Original Paper

Inhibition of Acid Sphingomyelinase by Antidepressants Counteracts Stress-Induced Activation of P38-Kinase in Major Depression

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Key Words

p38 Kinase • Neurogenesis • Ceramide • Acid sphingomyelinase • Antidepressants

Abstract

Background/Aims: Major depressive disorder is a common disease with serious morbidity, including increased risk of death from suicide. Major depressive disorder is treated with antidepressants. However, the molecular targets of antidepressants remained ill-defined and require further elucidation. ***Methods:*** Mice were treated with corticosterone to induce stress, amitriptyline and the p38-kinase (p38K) inhibitor SB239063 or a combination of these drugs. Phosphorylation of p38K in hippocampal neurons was determined by immunostaining with a phospho-specific antibody, neuronal proliferation using BrdU-labelling and behaviour employing a set of behavioural tests. ***Results:*** Corticosterone induced phosphorylation/ activation of p38K in the hippocampus *in vivo*. Antidepressants reversed the effect of corticosterone on p38K activation in wildtype mice, but had no effect in acid sphingomyelinase-deficient animals. Corticosterone also reduced neurogenesis and triggered depression-like behavioural changes, effects that were prevented by pharmacological inhibition of p38K. ***Conclusion:*** Stress induces p38K phosphorylation/activation in the hippocampus and thereby reduces neurogenesis and induces depression-like symptoms, events that are prevented by antidepressants via inhibition of the acid sphingomyelinase/ceramide system.

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Introduction

Major depressive disorder is a severe and chronic disease with a lifetime prevalence of more than 10%. It is a life-threatening illness, since approximately 10% of patients with severe MDD commit suicide. Recent concepts of the pathogenesis of major depressive disorder indicate a central role of neurogenesis in the hippocampus in the pathogenesis of the disease [1-4]. These concepts suggest that major depressive disorder can be triggered by a reduction of neurogenesis in the hippocampus [1-3]. Studies in rodents showed that stress induces a marked inhibition of neurogenesis that correlates with behavioural changes in these animals [1-3]. Further, chronic stress and depression result in hippocampal atrophy [5] in rodents and humans, suggesting a lack of neurogenesis and/or an increased cell death in the pathogenesis of major depressive disorder. It seems that in major depressive disorder the delicate balance between proapoptotic, antiapoptotic, and neuroneogenetic events is shifted toward anti-neuroneogenetic and possibly also pro-apoptotic stimuli that reduce neurogenesis and finally cause neurodegeneration and hippocampal atrophy [3, 5]. Remarkably, antidepressants trigger neurogenesis and prevent or even reverse the hippocampal atrophy observed in major depression [1-3, 6]. These insights were mostly obtained in stress-induced animal models of depression and anxiety [1-3]. In these paradigms treatment with antidepressants increased cell proliferation and neurogenesis in the hippocampus of adult rodents *in vivo*, a change that correlated with improvement of anxious and depressive behavior [1-3, 7]. Blockade of neurogenesis by irradiation of the hippocampus abolished most of the behavioral effects of antidepressants such as imipramine and fluoxetine, an observation highlighting the importance of neurogenesis for the biological effects of antidepressants [3]. At present, it is unknown whether the integration of newborn neurons into hippocampal neuronal networks is required for the antidepressive action of these drugs or whether the increased generation of immature neurons induced by antidepressants also buffers stress responses even prior to the formation of mature neuronal networks.

