ACS Chemical Neuroscience

Article

Rapid and prolonged antidepressant-like effect of crocin is associated with GHSR mediated hippocampal plasticity-related proteins in mice exposed to prenatal stress

Ruyan Wu, Dong Xiao, Xin Shan, Yu Dong, and Wei-Wei Tao

ACS Chem. Neurosci., **Just Accepted Manuscript** • DOI: 10.1021/acschemneuro.0c00022 • Publication Date (Web): 23 Mar 2020

Regular research article

Rapid and prolonged antidepressant-like effect of crocin is associated with GHSR mediated hippocampal plasticity-related proteins in mice exposed to prenatal stress

Ruyan Wu, Ph.D.[1,2]*, Dong Xiao, B.A[3], Xin Shan, B.A[3], Yu Dong, B.A[3], Wei-Wei Tao, Ph.D.[3]*

[1] School of Medicine, Yangzhou University, Yangzhou, 225000, China

[2] Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, 14203, USA

[3] Key Laboratory of Integrative Biomedicine for Brain Diseases, School of Basic Biomedical Science, Nanjing University of Chinese Medicine, Nanjing, 210023, China

Abstract:	245 words
Introduction:	516 words
Results and Discussion:	3335 words
Methods:	1045 words
Figures:	6

* Corresponding authors:
Dr. Ruyan Wu, email: ruyanwu0908@gmail.com
School of Medicine, Yangzhou University, Yangzhou, 225000, China and
Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, 14203, USA TEL: 1-716-829-2677 or
Dr. Weiwei Tao, email: tw845@163.com
Key Laboratory of Integrative Biomedicine for Brain Diseases, School of Basic Biomedical Science, Nanjing University of Chinese Medicine, Nanjing, 210023, China

Abstract

Prenatal stress (PNS) has a prolonged and adverse effect on offspring, leading to a significantly increased vulnerability to developing depression in their later life. Traditional therapies have delayed onset and limited efficacy, thus it remains an urgent need to find novel medications with fast-onset and high efficacy potentials. Crocin, with its structure clearly examined, has shown antidepressant-like effects. However, less studies extensively investigated its effect especially in mice exposed to PNS. Using an established PNS model, we tested whether crocin could have a rapid and persistent antidepressant-like effect in PNS mice. Growth hormone secretagogue receptor (GHSR) and Phosphoinositide 3-kinase (PI3K) inhibitors were used to test their effects in antidepressant-like effect of crocin. Hippocampal GHSR-PI3K signaling was examined both in PNS mice treated with a single dose of crocin and in combination of GHSR inhibitor. PNS mice showed depression-like behaviors at juvenile and adulthood, and crocin induced an instant and persistent antidepressant-like response in PNS mice in a dose-dependent manner. Moreover, crocin increased the expression hippocampal synaptic plasticity-associated proteins through the restoration of GHSR-PI3K signaling. Inhibitions of both GHSR and PI3K abolished the effect of crocin in alleviating depressive-like behaviors. More importantly, GHSR inhibitor JMV2959 blocked the enhanced expression of hippocampal plasticity-related proteins induced by crocin. The present study demonstrated that crocin induced a fast-onset and prolonged antidepressant effect in PNS mice, and suggested that GHSR-PI3K signaling may play a key role in crocin's effect at least partially by a restoration of hippocampal synaptic plasticity-associated proteins.

Key words: Prenatal stress, crocin, fast, antidepressant, GHSR, synaptic plasticity-associated proteins

1 Introduction

Prenatal stress (PNS) refers to the exposure of an expectant mothers to multiple stress which includes stressful life challenges from the transition to motherhood[1]. The resulting physical and psychological changes to mother also have a damaging and prolonged effect on the nervous system development in offspring [2, 3], leading to a significantly increased vulnerability to develop psychiatric disorders in their later life [4, 5]. Among different kinds of prenatal psychopathological stress, maternal depression ranks the top in incidence rates because of its high prevalence and pervasive effects [6, 7]. It is well established that children of mothers with depression have much higher risk of developing depression than children of mothers without depression [8, 9]. Meanwhile, they have more emotional and behavioral problems when they grow older and this adverse effect can even last to next generation [10, 11]. Unfortunately, the early onset of depression in subjects exposed to PNS also predicts treatment resistance and relapse in this disorder[12].

Traditional treatments of depression have been widely known for limited efficacy, undesirable side effects and delayed onset [13, 14]. What's more, women experienced depression during pregnancy remain largely under-recognized and undertreated owing to the balance between the risk reproductive toxicity and severity of illness [15-17]. Consequently, our need for safer and more effective medications remains profoundly unmet. Crocin, with its chemical structure clearly identified[18], is largely enriched in Gardenia yellow pigment (GYP) from *Gardenia jasminoides Ellis* (GJ)[19]. Importantly, it has been shown to have antidepressant potentials [20-22]. Consistently, our previous studies showed that both GYP and GJ demonstrated rapid onset of antidepressant-like effects in mice[11, 19, 23], which strongly suggests crocin as a promising rapid antidepressant agent. However, less studies extensively examined the rapid and long-lasting antidepressant-like potential of crocin, especially in mice exposed to PNS.

