 125 mL water at 20 °C (about 1 cm water) for 30 min;
- *cage tilting* - cages were tilted backwards (45°) for 3 h;
- *cycle disturbances* - the light/dark cycle was changed (2 h of dark during the light phase).

CMS and NO CMS groups were treated with vehicle, or the new compound, AF3581 (10 mg/kg p.o. twice a day), or a reference compound, fluoxetine (16 mg/kg i.p. once a day). AF3581 was administered at the same time when the stressor procedure started (preventive protocol), or 3 weeks after the beginning of the CMS procedure (therapeutic protocol).

The behavioral effects of stress were evaluated at the end of each week using the Sucrose Preference Test and the Splash Test. At the end of the whole procedure, plasma corticosterone (index of stress) and hippocampal BDNF content (index of neurogenesis) were also measured.

2.3.1. Sucrose preference test

Sucrose preference test was measured weekly. A decline of performance in this test is considered an index of anhedonia (loss of pleasure).

Mice were food and water deprived at 5 p.m. of the previous day. Then, at 9 a.m. of the test day, they were given a free choice between two identical and randomly positioned bottles, one with a freshly prepared 1% sucrose solution and another with tap water. Bottle choice exposure lasted for the subsequent 24 h. The total water/sucrose intake was measured at 9 a.m. of the next day. The percentage of preference was defined as ration sucrose intake (ml) over total liquid intake.

2.3.2. Splash test

This test is also considered a measure of anhedonia (loss of self-esteem). Mice were squirted on the snout with a 10% sucrose solution and the grooming frequency was then recorded. A decline of self-

grooming is considered an index of reduced self-esteem (anhedonia)

2.3.3. Plasma corticosterone levels

Measurement of plasma corticosterone was performed using a commercially available enzyme-ELISA kit (Cortisol assay, Enzo Life Science) according to the manufacturer's instructions. All measurements were performed in duplicate. Plasma samples were diluted 1:20.

2.3.4. Hippocampal BDNF levels

Hippocampal BDNF levels was measured using a commercially available ELISA kit (BDNF Emax ImmunoAssay System, Promega) following the manufacturer's protocol. Tissues were homogenized in Ripa buffer supplemented with protease inhibitors and diluted 1:5 in Block & Sample buffer.

2.4. Resident-intruder test

The experiment was performed according to Einat [16]. 10 C57BL/6 J male mice, 8 weeks old at the beginning of the study, were kept in isolation for 6 weeks in their home cage (RESIDENT). In this period, animals established a strong territorial supremacy. In order to maximize territoriality, an ovariectomized female was placed in the resident's home cage starting from 1 week before the test until 1 h prior testing. Bedding was not changed in the 7 days preceding the experiment.

In a different room of the animal facility, 10 C57BL/6 J male mice, 7 weeks old at the time of the study, were group-housed together and served as INTRUDER.

The test was carried out in the resident's home cage. Following a 2 min adaptation period, an intruder mouse was placed in the cage with the resident for 10 min and videotaped. Social behavior (time spent sniffing and grooming) was measured along with aggressive behavior of the resident towards the intruder (number of attacks, latency to first attack, time spent attacking). Lithium was mixed with food at the concentration of 2.4 g/kg corresponding to a daily intake of 10 mg/kg calculated on food consumption. AF3581 was given in the drinking water at the concentration of 90 mg/l corresponding to final daily intake 20 mg/Kg/day based on water consumption. Treatments were started at the beginning of isolation procedure and were continued for 6 weeks consecutively.

2.5. Target engagement studies

2.5.1. Enzyme-linked immunosorbent assay kit (Elisa)

Phospho-GSK-3β (pSer9-GSK-3β) and total GSK-3β levels were measured in hippocampal preparation by using ELISA kit by Invitrogen (Polyclonal Antibody). The measurement was carried out according to the manufacturer's instructions. pSer9-GSK-3β concentration was normalized by the total protein content for a given sample, as measured by using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA).

2.6. Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using Prism-8 software (version 8.00, Graph Pad, San Diego, CA). Unpaired *t*-test, One or Two-way analysis of variance (ANOVA, multiple comparisons) or Mixed effect analysis were used as a statistical analysis, as appropriate: Two-way ANOVA followed by Tukey post hoc test was used for chemical analyses; Two-way ANOVA followed by Bonferroni post hoc test was used for Amphetamine locomotor sensitization; Mixed effect analysis followed by Bonferroni post hoc test was used for Sucrose preference and Splash tests; One-way ANOVA followed by Tukey post hoc test was used for the target engagement; Unpaired *t*-test was used for Resident intruder model evaluations. Results were considered significant at $p < 0.05$.