Since stimulation of neurogenesis seems to be one of the major effects of antidepressants, it is very important to identify molecular mechanisms that mediate the effects of antidepressants. At present, only a few molecular mechanisms controlling neuronal proliferation, migration, maturation, and integration of neurons into the functional network of the hippocampus are known. It is also unknown how these factors are regulated by antidepressants. It was previously shown that reactive oxygen species and the Akt signaling pathway regulate neurogenesis [8]: In particular lower doses of reactive oxygen species generated by NADPH oxidases and an activation of the phosphatidylinositol-3-kinase (PI3K) - Akt axis was shown to trigger neurogenesis [8, 9]. PI3K and Akt also regulate release of reactive oxygen species in neurons [8], but the exact interaction of the two pathways in neurogenesis is unknown. Antidepressants such as amitriptyline and fluoxetine also activate Akt in neural stem cells [2] providing a potential pathway by which these drugs may act. We have previously shown that the acid sphingomyelinase (human protein: ASM, EC 3.1.4.12, sphingomyelin phosphodiesterase; optimum pH, 5.0; murine protein: Asm, gene symbol *Smpd1*) is directly targeted by the antidepressants amitriptyline and fluoxetine, which reduce acid sphingomyelinase activity and ceramide concentrations in the hippocampus [2]. Employing several genetic models we demonstrated that the acid sphingomyelinase/ceramide system mediates the effects of antidepressants on neuronal proliferation, maturation, and survival as well as behaviour in models of stress-induced depression [2]. Genetic deficiency of acid sphingomyelinase was already sufficient to partially mimic the effects of antidepressants and abrogated any further effect of antidepressants on neurogenesis and behaviour, while an increase of ceramide in the hippocampus decreased neuronal proliferation, maturation, and survival, and induced depression-like behavior even without stress [2]. The acid sphingomyelinase is a ubiquitously-expressed enzyme that releases ceramide from sphingomyelin [10]. Ceramide molecules dramatically change the

biophysics of the plasma membrane and spontaneously form ceramide-enriched membrane domains that serve to trap and cluster receptor and signalling molecules [11-14]. However, at present it is unknown how ceramide-enriched membrane domains signal and how a change of ceramide by antidepressants serves to treat major depression.

Here, we aimed to identify acid sphingomyelinase/ceramide-controlled targets of antidepressants. We demonstrate that stress induces phosphorylation/activation of p38-Kinase (p38K) in the hippocampus *in vivo*, an effect that is prevented by antidepressants. Genetic deficiency of the acid sphingomyelinase abrogates the effects of amitriptyline on p38K phosphorylation/activation, neurogenesis and behaviour. Direct pharmacological inhibition of p38K corrects stress-induced inhibition of neurogenesis and stress-mediated behaviorial changes, an observation underscoring the significance of p38K in major depression. In conclusion, our data demonstrate that p38K is an important molecule in the pathogenesis of major depressive disorder and that antidepressants prevent stress-induced p38K phosphorylation/activation by inhibition of the acid sphingomyelinase/ceramide system.

Materials and Methods

Mice and treatments

Asm-deficient mice ($Smpd1^{-/-}$) were used up to a maximum age of 10 weeks to avoid sphingomyelin accumulation [2-10]. Wildtypes were age-matched C57BL/6 littermates. Amitriptyline was administered to mice at 180 mg/L via their drinking water for 9 days. The p38K inhibitor SB239063 was dissolved in DMSO and further diluted in 0.9% NaCl. The inhibitor was intraperitoneally injected at 1 mg/kg once daily. Corticosterone was administered at 0.25 mg/mL in the drinking water for 9 days. Corticosterone application is an often used, well defined-model to induce major depression and mimics major depression in many aspects [1, 15]. Animals were individually housed, provided with food and water ad libitum, and kept on a 12:12 hour light: dark cycle (lights on at 7.00 am). Behavioral tests were performed during the light cycle between 14:00 and 17:00 h. Room temperature was maintained between 19°C and 22°C at a humidity of 55% (±10%).

Animal experiments were performed with permission of the local authorities.

Hippocampal acid sphingomyelinase activity

The hippocampus was removed, shock frozen, and lysed in 250 mM sodium acetate (pH 5.0), 1% NP40 for 15 min followed by 3 rounds of tip sonication, 10 sec each. Aliquots of the lysates were diluted to 250 mM sodium acetate (pH 5.0) and 0.1% NP40 and incubated with 50 nCi per sample [^{14}C]sphingomyelin for 60 min at 37°C. The samples were then extracted in $CHCl_3$:CH_3OH (2:1, v/v), vortexted, centrifuged and an aliquot of the upper phase was scintillation counted to determine the release of [^{14}C]phosphorylcholine as a measurement for acid sphingomyelinase activity.

Histologies

Mice were sacrificed, brains were removed, shock frozen, sectioned and the frozen sections were fixed for 10 min in acetone. The samples were washed, blocked for 10 min with PBS, 5% fetal calf serum (FCS) and then immunostained using an anti-phospho-p38K antibody (Cell Signalling, diluted 1:200 in HEPES/Saline, H/S; 132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$). Samples were washed 3-times in PBS and stained with a Cy3-coupled anti-rabbit antibody (Jackson ImmunoResearch, Newmarket, UK), embedded in Mowiol and analysed using a Leica fluorescence microscopy (Leica, Mannheim, Germany).