Ghrelin is a 28-amino-acid peptide feeding peptide[22], which was recognized as an endogenous ligand for the growth hormone secretagogue receptor 1a (GHSR-1a)[24, 25]. It is identified as an important mediator in the pathology of mood disorders [26-28]. A pioneering study showed that increasing ghrelin levels in mice produced antidepressant-like response in forced swim test [29], with further support from the results that GHSR-1a-KO mice showed remarkably more social avoidance than wide-type littermates in chronic social defeat stress (CSDS) model[30]. The possible mechanism underlying GHSR's effects has been linked to its pro-neurogenic properties [31], and a role in mediating vasopressin (VAP) [32], serotonergic [33, 34] and noradrenergic transmission [35, 36]. However, the picture is not clear that by which mechanism this antidepressant-like effect mainly is modulated.

In the present study, we aimed to examine the adverse effect of prenatal stress on offspring by our established model in which dams experienced chronic stress before pregnant [11, 37]. We assessed the rapid and long-lasting antidepressant-like effect of crocin in PNS mice and explored whether and how GHSR mediated crocin's effect. We found that crocin induced an instant and enduring antidepressant-like response in PNS mice in a dose-dependent manner. Crocin enhanced the hippocampal plasticity-associated proteins through GHSR-PI3K signaling, and inhibitions of both GHSR and PI3K abolished the rapid antidepressant effect and enhanced expression of hippocampal plasticity-associated proteins induced by crocin.

2 Results and Discussion

PNS mice showed depression-like behaviors at juvenile and adulthood.

At postnatal day 28, PNS mice had significantly lower body weight than control group (Figure 1a, $t=11.47$, $p<0.05$), demonstrating an adverse effect from PNS at early lifetime. However, there was no difference in the body weight between these two groups at postnatal day 60. PNS

mice also demonstrated increased immobility in tail suspension test (TST) test compared with control group (Figure 1b, t=2.268, p<0.05), and this abnormal behavior lasted to postnatal day 60 (Figure 1b, t=2.155, p<0.05). Consistent with results from TST test, PNS group also showed a significant increase in immobility time in forced swimming test (FST) test both at postnatal day 28 (Figure 1c, t=2.110, p<0.05) and day 60 (Figure 1c, t=2.454, p<0.05). For the novelty suppressed feeding (NSF) test, there was no difference in the latency to feed between PNS and control group either at postnatal day 28 (Figure 1d, t=0.793, p>0.05) or day 60 between two groups (Figure 1d, t=1.491, p>0.05). However, PNS mice had a higher food consumption than control group at postnatal day 28 (Figure 1e, t=4.276, p<0.05), with this difference disappeared at day 60 (Figure 1e, t=0.329, p>0.05).

Crocin alleviated the depression-like behaviors in PNS mice at a dose-dependent manner.

To test the antidepressant-like effects of crocin, we utilized a list of behavioral tests including OFT, TST, FST and sucrose preference test (SPT) after crocin treatment. Mice at postnatal day 60 are used for evaluating the effects of crocin. There was no difference in total distance traveled and time spent in central zone among all groups in the OFT test (Figure 2a&b). PNS mice treated with vehicle showed increased immobility time compare with control group in TST test (Figure 2c, t=2.201, p<0.05). Moreover, there was a significant main effect for the treatment (Figure 2c, $F_{(4.42)}$=2.704, p<0.05) in the TST test. Pos hoc analysis showed that high dose of crocin (40mg/kg) significantly decreased the immobility time (p<0.05) in PNS mice, and this effect is similar with ketamine (p<0.05). In FST test, PNS mice treated with vehicle still maintained increased immobility time (Figure 2d, t=2.277, p<0.05). A significant effect for treatment was observed in FST test (Figure 2d, $F_{(4.36)}$=3.932, p<0.05), with high dose of crocin (40mg/kg) as well as ketamine significantly decreased the elevated immobility time in PNS mice (p<0.05). As a behavior model trying to test the core symptom of depression, sucrose

preference test was also used to determine the antidepressant-like effect of crocin. The vehicle group showed a decreased preference (Figure 2e, t=4.514, p<0.05) while 40mg/kg crocin and ketamine significantly reversed the decreased sucrose preference in PNS mice (Figure 2e, $F_{(4.36)}$=5.119, p<0.05; pos hoc: p<0.05).

Acute treatment of crocin induced a rapid and long-lasting antidepressant-like effect in PNS mice.