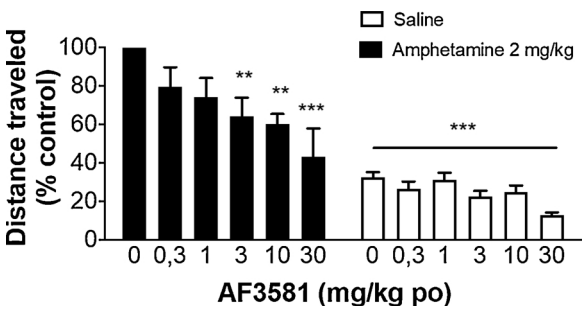

Fig. 1. Inhibition by the oral administration of AF3581 of amphetamine-induced hyperactivity in mice. Amphetamine was given at 2 mg/kg i.p. Saline or amphetamine were given after 20 min of habituation period (no recording). Data are the mean ± SEM (n = 12) of the distance travelled after 90 min recording. $^{**}p < 0.01$, $^{***}p < 0.001$ veh + amph.

3. Results

3.1. Amphetamine challenge

Since the experimental models identified to assess the efficacy of AF3581 in the two phases of bipolar required prolonged oral administration of the compound, the amphetamine-induced hyperactivity assay was used to identify the suitable orally effective dose, based on previous results by Furlotti et al. [13].

As shown in Fig. 1, a range of doses between 0.3 and 30 mg/kg p.o. was tested and a significant reduction of hyperactivity was seen starting at a dose as low as 3 mg/kg, while full effectiveness was observed at 30 mg/kg ($p < 0.001$, $F_{(5,145)} = 6328$). The dose of 10 mg/kg was selected as the most suitable dose for next studies because at 30 mg/kg a trend towards sedation was observed in naive mice.

In a subsequent pharmacokinetic study, we established the dose regimen by measuring blood and brain levels of AF3581 after the oral dosing of 10 mg/kg. The oral administration of AF3581 proved highly bioavailable (F = 68%) and poorly metabolized (half-life = 240 min). Moreover, no enzymatic induction was observed following repeated dosing (data not shown). Based on these findings, we selected 10 mg/kg p.o. twice a day as the optimal drug regimen of AF3581 administration for the entire duration of study.

3.2. Chronic mild stress

Anhedonia is a typical symptom associated to depression, Repeated, mild stressful stimuli causes a slow development of anhedonia both in humans and in rodents as measured by decline of sucrose preference (loss of pleasure) and absence of grooming behavior after squirt of sucrose (loss of self-esteem).

3.2.1. Sucrose preference test

As shown in Fig. 2A, B, C (time 0), NO CMS mice displayed an initial 80–90% of sucrose preference but, when mice started receiving the CMS procedure, a progressive decline of sucrose preference can be recorded. This decline was interpreted as a gradual loss of interest in normally enjoyable activities (*sucrose drinking*) and starting from week 4 a significant difference of sucrose preference in CMS mice was observed ($p = 0.0016$; $F_{(5,42)} = 4.729$).

In the preventive protocol, the compounds were administered at the selected dose regimen starting at time 0 (Fig. 2A for fluoxetine, 16 mg/kg i.p. once a day and Fig. 2B for AF3581, 10 mg/kg p.o. twice a day). When the tested compounds were given to NO CMS mice, sucrose preference remained stable (no unspecific performance alteration) while, when they were given to CMS mice, both AF3581 (Fig. 2B; $p < 0.0001$; $F_{(1,41)} = 25.88$) and fluoxetine (Fig. 2A; $p = 0.0157$; $F_{(1,39)} = 6.383$) exhibited a preventive blockade of CMS-induced