Alternatively, mice were perfused via the left heart for 2 min with 0.9% NaCl followed by perfusion with 4% paraformaldehyde (PFA) buffered in PBS (pH 7.3) for 15 min. After the brains were removed they were fixed for an additional 36 h in 4% buffered PFA/PBS, embedded in paraffin and the hippocampus was serially sectioned. The sections were dewaxed, antigens were retrieved by incubation for 30 min with pepsin (Digest All, Invitrogen) at 37°C, followed by the procedure described above. Both methods yielded equivalent results.

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BrdU stainings

Mice were injected with BrdU at 75 mg/kg 4-times (every 2 hrs) 1 day before the mice were sacrificed. Brains were shock frozen and sectioned. The sections were fixed in acetone for 10 min, incubated for 2 h with 50% formamide in saline-sodium citrate buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.0) at 65°C, and washed twice in saline-sodium citrate buffer. The DNA was denatured for 30 min at 37°C with 2 M HCl. Sections were then washed, neutralized for 10 min with 0.1 M borate buffer (pH 8.5), washed again, blocked with 0.05% Tween 20 and 5% FCS in PBS (pH 7.4), stained with 5 µg/mL anti-BrdU antibodies (Roche) for 45 min at 22°C, washed, and stained with Cy3-coupled F(ab)$_2$ anti-mouse IgG (Jackson ImmunoResearch, Newmarket, UK). A blinded investigator then counted BrdU-positive cells in serial sections from the hippocampus.

Behavioral studies

These studies were performed as previously described [2]. Briefly, for the novelty-suppressed feeding test, mice were deprived of food for 24 hours and then transferred into a new cage with one piece of food on a small piece of paper in the center of the cage. The time during which the mice explored the new environment before they began eating was measured. In the open-field test the animals were released near the wall of a cage and observed for 30 minutes. The time they were present in the open field, which was defined as 10 cm away from the wall, was measured. The light/dark box test consisted of a dark and safe compartment and a brightly illuminated, open, and thus aversive area. Each mouse was released in the dark compartment and observed for 5 min. The time the animal was present in the open light area was recorded. Coat state was scored on the head, neck, back, and ventrum. A normally groomed coat was rated 0 and an unkempt coat at each site was rated 1.

Statistical analysis

Data were examined with analysis of variance (ANOVA) and the appropriate post hoc tests. A *P* value of 0.05 or less (two-tailed) was considered indicative of statistical significance.

Results

To define the role of p38K as target of amitriptyline and to test whether a potential effect of amitriptyline on p38K is mediated by the acid sphingomyelinase/ceramide system, we treated mice with corticosterone, a typical stress inducer [15], amitriptyline, corticosterone plus amitriptyline or left the mice untreated. We then determined phosphorylation of p38K as a measurement for its activity by staining hippocampal sections with Cy3-labelled anti-phospho-p38K antibodies. The stainings revealed a marked phosphorylation of p38K in neurons of the hippocampus after administration of corticosterone (Fig. 1A, B). Amitriptyline prevented the increase of p38K phosphorylation induced by corticosterone (Fig. 1A, B). Interestingly, amitriptyline already induced a significant decrease of p38K phosphorylation in resting, not-stressed animals suggesting that p38K phosphorylation and activity, respectively is controlled by the acid sphingomyelinase/ceramide system.

Acid sphingomyelinase-deficient mice also showed an increase of p38K phosphorylation after corticosterone-induced stress (Fig. 1C, D). However, in contrast to wildtype mice, in acid sphingomyelinase-deficient mice amitriptyline did not prevent this stress-induced increase of p38K phosphorylation (Fig. 1C, D) indicating that the effect of amitriptyline on p38K depends on expression of the acid sphingomyelinase.