We also examined the time-course of antidepressant-like effects of crocin (40mg/kg). Firstly, we tested the TST 30 minutes after crocin treatment. There was a significant effect for treatment as shown in Figure 3a ($F_{(2.17)}$=5.039, p<0.05). PNS mice treated with vehicle still showed an increased immobility time in TST compared with control group (p<0.05), while crocin remarkably reduced the immobility time in PNS mice (p<0.05). Two hours after treatment, crocin also blocked the elevated immobility time in PNS mice (Figure 3b, $F_{(2.19)}$=6.082, p<0.05; pos hoc: p<0.05). We also tested the FST and SPT test 24 hours after crocin treatment. Vehicle group still maintained a depressive-like behaviors as shown by the increased immobility time in FST test (Figure 3c, $F_{(2.16)}$=7.008, p<0.05; pos hoc: p<0.05), and decreased preference for sucrose (Figure 3d, $F_{(2.27)}$=4.349, p<0.05; pos hoc: p<0.05). However, crocin significantly attenuated the immobility time (p<0.05) and increased sucrose preference in PNS mice (p<0.05), demonstrating a rapid antidepressant effect in mice.

To test whether crocin has a long-lasting antidepressant-like effect in PNS mice, we also measured the FST and SPT test 3 days after the acute treatment. Similarly, PNS mice treated with vehicle showed increased immobility time in FST test (Figure 3e, $F_{(2.18)}$=5.832, p<0.05; pos hoc: p<0.05)and decreased sucrose preference (Figure 3f, $F_{(2.22)}$=4.762, p<0.05; pos hoc: p<0.05). Importantly, crocin reversed the increased immobility time (p<0.05) and increased the preference for sucrose (p<0.05) in PNS mice 3 days after the treatment, demonstrating a long-lasting antidepressant-like efficacy.

Crocin increased the hippocampal expression of synaptic plasticity-associated proteins through the restoration of GHSR-PI3K signaling in PNS mice.

Studies have shown that depression is associated with decreased volume of prefrontal cortex and hippocampus [38-40]. Moreover, exposure to stress resulted in hippocampal neuronal atrophy and loss[41]. Thus, we chose hippocampus as a central brain region to study the antidepressant-like effect of crocin in PNS mice. To determine whether GHSR-PI3K signaling was involved in crocin's effect, we examined the expression levels of this signaling after 40mg/kg crocin treatment. PNS mice treated with vehicle showed significantly reduced GHSR levels (Figure 4a, $F_{(4.17)}$=3.663, p<0.05, pos hoc: p<0.05), while crocin significantly increased the GHSR levels in hippocampus of PNS mice (p<0.05). Crocin also restored the phosphorylation level of PI3K (Figure 4b, p<0.05), this effect was similar with ketamine (p<0.05). As downstream effectors of PI3K, the phosphorylation levels of AKT and mTOR were also decreased in the vehicle group (Figure 4c, AKT: $F_{(4.13)}$=3.875, p<0.05; Figure 4d, mTOR: $F_{(4.15)}$=7.659, p<0.05). Moreover, crocin and ketamine significantly increased the phosphorylation levels of these two effectors (p<0.05). We also found crocin and ketamine significantly reversed the decreased BDNF expression level in PNS mice (Figure 4e, $F_{(4.13)}$=4.021, p<0.05, pos hoc: p<0.05). Crocin also restored the reduced expression levels of GluR1 and PSD95 (Figure f&g, GluR1: $F_{(4.17)}$=4.141, p<0.05; pos hoc: p<0.05; PSD95: $F_{(4.12)}$=3.804, p<0.05, pos hoc: p<0.05), while the expression level of synapsin1 remained unchanged (Figure 4h).

Blockade of GHSR and PI3K abolished the antidepressant-like effect of crocin in PNS mice.

To determine the mechanism underlying crocin's antidepressant-like effect, we tested the effect of GHSR antagonist JMV2959 in crocin's effect. Thirty minutes before crocin treatment, a group

of mice were pretreated with JMV2959. Two-way ANOVA showed that there was a significant effect for the interaction of crocin and JMV2959 (Figure 5a, $F_{(1.40)}$=5.685, p<0.05) and crocin treatment ($F_{(1.40)}$=7.097, p<0.05). Furthermore, there was no significant effect for JMV2959 treatment. Crocin treatment significantly reversed the higher immobility time in PNS mice (p<0.05). More importantly, JMV2959 alone did not change the increased immobility in PNS mice. However, pretreatment of JMV2959 almost significantly abolished the antidepressant-like effect of crocin (p=0.05). Consistently, for FST test, there was a significant effect for crocin treatment (Figure 5b, $F_{(1.32)}$=4.827, p<0.05). Crocin significantly reduced the increased immobility time in PNS mice in FST test (p<0.05), and pretreatment of JMV2959 almost significantly blocked the antidepressant-like effect of crocin (p=0.05) while JMV2959 alone did not change this abnormal behavior.

We also tested whether PI3K was involved in the antidepressant-like effect of crocin. In TST test, two-way ANOVA showed that there was a significant effect for the interaction of crocin and LY294002 (Figure 5c, $F_{(1,32)}$=5.939, p<0.05) and crocin treatment ($F_{(1,32)}$=7.471, p<0.05). Pos hoc tests showed that mice treated with crocin showed a decrease immobility time in FST (p<0.05). PI3K antagonist LY294002 alone did not change the increased immobility time in PNS mice. However, pretreatment of LY294002 significantly attenuated the reduced immobility time induced by crocin (p<0.05). In FST test, there was also a significant effect for crocin treatment (Figure 5d, $F_{(1,35)}$=5.354, p<0.05) and this effect was almost significant for interaction between crocin and LY294002 treatment ($F_{(1,35)}$=3.402, p=0.07). Pos hoc tests showed that crocin significantly decresed the immobility time in PNS mice (p<0.05), and pretreatment of LY294002 abolished the antidepressant-like effect of crocin in FST test (p<0.05).