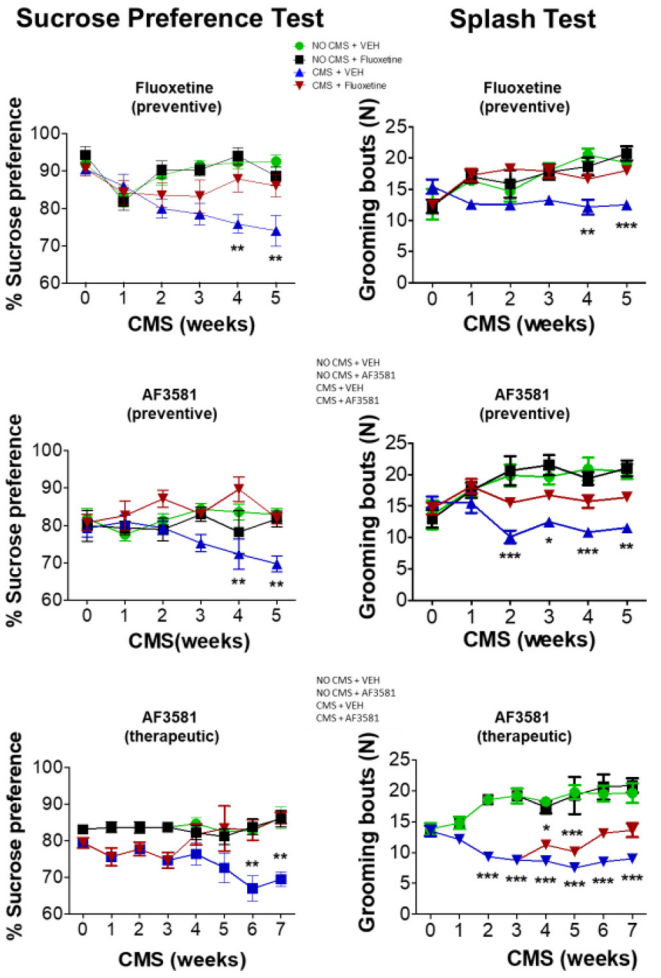

Fig. 2. Effect of AF3581 administration on Chronic Mild Stress-induced decrease of sucrose consumption in mice (anhedonia) and reduction of grooming in the splash test (loss of self-esteem). Mice (n = 8) were exposed to a Chronic Mild Stress protocol for 6 weeks. The following experimental groups were included: (A; D) fluoxetine preventive treatment. The compound was administered once a day at 16 mg/kg i.p. starting from day 0; (B, E) AF3581 preventive treatment. The compound was administered twice a day at 10 mg/kg p.o. starting from day 0; (C, F) AF3581 therapeutic treatment. The compound was administered twice a day at 10 mg/kg p.o. starting at week 3. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs NO CMS + veh.

decline of sucrose consumption (blockade of anhedonia).

In the therapeutic protocol (Fig. 2C), only AF3581 was studied. The administration of the compound started at week 3 after the beginning of CMS procedure. At this time in non-drug treated CMS mice, the decline of sucrose consumption continued, as expected, while treatment with AF3581 initially halted and then significantly reversed the reduction of sucrose preference ($p < 0.0001$; $F_{(1,81)} = 66.69$). After two additional weeks of treatment (*i.e.* at week 5) sucrose preference was back to normal although the CMS procedure the reduced preference was still in place.

3.2.2. Splash test

The Splash Test was measured as additional read-out and was assessed in the same CMS mice as for the sucrose preference test. As shown in Fig. 2D–F, after squirt of sucrose on the snout, a clear grooming behavior occurred in NO CMS mice to eliminate the sucrose, while a reduced grooming behavior was gradually observed in CMS mice. This gradual reduction of grooming behavior suggested a progressive loss of self-esteem (anhedonia) already at week 2 from the beginning of the stressful procedure with the maximal statistically significant effect at week 4 and 5 ($p = 0.0009$; $F_{(5,42)} = 5.140$). In the preventive protocol (Fig. 2D and E), either fluoxetine or AF3581 did not change the grooming performance in NO CMS mice (no unspecific performance alteration), while both fully prevented the performance decline in CMS mice (Fig. 2D, $p = 0.0002$, $F_{(139)} = 17.34$; Fig. 2E, $p = 0.0027$; $F_{(1, 38)} = 10.32$).

In the therapeutic protocol, AF3581 treatment was started at week 3 when the grooming behavior performance in the CMS group was already highly compromised (Fig. 2F, $p < 0.0001$, $F_{(7,88)} = 12.26$). Nonetheless, AF3581 significantly reversed the declining grooming, bringing quickly the performance close to normal ($p < 0.0001$, $F_{(1,84)} = 22.09$).

3.2.3. Plasma corticosterone levels

Increased plasma corticosterone levels are an index of stress and measure of this index was carried out at the end of the CMS study (12 h after the last AF3581 administration).

As shown in Fig. 3A and B, regardless the protocol used (*i.e.* preventive or therapeutic), CMS caused a stable increase of corticosterone level in no treated mice ($p = 0.0002$; $F_{(1,28)} = 1804$).

AF3581 had no effect on corticosterone levels in NO CMS mice suggesting an absence of unwanted sedative action *per se* (Fig. 3A and B), but it prevents the increase of plasma corticosterone levels in stressed mice in the preventive ($p < 0.001$; $F_{(1,28)} = 26.88$ in Fig. 3A) and in the therapeutic ($p = 0.0192$; $F_{(1,26)} = 6.237$ in Fig. 3B) protocols. Since AF3581 is devoid of sedative effect, this result was interpreted as the consequence of the prevention of CMS-induced lowered mood.