Next we tested whether hippocampal neurogenesis, which has been shown to be critical to prevent the development of major depression [1-3], is affected by inhibition of p38K in stressed or non-stressed mice. Treatment of wildtype mice with corticosterone resulted in a marked decrease of neurogenesis in the hippocampus (Fig. 2) consistent with previously published data [2]. Treatment with a p38K-inhibitor prevented the inhibitory effect of corticosterone on neurogenesis and at least partially restored neurogenesis in the brain of stressed wildtype mice (Fig. 2). Neurogenesis in acid sphingomyelinase-deficient mice was
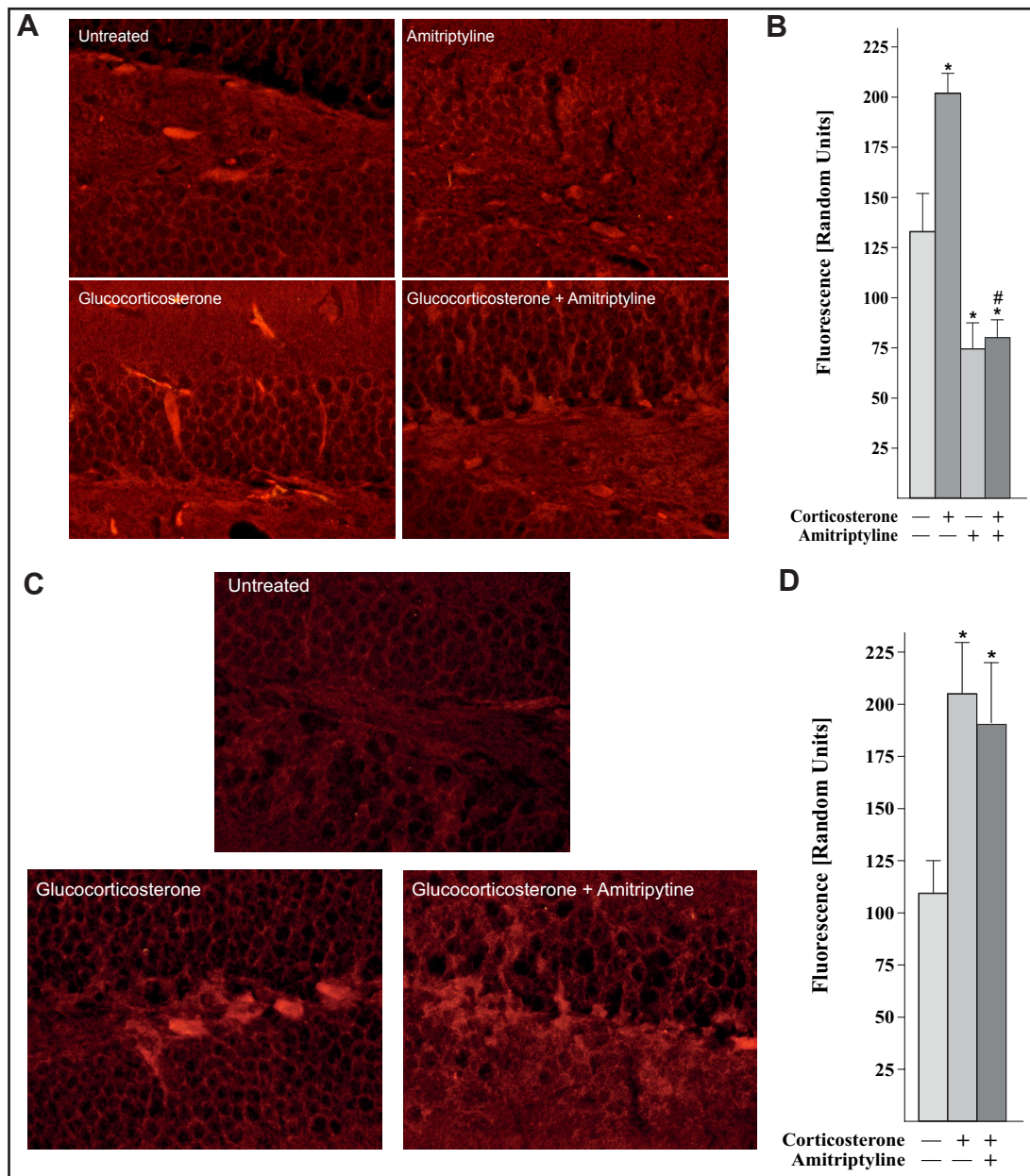
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Fig. 1. Glucocorticoid stress-induced phosphorylation/activation of p38K in the hippocampus is prevented by antidepressants via inhibition of the acid sphingomyelinase. Wildtype (A, B) and acid sphingomyelinase-deficient (C, D) mice were treated with corticosterone, amitripytline, corticosterone plus amitriptyline or left untreated. The phosphorylation of p38K as measurement for its activity was then determined by staining hippocampal sections with Cy3-labelled anti-phospho-p38K antibodies. Typical results from group are shown (n=6 per group). Panel B and D show the quantitative analysis of the fluorescence in at least 200 hippocampal neurons from 6 different mice. Shown is the mean ± SD, *p < 0.05 compared to untreated controls, # p<0.05 compared to corticosterone alone, ANOVA.