Blockade of GHSR abolished the enhanced hippocampal expression of synaptic plasticity-associated proteins induced by crocin in PNS mice.

To confirm whether GHSR-PI3K signaling modulated crocin's effect in strengthening the expression of synaptic plasticity-associated proteins, we further assessed the change of this signaling after the treatment of GHSR inhibitor. Crocin restored the lower phosphorylation level of PI3K in PNS mice (Figure 6a, two-way ANOVA: $F_{(1.15)}$=4.606, p<0.05, pos hoc: p<0.05). More importantly, GHSR antagonist JMV2959 reversed the enhanced effect of crocin on phosphorylation level of PI3K (p<0.05). Consistently, crocin also increased the phosphorylation levels of AKT and mTOR while the inhibition of GHSR significantly abolished this effect (Figure 6b&c, AKT: $F_{(1.9)}$=9.126, p<0.05, pos hoc: p<0.05; mTOR: $F_{(1.10)}$=6.450, p<0.05, pos hoc: p<0.05). Inhibition of GHSR also decreased the enhanced expression level of BDNF in PNS mice treated with crocin (Figure 6d, $F_{(1,10)}$=7.951, p<0.05, pos hoc: p<0.05), suggesting a decrease in the expression of synaptic plasticity-associated proteins in the hippocampus. Furthermore, the expression level of PSD95 were also increased after the crocin treatement, while GHSR inhibitor reversed the enhanced the expression of synaptic plasticity-associated proteins of crocin treatment (Figure 6e, $F_{(1,13)}$=6.513, p<0.05, pos hoc: p<0.05).

In the present study, we observed that mice exposed to PNS demonstrated more depressive-like behaviors as shown by increased immobility time at TST and FST test, and reduced sucrose preference in SPT test both at juvenile and adulthood, which is consistent with our previous study [11]. We also determined that crocin induced a rapid-onset and long-lasting antidepressant-like effect in PNS mice, which probably through the modulation of hippocampal neuroplasticity via GHSR-PI3K signaling. More importantly, inhibiton of GHSR or PI3K reversed the antidepressant-like effect of crocin. This study represents the first to carefully examine the rapid and enduring antidepressant-like effect of crocin in PNS mice, and further delineates a causal role of GHSR in this particular behavioral effect.

Interestingly, we found that PNS mice showed increased food consumption at postnatal day 28 but not day 60 in NSF test. However, PNS mice showed no difference in latency to feed, which

is a key marker of the anxiety-like behaviors[42]. Based on these conflicting results, it is relatively hard to confirm that PNS mice showed more anxiolytic-like behaviors. Moreover, we found that PNS mice had lower level of GHSR expression in the hippocampus, suggesting that they may have altered ghrelin level. In light of ghrelin signaling in hypothalamus plays important role in feeding behavior[43-45], it is possible that PNS mice had abnormal feeding behavior and glucose metabolism as shown by the increased food consumption and lower body weight at postnatal day 28. Studies have shown that mice lacking ghrelin or GHSR showed decreased body weight when they were exposed to a high-fat diet at early life time [46, 47]. This is possibly due to the increased glucose disposal and insulin sensitivity [48]. Interestingly, studies have also shown that ghrelin deficiency did not alter feeding behavior or body weight at adulthood [45, 49], which is in congruent with our findings that PNS mice had no difference in food consumption and body weight at postnatal day 60 compared with control group.

Recent studies have shed light on the antidepressant-like potential of crocin both preclinically and clinically. For example, repeated treatment of crocin for 14 days attenuated the depressive-like behaviors induced by malathion in rats[50]. Moreover, Amin and colleagues reported that acute treatment of crocin decreased the immobility time in FST at the dose of 40mg/kg in mice[22], which is in line with our findings. We found that only 40mg/kg crocin could alleviate the depressive-like behaviors in PNS mice without affecting the locomotor activity while the lower doses (10 and 20mg/kg) did not change these abnormal behaviors. However, less study examined the rapid-onset and long-lasting effect of crocin. Here, we tested the effect from 30 minutes to 3 days after a single administration of crocin. We found that crocin produced the antidepressant-like effect since 30 minutes after the treatment and this effect lasted at least 3 days, which has the similar effect as ketamine. After oral administration of crocin, it is rapidly detected as crocetin with a half-life of 6-7 hours after the administration[51, 52]. Thus the persistent antidepressant-like effects of crocin in PNS mice are probably due to the activation of GHSR

signaling which subsequently enhanced the expressions of synaptic plasticity-related proteins but not plasma pharmacokinetics of crocin. These results provided an important knowledge that crocin has rapid-onset effect and fill the gap in the time-course study of crocin. Previous study also found that chronic oral administration of crocin for 21 days produced similar effect only at a higher dose of 100mg/kg in mice [22]. This discrepancy may be due to the difference in duration which crocin was administered since our study used the acute injection. However, it merits study to explore the optimal doses and duration for the oral administration of crocin.