3.2.4. Hippocampal BDNF levels

Hippocampal BDNF content is taken as a marker of neurogenesis and was measured at the end of the CMS study, 12 h after the last AF3581 administration. As previous reported [17], different chronic mild stress protocols induce a decline of hippocampal BDNF levels. As shown in Fig. 3C, AF3581, administrated either at the beginning of the trial (preventive protocol) or 3 weeks from the beginning of CMS protocol (therapeutic protocol) was able to reverse the BDNF content decline due to the CMS procedure in a statistically significant manner ($p = 0.0009$, $F_{(1,40)} = 12{,}88$, preventive protocol; $p = 0.0265$, $F_{(1,47)} = 5.245$, therapeutic protocol). Moreover, in mice receiving both preventive and therapeutic AF3581 administration, a trend of increased BDNF content was observed in NO CMS mice although the effect was not significant.

3.3. Resident intruder test

Resident intruder test started with 6 weeks of total isolation. After this period, the resident mouse was exposed to an unknown intruder mouse for a test time of 600 s. As shown in Fig. 4, during the test time, a non-treated mouse spent about half of the time ignoring the intruder (350–400 seconds), while the remaining time was spent to engage interaction with the intruder.

The distribution of the interaction time between resident and intruder in non-drug treated resident mice was as follows:

- quiet and friendly social interaction for about 150 s (Fig. 4A and E),
- in the remaining time, some non-natural hostile behaviors such as abnormal aggressiveness and attacks towards the intruder accompanied by strong fighting were seen against the intruder that normally would have not occurred (Fig. 4B–D, F–H).

When resident mouse received lithium mixed with food starting from the beginning of the isolation period (average daily intake of 10 mg/kg), a profound redistribution in the time spent interacting with the intruder was observed. In fact, as reported in Fig. 4, mice showed reduction of aggressiveness behavior as indicated by a low number of