also affected by corticosterone, in accordance with our previous findings [2]. The treatment with a p38K inhibitor also restored neurogenesis in acid sphingomyelinase-deficient mice (Fig. 2), while treatment with amitriptyline only restored neurogenesis in wildtype, but not in acid sphingomyelinase-deficient mice (Fig. 2). This indicates that p38K functions downstream of the acid sphingomyelinase/ceramide system.

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Fig. 2. Amitriptyline targets p38K via inhibition of the acid sphingomyelinase/ ceramide system to increase neurogenesis. Corticosterone-induced stress reduces hippocampal neurogenesis. This effect is prevented by amitriptyline in wildtype mice, whereas acid sphingomyelinase-deficient mice did not respond to the antidepressant. Direct inhibition of p38K abrogates the inhibitory effects of corticosterone on neurogenesis in wildtype and acid sphingomyelinase-deficient mice. Shown are the BRDU-positive neurons from serial hippocampal sections with the mean ± SD from 6 mice each, *p < 0.05 compared to untreated controls, # p < 0.05 compared to corticosterone alone, Δ comparison between amitriptyline and p38K inhibitor, ANOVA.
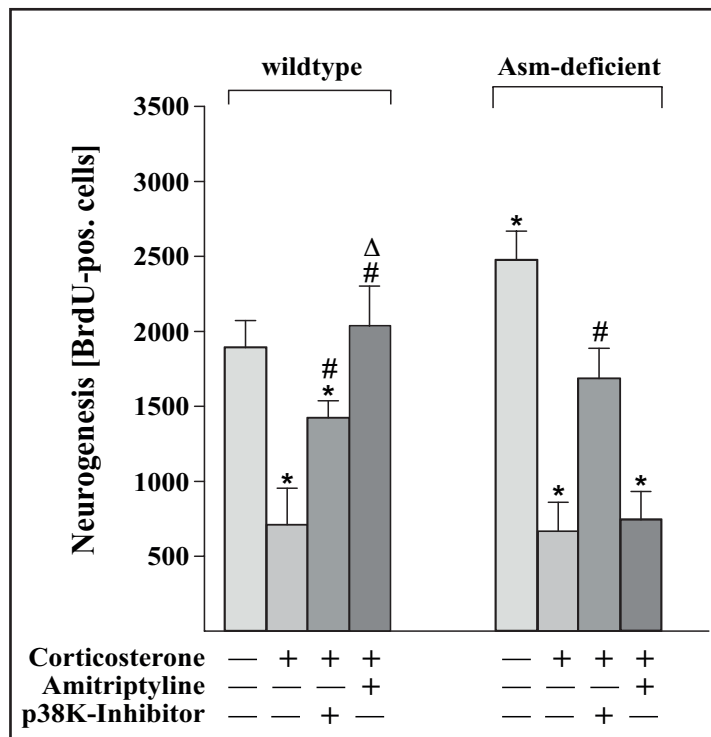
Stress-induced inhibition of neurogenesis has been shown in several studies to correlate with behavioural changes of mice that are typical for major depression and anxiety [1-3]. Thus, we tested whether inhibition of p38K reverses stress-induced behavioural changes in wildtype mice and whether this effect depends on expression of the acid sphingomyelinase. Since our data suggest that p38K acts downstream of the acid sphingomyelinase we also predicted that acid sphingomyelinase-deficiency would not prevent the effects a p38K inhibitor on behaviour. To perform these experiments we treated mice with corticosteroids and performed a set of behavioural tests, i.e. the novelty-suppressed feeding, open-field, the light/dark box and the coat state test. The tests showed a severe impact of corticosterone on behaviour, which was completely reversed by inhibition of p38K, both in wildtype and acid sphingomyelinase-deficient mice (Fig. 3A-D). Amitripytline-treatment also reversed stress-induced behavioural changes in wildtype mice, but was without effect in acid sphingomyelinase-deficient mice (Fig. 3A-D), consistent with previous data.