Fukumoto et al reported that both (S)-ketamine and (R)-ketamine reduced the immobility time in forced-swimming test (FST) and tail-suspension test (TST) at 30 minutes and 24 hours after the acute i.p. injection in naïve animals [53]. In the present study, we found that crocin induced a rapid and persistent antidepressant-like effects in PNS-related depression-like behaviors. However, we did not examine the effects of crocin on naïve mice, thus it remains unclear whether crocin could have antidepressant response in naïve animals exposed to acute inescapable stress like TST test. Nevertheless, our previous study has shown that Gardenia yellow pigment (GYP) induced antidepressant-like effect in naïve mice at 30 minutes after a single administration by decreasing the immobility time in TST, and this effect lasted at least 72 hours. Moreover, GYP showed antidepressant response in an inescapable learned helplessness (LH) paradigm [19]. These results suggested that GYP have antidepressant-like effects on depression-like behaviors induced by TST test as well as in an animal behavioral assay (LH) trying to model depression. Consequently, it is possible that crocin, which is highly enriched in GYP, also have effects on naïve mice exhibiting depression-like behavior as well as PNS-related depression-like behaviors.

Most theories of depression focus on the key role of stressful life event, thus stress animal models like chronic unpredictable mild stress (CUMS) and learned helplessness are widely used when modeling depression preclinically [54, 55]. These repeated and persistent stress models

including PNS model in the present study possibly induce a long-lasting change that could be interpreted as a “depressive state”, much closer to models of depression [56, 57]. Meanwhile, the FST and TST tests have shorter duration and frequency and the dependent variable (immobility time) is a direct manifestation to the test itself and dependent on the test situation[55]. Consequently, these tests are better considered as tests for antidepressants other than models of depression[55]. Although they are both widely used in the depression study [58, 59], there may be discrepancies in activity of antidepressants tested between the prolonged stress models and acute stress models. For example, a study has shown that ketamine (10 and 30 mg/kg) increased the immobility time in unstressed mice while decreased the prolonged the immobility time in CUS mice [60]. The differences in antidepressant responses in these two procedures are remained to be elucidated.

Clinical studies also support the antidepressant-like effect of crocin. In a randomized, double-blind pilot clinical study, crocin combined with SSRIs significantly improved the scores on multiple depression scales than the SSRIs alone [21]. In this study, the dose of crocin was 30mg/kg/d and the treatment duration is 4 weeks. Another study found that 8 weeks of crocin alone treatment improved depression in patients with coronary artery disease (CAD) [61]. This is probably the longest treatment period so far. However, since crocin has shown its unique properties in the treatment of depression, as supported by our study that crocin reversed the abnormal depressive-like behaviors in PNS mice, further studies should determine the optimal duration of treatment, the safety and efficacy for the long-term use of this promising antidepressant agent [18].

GHSR1a is broadly expressed throughout the brain including hippocampus [62-64]. It is widely suggested that activation of GHSR by ghrelin induced a significant antidepressant effect [29, 65]. For instance, GHSR1a-KO and hippocampal-specific GHSR knockdown mice showed worsened depressive-like behavior after the exposure to CSDS than wide-type littermates [29, 66],

and increased ghrelin level by caloric restriction failed to induce antidepressant-like effect in GHSR1a-KO mice [30]. In consistent with this study, GHSR1a-KO mice showed less depressive-like behaviors when ghrelin activated a specific catecholaminergic neurons which expressed GHSR1a[67]. Here, we reported that GHSR1a played a resistant role in the adverse effect of PNS in mice, as shown that GHSR level decreased in PNS mice and GHSR inhibitor abolished crocin's antidepressant-like effects. All these findings are in agreement with the aforementioned studies that the activation of GHSR could have an antidepressant-like potential. Moreover, these results expand the key role of GHSR in the influence of multiple stress models as PNS have more profound harmful effect through the offspring and even to next generation.

However, there are still debates on GHSR's role in depression[68]. A most recent study demonstrated that GHSR1a-KO mice showed less depressive-like behaviors with increased hippocampal BDNF level [69], thus suggesting that GHSR1a deficiency played a protective role in depression. Another studies also implied the depressogenic propertity of GHSR activation in which central administration of ghrelin decreased serotonergic transmission [34]. In addition, ghrelin anti-sense DNA in lateral ventricle produced antidepressant effects which is in contrast with our results [70]. Arguments about these findings would be the lack of knowledge whether ghrelin is produced in the brain[71]. The authors did not measure the corresponding concentration of ghrelin[68] while it is reasonable that there might be a dose-effect curve of ghrelin with the high dose producing a differing effect.