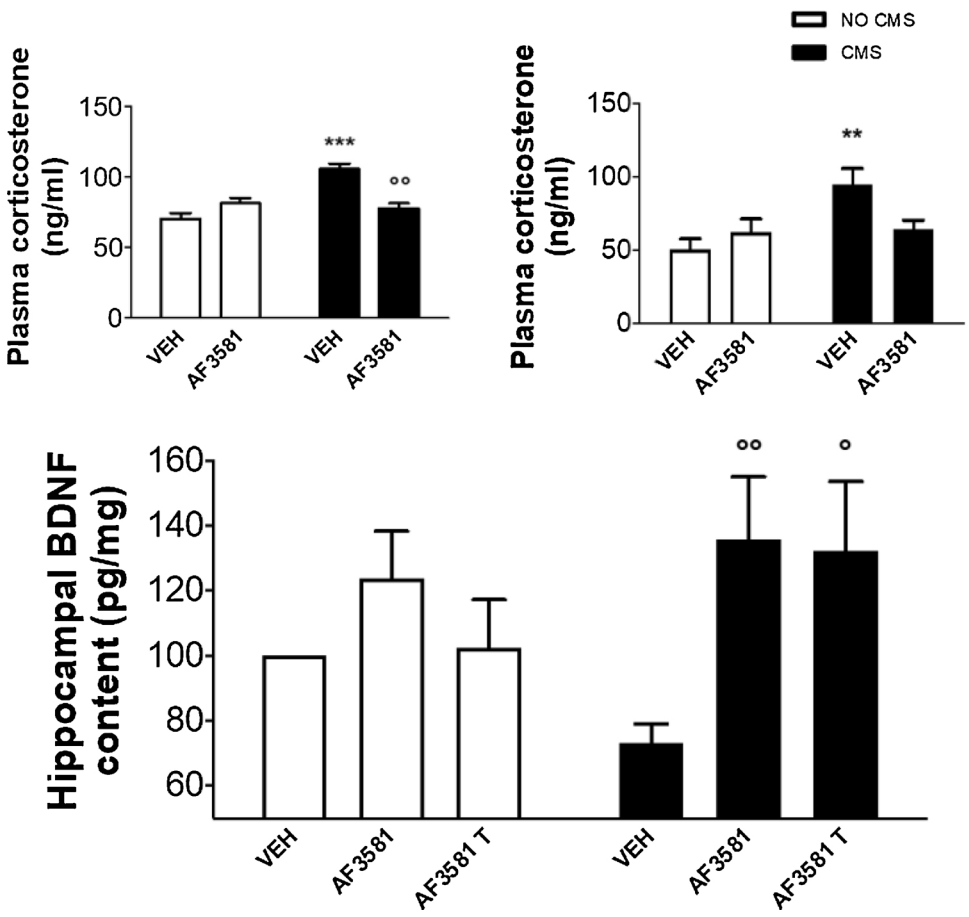

Fig. 3. Effect of AF3581 administration on plasma corticosterone and hippocampal BDNF levels during Chronic Mild Stress. Mice (n = 8) were exposed to a Chronic Mild Stress protocol for 6 weeks. Plasma corticosterone levels (A, preventive treatment and B, therapeutic treatment) and hippocampal BDNF content (C) were measured at week 6 when all the behavioral assays were concluded. Basal BDNF in vehicle treated animals was 50–60 pg/mg prot. AF3581 was given orally at the dose of 10 mg/kg twice a day starting from Day 0 (preventive protocol) or at week 3 (therapeutic protocol). $^{***}p < 0.001$, $^{**}p < 0.01$ vs veh + NO CMS; $^{°°}p < 0.01$, $^{°}p < 0.05$ vs veh + CMS.

attacks (*t*-test $p = 0.042$; $t_{(17)} = 2.200$; Fig. 4C) and very little time spent for each attack (*t*-test $p = 0.0329$; $t_{(17)} = 2.322$; Fig. 4B). Interestingly, the effect of lithium was highly specific on aggressiveness since no reduction of quiet social interaction was observed (Fig. 4A). This latter finding and the missing effect of lithium on latency of first attack, indicates that, at least at this dose, lithium does not display unwanted sedative effects (i.e. sedation and anti-aggressiveness effects can be split).

In this model, AF3581 could not be administered by oral route by using the classical gavage protocol because this would require animal manipulation, thus causing a severe violation of the 6 weeks isolation protocol. Therefore, the compound was diluted in the drinking water up to a final concentration of 90 mg/l, corresponding to a final oral dosing of about 20 mg/kg/day (based on the average daily water consumption). Parallel pharmacokinetic studies confirmed that this AF3581 regimen of administration can provide drug exposure coverage for 24 h as well as brain concentrations enough to achieve GSK-3β engagement (data not shown). Finally, side measurements confirmed that at this concentration AF3581 did not affect regular water intake (data not shown).

As shown in Fig. 4 and similarly to lithium, AF3581 dissolved in the drinking water provided a reduction of time spent in hostile behavior towards the intruder (*t*-test $p = 0.0268$; $t_{(17)} = 2.424$; Fig. 4F). This was accompanied by a reduction of the number of attacks although the effect was not significant ($p = 0.07$, Fig. 4G).

As for lithium, the effect of AF3581 administration had no effect on positive social interaction thus suggesting specificity on aggressiveness (Fig. 4E); at the same time our compound does not display unwanted sedative effects, as demonstrated by the absence of effect on latency to first attack (Fig. 4H).

3.4. GSK-3β engagement

In vivo induction of pSer9-GSK-3β expression was taken as index of GSK-3β target engagement by AF3581. Hippocampal content of Total GSK-3β and pSer9-GSK-3β was measured with a specific enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 5, when AF3581 was given at increasing concentrations, an increase of pSer9-GSK-3β /Total GSK-3β RATIO was observed even at a dose as low as 0.3 mg/kg. However, the first statistical significant difference was seen at 3 mg/kg and confirmed at 10 mg/kg. At 30 mg/kg the RATIO was more than double compared to vehicle alone ($p < 0.0001$; $F_{(5,29)} = 10.50$,). Since no change of total GSK-3β expression was observed this difference must be entirely due to changes of pSer9 form indicating target engagement.

4. Discussion

The present study was planned/designed and carried out to fully explore the therapeutic potential of the potent and selective GSK-3β inhibitor AF3581 in BDs. The compound was originated from our previous medicinal chemistry effort on GSK-3β inhibitors [13] and represents a significant improvement of *druggability* over the existing in view of its high plasma stability and overall drug-like properties.

Due to the experimental limitations to mimic the complex human disease condition in a single model, AF3581 was studied separately on the two main disease features of BDs, i.e. depression and hyperactivity (aggressiveness) by using different models mimicking the two different phases.