Discussion

Our studies demonstrate a critical role of p38K in the hippocampus to mediate stress responses, in particular corticosterone-mediated stress. Corticosterone reduces neurogenesis and severely impacts on murine behaviour. The behavioural changes manifest as lack of social interaction, anxiety, and depressive-like behaviour. Both neurogenesis and behaviour were normalised upon pharmacological inhibition of p38K with the pharmacological inhibitor SB239063. This indicates that p38K is an important regulator of neurogenesis and altered behaviour, hallmarks of major depression. Stress activates p38K, an event that results in inhibition of neurogenesis in the hippocampus. The acid sphingomyelinase seems to function upstream of p38K as indicated by the findings that (i) amitriptyline, which blocks the acid sphingomyelinase, normalizes p38K phosphorylation/activity in stressed animals, (ii) amitriptyline has no effect on p38K phosphorylation/activity in acid sphingomyelinase deficient mice and (iii) direct inhibition of p38K normalises neurogenesis and behaviour in wildtype and acid sphingomyelinase-deficient mice.
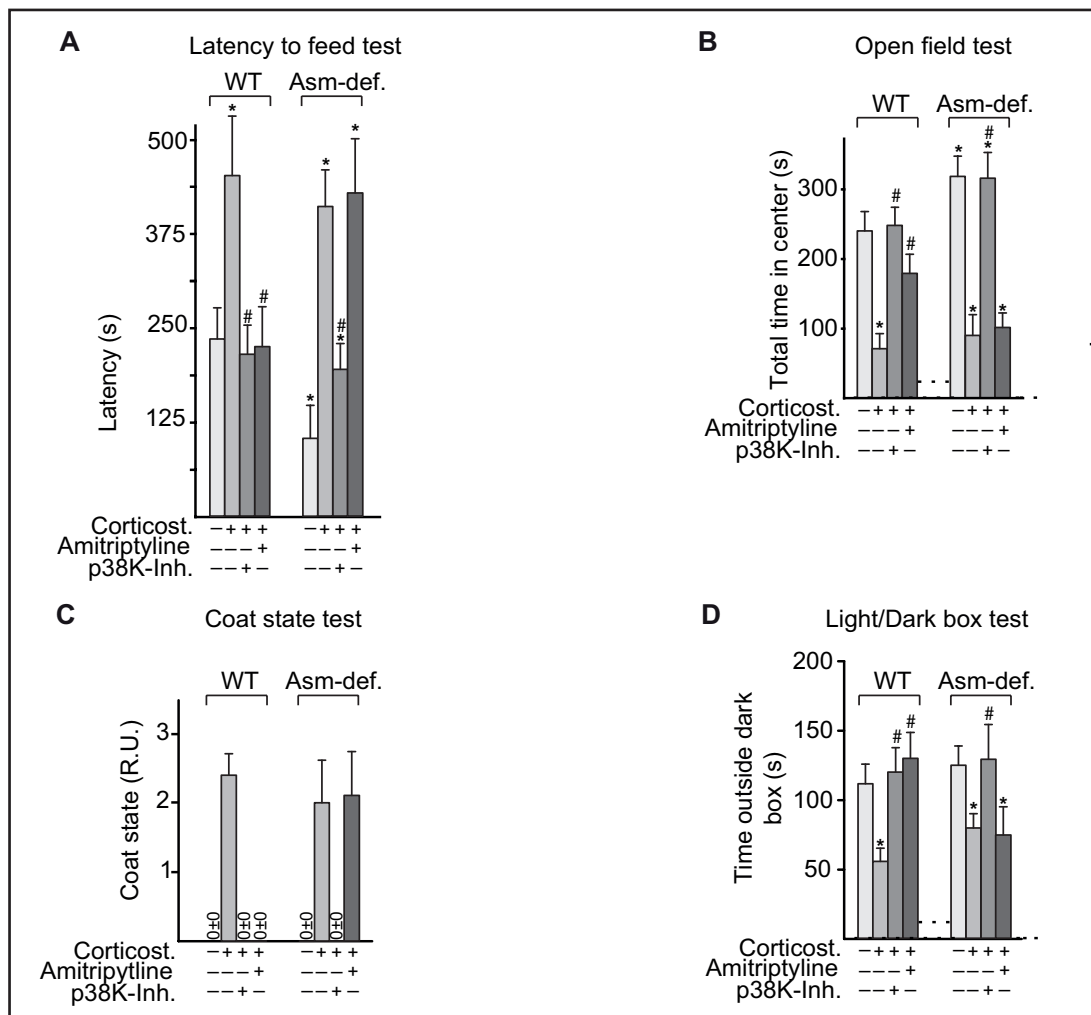
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Fig. 3. Stress-induced behavioural changes of mice are reverted by direct inhibition of p38K in wildtype and acid sphingomyelinase-deficient mice. A set of behavioural tests, i.e. the novelty-suppressed feeding test (A), open-field test (B), the coat state test (C) and the light/dark box test (D) reveals that inhibition of p38K (p38K-Inh = p38K-inhibitor) reversed the severe behavioural changes induced by corticosterone (Corticost.), both in wildtype (WT) and acid sphingmyelinase (Asm)-deficient mice. Amitripytline-treatment also prevented glucocorticoid-induced behavioural changes in wildtype mice, but was without effect in acid sphingomyelinase-deficient mice. Displayed are the mean ± SD from 6 mice each, *$p < 0.05$ compared to untreated wildtype controls, # $p < 0.05$ compared to corticosterone-treated animals, ANOVA.