While it is reasonable that PNS could alter ghrelin level and crocin may normalize it to achieve its antidepressant-like effects, unfortunately, there are no studies investigated this question [72]. However, a study reported that rats exposed to early life stress showed increased ghrelin receptor expression in the paraventricular nucleus (PVN) [73], which is in contrast with our findings. Ghrelin levels in healthy-weight depressive patients have been reported to be increased, decreased and unchanged [74, 75]. Considering the controversial role of ghrelin in

depression, future studies specifically into ghrelin levels in PNS-related depression and crocin's effect in modulating ghrelin levels are needed. Also, it would be helpful to test the GHSR levels in hypothalamus as ghrelin signaling in this particular brain region mediates feeding behavior.

Synaptic plasticity is a fundamental brain function that has been widely implicated in the pathophysiology and treatment of depression [76, 77]. Selective serotonin reuptake inhibitors produced increase in hippocampus volume in patients [78] and ketamine increased glutamatergic neurotransmission and activation of BDNF and subsequently elevated synaptic plasticity [79, 80]. In consistent with these findings, we found that crocin activated GHSR-PI3K signaling and increased the expression of synaptic plasticity-associated proteins like BDNF and PSD95, which may be the underlying mechanism of its antidepressant response in PNS mice. Crocin reversed the lower phosphorylation levels of AKT and mTOR, and strengthened the expression of BDNF and post-synaptic proteins like GluR1 and PSD95 in PNS mice. Moreover, GHSR inhibitor abolished the hippocampal synaptic changes induced by crocin, further suggesting the central role of GHSR in this signaling. This study remains the first to link GHSR-PI3K pathway to crocin's rapid and long-lasting antidepressant-like effect in PNS mice. It is in consistency with our previous study that offspring of dams demonstrating depression-like behaviors had a deficiency in the hippocampal AKT-mTOR signaling [11], while the present study further underlined the importance of GHSR in this particular effect. As an endogenous ligand of GHSR, ghrelin also has been reported to activate the PI3K signaling to produced its neuroprotective effects [81, 82]. Here, we showed that crocin activated the GHSR and the following PI3K signaling and induced a rapid and enduring antidepressant effect, which is similar with the effect of ghrelin.In conclusion, the present study demonstrated the adverse effect of prenatal stress as evidenced by significant depressive-like behaviors in mice. Crocin, a natural chemical, produced a fast and long-lasting antidepressant-like effect in PNS mice and restored the impaired the expression hippocampal of synaptic plasticity-associated proteins via modulation of GHSR-PI3K

signaling. This signaling may at least partially contributed to the antidepressant properties of crocin as the inhibition of GHSR abolished its unique effect. The present study remains the first to examine the rapid and long-lasting antidepressant potential of crocin and further suggest the GHSR-PI3K signaling as a part of underlying mechanism of crocin's effect. These findings illuminated the promising antidepressant-effect of crocin as a novel antidepressant agent, further studies concerning long-term efficacy and safety of crocin are warranted.

3 Methods

Animals

Balb/cJ female and male mice aged 6-8 weeks old (18-24 g) were housed in the animal facilities for 1 week to habituate before the experiment. Mice were kept on a 12h/12h light dark cycle and were given free access to food and water. All animal procedures were followed by the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Nanjing University of Chinese Medicine.

Experimental design

The experimental design was similar with our previous studies [11, 37]. Female mice were randomly divided into stressed and non-stressed groups. The stressed mice received a chronic mild stress procedure which consisted of daily 6 h restraint stress in a 50-ml centrifuge tube, combined with overnight illumination twice a week for 3 weeks. Four days later, all females were mated with naïve males. Once the females became pregnant, the males were removed from the cages. About 4 weeks later, females gave birth to offspring. Stressed females develop depression-like behavior postpartum [37]. The offspring of pre-pregnancy stressed females were defined as PNS mice. In contrast, the offspring of pre-pregnancy non-stressed (naïve) females were defined as control group. The two groups of offspring were housed with dams until 3weeks

postnatal. PNS and control group were tested with a battery for depression-like behaviors at postnatal day (PND) 28 (juvenile) [83] and 60 (adulthood) [84].

Behavior testing

Open field test (OFT): OFT was used to assess the locomotor as well as the exploratory behavior in an open area. Spontaneous locomotor activity was measured in a square arena (40 X 40 X 15cm) as the total distance traveled. Mice were tested in a well-illuminated (~300 lx) transparent acrylic cage for 5 min. Activity of mice in the two compartments, near the bulkhead and central regions, was tracked. Distance (cm) and time spent in the central zone were analyzed. A camera was placed in the top of the box for recording the activity. Both the distance traveled (cm) and time spent in central area(s) were analyzed by a computer-based tracking system. The device was thoroughly cleaned before each animal using 75% ethanol.

Tail Suspension Test (TST): In acoustic and visual isolated chambers, a single mouse was suspended in 50 cm above the floor, with a tape placed at about 1 cm of the tail. Activities of the animals were videotaped. The computer calculated the total duration of immobility during the last 4 min in a 6 min testing time. Immobility was defined when the animals hung passively without any struggling movements.