The first main finding of the study is the capacity of the GSK-3β inhibitor AF3581 to fully prevent CMS induced anhedonia (down phase). This protection was observed in two protocols: CMS induced

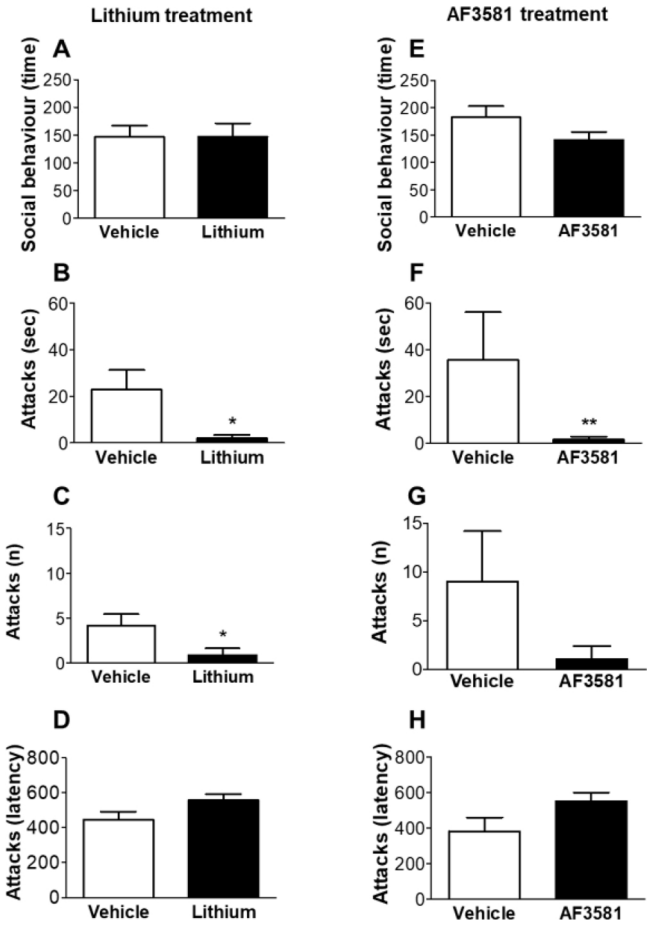

Fig. 4. Effects of repeated assumption of lithium and AF3581 on mice aggressiveness in the Resident Intruder model. Mice (n = 10) were isolated for 6 weeks. During this time, they received standard diet only (vehicle) or mixed diet with (A–D) 2.4 g/kg/day of LiCo3 (based on food consumption) and (E–H) 20 mg/Kg/day of AF3581 (based on water consumption). Test time was 600 s. (A, E) time spent in positive interaction; (B, F) number of attacks; (C, G) time spent in hostile behavior during each attack; (D, H) time spent ignoring each other. $^{**}p < 0.01$, $^{*}p < 0.05$ vs vehicle treated mice.

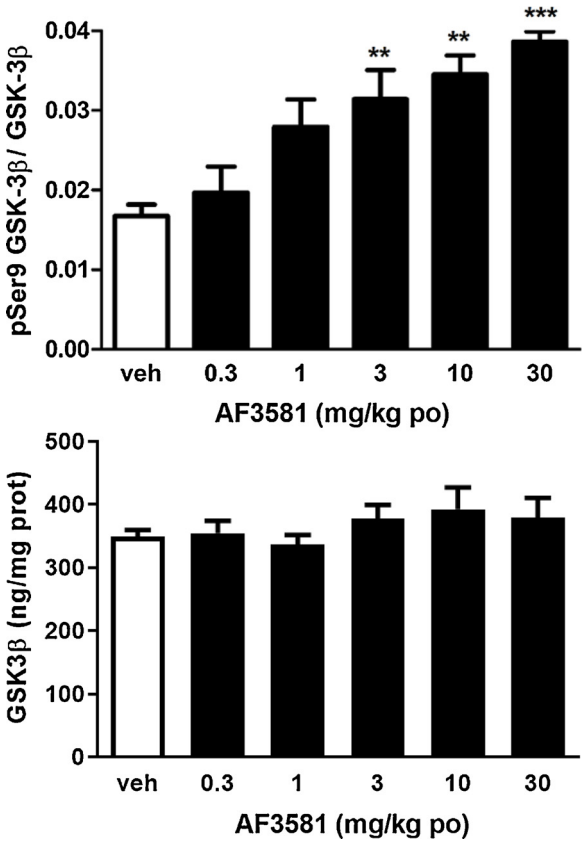

Fig. 5. pSer9-GSK-3β expression after treatment with AF3581. Elisa analysis after AF3581 administration at 0,3, 1, 3,10, 30 mg/kg p.o. $^{*}p < 0.01$, $^{**}p < 0.01$ vs vehicle.

reduction of sucrose consumption in the Sucrose Preference Test (loss of pleasure) and reduced grooming behavior in the Splash Test (loss of self-esteem).

Moreover, it is worth stressing that a significant effect of AF3581 on CMS induced behavior was seen also when compound was administered after the CMS procedure start and anhedonia was already in place (therapeutic effect). Remarkably, AF3581 not only halted disease development, but also reverted mice anhedonia bringing back to normal state the affected behavioral performance (normalization effect).

Interestingly, we found that CMS decreases the hippocampal content of BDNF (index of decreased neurogenesis) and increases plasma corticosterone (index of anxiety).