Amitriptyline increased a larger increase of neurogenesis than p38K inhibition in wildtype mice, while the effect of amitriptyline and the p38K inhibitor on behaviour was very similar. This surprising result might indicate that a certain level of neurogenesis is sufficient to maintain the function of the hippocampus and restore behaviour, while an increase of neurogenesis might not be translated into biological consequences.

An increase of neurogenesis upon inhibition of p38K has also been previously shown in neurons of hippocampal slices that were deprived of glucose and oxygen [16]. Further, inhibition of p38K prevented cell death and an inflammatory response of the microglia in these experiments [16]. In accordance with our data preclinical and clinical studies showed an anti-depressive effect of the p38K inhibitors trazodone and iosmapimod and p38K inhibitors are under further investigation and clinical studies for the treatment of major depression [17, 18, https://clinicaltrials.gov/ct2/show/NCT02145468].

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On the other hand, the effect of stimuli that trigger proliferation of neural progenitor cells, for instance curcumin or ketamine, was neutralized by inhibition of p38K [19, 20]. The differential effects of p38K might be explained by the context of signalling events triggered by these stimuli. Curcumin and ketamine also activate MAPK/ERK [19, 20], which is known to trigger proliferation. Since p38K and MAPK/ERK are structurally related, inhibitors of p38K might also affect MAPK/ERK *in vivo* abrogating proliferation. Further, since corticosterone does not activate MAPK/ERK (E. Gulbins, unpublished data), it is very well possible that an isolated activation of p38K mediates stress, while a coordinated activation of MAPK/ERK and p38K and possibly also other kinases may result in proliferation.

At present it is unknown how p38K mediates cellular stress and how, vice versa, inhibition of p38K prevents the effects of stress. P38K has been previously shown to play a role in apoptotic cell death [21]. It is possible that inhibition of p38K prevents death of newborn neurons and therefore promotes neurogenesis. Further, p38K has been shown to regulate oxygen radicals [22]. Oxygen radicals have a differential effect on neuronal proliferation and neurogenesis: While high concentrations of oxygen radicals in superoxide-deficient cells severely reduce neurogenesis, lower doses of reactive oxygen species generated by NADPH oxidases trigger neurogenesis [15]. A differential biological effect mediated by the strength of p38K activation might explain the conflicting results regarding the effects of p38K inhibition on neurogenesis. Further, in inflammatory cells p38K is coupled to the inflammasome [23], a stress response system [24] that might be also involved in inhibition of neurogenesis and the induction of major depression.

In summary, our studies demonstrate that glucocorticoid-mediated stress induces phosphorylation/activation of p38K in the hippocampus and thereby reduces neurogenesis as well as induces alterations in the behaviour of mice that are similar to typical symptoms of major depressive disorder. These events are abrogated upon direct inhibition of p38K. Inhibition of the acid sphingomyelinase by antidepressants prevents stress-induced phosphorylation/activation of p38K indicating that antidepressants indirectly target p38K via the acid sphingomyelinase/ceramide system. Direct inhibitors of p38K might thus be promising drugs to treat major depressive disorder.

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Disclosure Statement

The authors declare to have no conflict of interest.

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