Forced swim test (FST): The FST was carried out following the method of Porsolt et al. [85]. In brief, each mouse was placed into a transparent Plexiglass cylinder (25 cm high, 10 cm in diameter) containing 10 cm of water maintained at 23–25 °C. Animals were left in the cylinder for 6 min. The total immobility time was measured during the last 4 min. The animals were considered to be immobile when they floated passively in the water. For TST and FST, total immobility time during the last 4 min was analyzed by ANY-maze software.

Sucrose preference test (SPT): SPT was performed following the procedure of Opal et al. [86]. All mice were trained to consume two bottles of sucrose solution (2%) for 3 days to establish

baseline preference levels. After 18 h of food and water-deprivation, mice were single-housed and presented with two pipettes containing 2% sucrose solution or tap water for 1 h. Sucrose preference was calculated by the formula: (sucrose preference) = ((sucrose intake) / (sucrose intake +water intake)) X 100, as previously described.

Novelty suppressed feeding (NSF) test: 24 h before the NSF test, the mice were deprived of food but not water. On the day of the test, mice were moved to a quiet room with dim lighting. They were placed at the edge of the Plexiglass test chamber (30 cm X 60 cm) with a single food pellet in the center. Latency to feeding was measured for 5 min; non-feeding behaviors (e.g., touching, smelling) were ignored. If food was not eaten within 5 min, feeding latency was recorded as 5 min. Food pallets were weighed before and after test.

Drug administrations

Ketamine HCl (Gutian Pharmaceuticals, China) and crocin (Selleck Chemicals) was dissolved in saline [87]. Ketamine (30 mg/kg, i.p.) or crocin (10, 20, 40mg/kg, intragastrically) or saline control was administered i.p. 24 h before the behavior tests. In the time-course test, crocin (40mg/kg) was administered 30 minutes, 2, 24 and 72 hours prior to the behavioral tests respectively. JMV2959 (12mg/kg, i.p.) and LY294002 (50mg/kg, i.p.) were administered 30 minutes before crocin (40mg/kg) treatment.

Western blot

After the behavioral tests, mice were sacrificed by cervical dislocation to take the brain. The entire hippocampus (ventral and dorsal) was removed and lysed in RIPA buffer containing protease inhibitors and phosphatase inhibitors. Protein concentration was determined colorimetrically using a NANODROP 2000 (Thermo Scientific). Protein lysates were separated by 10% SDS-PAGE electrophoresis and were transferred onto polyvinylidenedifluoride (PVDF) membranes. After blocking with 5% BSA for 1 h, the following antibodies were used: GHSR

(Abcam, ab85104, 1:500), BDNF (Abcam, ab108319, 1:500), Akt/p-Akt (Cell Signaling Technology, 9272S, 9271s, 1:500), mTOR/p-mTOR (Cell Signaling Technology, 2983S, 2971S, 1:500), PI3K/ p-PI3K (Cell Signaling Technology, 4249S, 4228S, 1:500), GluR1 (Cell Signaling Technology, 13185S 1:500), PSD95 (Cell Signaling Technology, 3450S 1:500), β-tubulin (Epitomics, 1879-1, 1:2000) and GAPDH (Abcam, ab181602, 1:2000). After the blots were incubated with antibodies overnight at 4 °C, they were incubated with horseradish peroxidaseconjugated secondary antibodies for 1 h. The blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). All experiments were performed in triplicate. The final data are expressed as a ratio of the relative optical density (ROD) of the protein of interest to the ROD of β-tubulin or GAPDH.

Data analysis

All results were analyzed by GraphPad Prism 8 software. Two-sample comparisons were carried out using a two-tailed Student's t-test; the Mann-Whitney test was employed when the data was not normally distributed. Multiple comparisons were made using ANOVAs (specifically stated in figures) followed by the Bonferroni post hoc test. Two-way ANOVA were used to analyze the data from inhibitor study. A p-value < 0.05 was considered significant. All results are indicated as the mean ± SEM.

Abbreviations

PNS: prenatal stress, GYP: Gardenia yellow pigment; GJ: Gardenia jasminoides Ellis; GHSR: growth hormone secretagogue receptor; CSDS: chronic social defeat stess; PND: postnatal day; OFT: open field test; TST: tail suspension test; FST: forced swimming test; SPT: sucrose preference test; NSF: novelty suppressed feeding; PI3K: Phosphoinositide 3-kinase

Author information

Current addresses:

Dr. Ruyan Wu, email: ruyanwu0908@gmail.com

School of Medicine, Yangzhou University, Yangzhou, 225000, China and

Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, 14203, USA TEL: 1-716-829-2677

Dr. Weiwei Tao, email: tw845@163.com

Key Laboratory of Integrative Biomedicine for Brain Diseases, School of Basic Biomedical Science, Nanjing University of Chinese Medicine, Nanjing, 210023, China

Author Contributions

R.W. and W.T. designed the experiments. R.W., D.X., X.S., Y.D. and W.T. conducted the experiments. R.W., D.X. and W.T. analyzed the data. R.W. and W.T. wrote the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China [grant numbers 81701340, 81873096]; Natural Science Foundation of Jiangsu Province [grant number BK 20170517]. All these funding had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflict of Interest

None.