In the prevention protocol AF3581 blocked the dysregulation of both indexes as well as it reversed BDNF decrease in the therapeutic protocol (corticosterone was not measured).

Therefore, it can be inferred that AF3581 normalizes the depressive state by decreasing anxiety and possibly reactivating neurogenesis being the former a cause and the latter a consequence of the depressive state [18,19].

A second main result was that the GSK-3β inhibitor AF3581 reduced the aberrant hostile behavior towards intruder following long lasting isolation (up phase).

The aggressive behavior is a phenotype that can be associated to the manic phase of BDs (up phase). The prolonged administration of both AF3581 and lithium significantly reduced the aggressiveness of the resident against the intruder mice suggesting a potential for AF3581 as anti-manic effect.

Interestingly, the effect of AF3581, and also lithium, was specifically directed towards the attack phase without influencing the normal social behavior. This suggests that observed effect of both drugs is specific to aggressiveness and is not related to a non-specific sedative effect.

A third important finding was that the efficacy of AF3581 in the different models was always comparable to that of fully effective doses of the reference drugs used, fluoxetine and lithium, respectively. This is an extremely important observation because such a profile suggests a global stabilizing efficacy of AF3581 on both symptoms. In this respect, the compound is superior to current medications. For example, lithium (the most common drug stabilizer currently used) is, indeed, preferentially on the high mood phase only (antimanic).

A fourth important finding was that in the range of doses associated to the observed pharmacological activity, AF3581 fully engaged hippocampal GSK-3β at doses consistent with its in vivo efficacy.

Since the efficacy of drugs is strictly dependent upon the interaction with their molecular target, we dedicated efforts to study the link between in vivo efficacy of AF3581 and central GSK-3β and, to this purpose, in vivo content of pSer9-GSK-3β was measured. pSer9-GSK-3β is an inactive form of the native enzyme. An increased tissue content of pSer9 further to inhibitor treatment indicates a reduced enzymatic activity due to a molecular rearrangement following the inhibitor interaction with the target protein. Therefore, we assumed that an increased expression of pSer9 form is an indication of GSK-3β engagement by AF3581.

The mechanism by which GSK-3β inhibition could be effective in bipolar disorders is still rather unclear. Under resting conditions, the

GSK-3β system is tightly controlled (inhibited) by several upstream regulators such as the monoaminergic signaling. There is now a shared opinion that a reduction of upstream inhibition can disinhibit GSK-3β activity leading to disease. Serotonin has been reported to have a role in maintaining GSK-3β inhibition, thus serotonin deficiency, as in depression, can led to increased GSK-3β activity (reduced inhibitory input), which could favor the susceptibility to depression.

A key finding supporting the role of serotonin is that serotonin-deficient mice have depressive type [20]. Similarly, excessive dopaminergic input inhibits the Akt pathway (an additional upstream pathway) that normally keeps GSK-3β inhibited. Thus, an excess of dopamine, as it may occur in the up phase, can indirectly led to increased GSK-3β activity via disinhibition of the Akt pathway [21].

In this respect, genetically altered mice with an Akt resistant form of GSK-3β, display decreased anxiety, depressive type behaviors, hyperactivity and enhanced curiosity in various experimental conditions, affording a replicable model of mania [21].

In conclusion, BDs remain an area of large unmet medical need. Research in the field is still very active, but lithium remains the most clinically used molecule so far. In the present study, we have demonstrated that AF3581, a novel potent and selective GSK-3β inhibitor, has a therapeutic potential in bipolar disorders. This compound shows efficacy not only in *in vivo* models mimicking the up phase, but also in those endowed with behavioral characteristics typical of the down phase, where lithium efficacy is lacking. For this reason, AF3581 could become a promising candidate of a new class of drugs active on bipolar disorders

Declaration of Competing Interest

None.

References

[1] J.R. Geddes, S. Burgess, K. Hawton, K. Jamison, G.M. Goodwin, Long-term lithium therapy for bipolar disorder: systematic review and meta-analysis of randomized controlled trials, Am. J. Psychiatry 161 (2) (2004) 217–222.

[2] T.D. Gould, G. Chen, H.K. Manji, In vivo evidence in the brain for lithium inhibition of glycogen synthase kinase-3, Neuropsychopharmacology. 29 (2004) 32–38.

[3] E. Beurel, S.F. Grieco, R.S. Jope, Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases, Pharmacol. Ther. 148 (2015) 114–131.

[4] A. Wada, Lithium and neuropsychiatric therapeutics: neuroplasticity via glycogen synthase kinase-3beta, beta-catenin, and neurotrophin cascades, J. Pharmacol. Sci. 110 (2009) 14–28.

[5] W.T. O'Brien, A.D. Harper, F. Jovè, J.R. Woodgett, S. Maretto, S. Piccolo, P.S. Klein, Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium, J. Neurosci. 24 (2004) 6791–6798.

[6] M.K. Rowe, C. Wiest, D.M. Chuang, GSK-3 is a viable potential target for therapeutic intervention in bipolar disorder, Neurosci. Biobehav. Rev. 31 (2007) 920–931.

[7] A.R. Cole, Glycogen synthase kinase 3 substrates in mood disorders and schizophrenia, FEBS J. 280 (2013) 5213–5227.

[8] A. Muneer, Wnt and GSK3 signaling pathways in bipolar disorder: clinical and therapeutic implications, Clin. Psychopharmacol. Neurosci. 15 (2017) 100–114.

[9] O. Kaidanovich-Beilin, A. Milman, A. Weizman, C.G. Pick, H. Eldar-Finkelman, Rapid antidepressive-like activity of specific glycogen synthase kinase-3 inhibitor and its effect on beta-catenin in mouse hippocampus, Biol. Psychiatry 55 (2004) 781–784.

[10] H. Eldar-Finkelman, A. Martinez, GSK-3 inhibitors: preclinical and clinical focus on CNS, Front. Mol. Neurosci. 4 (2011) 32.

[11] Y. Mao, X. Ge, C.L. Frank, J.M. Madison, A.N. Koehler, M.K. Doud, C. Tassa, E.M. Berry, T. Soda, K.K. Singh, T. Biechele, T.L. Petryshen, R.T. Moon, S.J. Haggarty, L.H. Tsai, Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling, Cell. 136 (6) (2009) 1017–1031.

[12] D.H. Oh, Y.C. Park, S.H. Kim, Increased glycogen synthase kinase-3β mRNA level in the hippocampus of patients with major depression: a study using the stanley neuropathology consortium integrative database, Psychiatry Investig. 7 (2010) 202–207.

[13] G. Furlotti, M.A. Alisi, N. Cazzolla, P. Dragone, L. Durando, G. Magarò, F. Mancini, G. Mangano, R. Ombrato, M. Vitiello, A. Armirotti, V. Capurro, M. Lanfranco, G. Ottonello, M. Summa, A. Reggiani, Hit Optimization of 5-Substituted-N-(piperidin-4-ylmethyl)-1H-indazole-3-carboxamides: Potent Glycogen Synthase Kinase-3 (GSK-3) Inhibitors with in Vivo Activity in Model of Mood Disorders, J. Med. Chem. 58 (2015) 8920–8937.

[14] N. Elizalde, F.J. Gil-Bea, M.J. Ramírez, B. Aisa, B. Lasheras, J. Del Rio, R.M. Tordera, Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment, Psychopharmacology (Berl.) 199 (2008) 1–14.

[15] M. Nollet, A.M. Le Guisquet, C. Belzung, Models of Depression: Unpredictable Chronic Mild Stress in Mice, Curr. Protoc. Pharmacol, (2013) Chapter 5 Unit 5.65.

[16] H. Einat, Establishment of a battery of simple models for facets of bipolar disorder: a practical approach to achieve increased validity, better screening and possible insights into endophenotypes of disease, Behav. Genet. 37 (2007) 244–255.

[17] J. Grønli, C. Bramham, R. Murison, T. Kanhema, E. Fiske, B. Bjorvatn, R. Ursin, C.M. Portas, Chronic mild stress inhibits BDNF protein expression and CREB activation in the dentate gyrus but not in the hippocampus proper, Pharmacol. Biochem. Behav. 85 (2006) 842–849 9.

[18] J.A. Morales-Garcia, R. Luna-Medina, S. Alonso-Gil, M. Sanz-Sancristobal, V. Palomo, C. Gil, A. Santos, A. Martinez, A. Perez-Castillo, Glycogen synthase kinase 3 inhibition promotes adult hippocampal neurogenesis in vitro and in vivo, ACS Chem. Neurosci. 3 (2012) 963–971.

[19] A.J. Valvezan, P.S. Klein, GSK-3 and Wnt signaling in neurogenesis and bipolar disorder, Front. Mol. Neurosci. 5 (2012) 1.

[20] A.M. Polter, X. Li, Glycogen synthase Kinase-3 is an intermediate modulator of serotonin neurotransmission, Front. Mol. Neurosci. 4 (2011) 31.

[21] J.M. Beaulieu, R.R. Gainetdinov, M.G. Caron, Akt/GSK3 signaling in the action of psychotropic drugs, Annu. Rev. Pharmacol. Toxicol. 49 (2009) 327–347.