Acknowledgments

This work was supported by the National Natural Science Foundation of China [grant numbers 81701340; 81873096]; Natural Science Foundation of Jiangsu Province [grant numbers BK 20170517]. We thank Dr. Jianfeng Liu for providing language help and proof reading the article.

Reference

1. Aktar, E.; Qu, J.; Lawrence, P. J.; Tollenaar, M. S.; Elzinga, B. M.; Bogels, S. M., Fetal and Infant Outcomes in the Offspring of Parents With Perinatal Mental Disorders: Earliest Influences. *Front Psychiatry* **2019,** *10*, 391.
2. Drury, S. S.; Scaramella, L.; Zeanah, C. H., The Neurobiological Impact of Postpartum Maternal Depression: Prevention and Intervention Approaches. *Child Adolesc Psychiatr Clin N Am* **2016,** *25* (2), 179-200.
3. Pawluski, J. L.; Lonstein, J. S.; Fleming, A. S., The Neurobiology of Postpartum Anxiety and Depression. *Trends Neurosci* **2017,** *40* (2), 106-120.
4. Verbeek, T.; Bockting, C. L.; van Pampus, M. G.; Ormel, J.; Meijer, J. L.; Hartman, C. A.; Burger, H., Postpartum depression predicts offspring mental health problems in adolescence independently of parental lifetime psychopathology. *J Affect Disord* **2012,** *136* (3), 948-54.
5. Goodman, S. H.; Rouse, M. H.; Connell, A. M.; Broth, M. R.; Hall, C. M.; Heyward, D., Maternal depression and child psychopathology: a meta-analytic review. *Clin Child Fam Psychol Rev* **2011,** *14* (1), 1-27.
6. Wisner, K. L.; Zarin, D. A.; Holmboe, E. S.; Appelbaum, P. S.; Gelenberg, A. J.; Leonard, H. L.; Frank, E., Risk-benefit decision making for treatment of depression during pregnancy. *Am J Psychiatry* **2000,** *157* (12), 1933-40.
7. Le Strat, Y.; Dubertret, C.; Le Foll, B., Prevalence and correlates of major depressive episode in pregnant and postpartum women in the United States. *J Affect Disord* **2011,** *135* (1-3), 128-38.
8. Hammerton, G.; Zammit, S.; Mahedy, L.; Pearson, R. M.; Sellers, R.; Thapar, A.; Collishaw, S., Pathways to suicide-related behavior in offspring of mothers with depression: the role of offspring psychopathology. *J Am Acad Child Adolesc Psychiatry* **2015,** *54* (5), 385-93.
9. Bridge, J. A.; Goldstein, T. R.; Brent, D. A., Adolescent suicide and suicidal behavior. *J Child Psychol Psychiatry* **2006,** *47* (3-4), 372-94.
10. Netsi, E.; Pearson, R. M.; Murray, L.; Cooper, P.; Craske, M. G.; Stein, A., Association of Persistent and Severe Postnatal Depression With Child Outcomes. *JAMA Psychiatry* **2018,** *75* (3), 247-253.
11. Wu, R.; Zhang, H.; Xue, W.; Zou, Z.; Lu, C.; Xia, B.; Wang, W.; Chen, G., Transgenerational impairment of hippocampal Akt-mTOR signaling and behavioral deficits in the offspring of mice that experience postpartum depression-like illness. *Prog Neuropsychopharmacol Biol Psychiatry* **2017,** *73*, 11-18.
12. Anacker, C.; O'Donnell, K. J.; Meaney, M. J., Early life adversity and the epigenetic programming of hypothalamic-pituitary-adrenal function. *Dialogues Clin Neurosci* **2014,** *16* (3), 321-33.
13. Rush, A. J.; Trivedi, M. H.; Wisniewski, S. R.; Nierenberg, A. A.; Stewart, J. W.; Warden, D.; Niederehe, G.; Thase, M. E.; Lavori, P. W.; Lebowitz, B. D.; McGrath, P. J.; Rosenbaum, J. F.; Sackeim,

H. A.; Kupfer, D. J.; Luther, J.; Fava, M., Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *Am J Psychiatry* **2006,** *163* (11), 1905-17.

14. Fogaca, M. V.; Fukumoto, K.; Franklin, T.; Liu, R. J.; Duman, C. H.; Vitolo, O. V.; Duman, R. S., N-Methyl-D-aspartate receptor antagonist d-methadone produces rapid, mTORC1-dependent antidepressant effects. *Neuropsychopharmacology* **2019,** *44* (13), 2230-2238.

15. Scholle, S. H.; Haskett, R. F.; Hanusa, B. H.; Pincus, H. A.; Kupfer, D. J., Addressing depression in obstetrics/gynecology practice. *Gen Hosp Psychiatry* **2003,** *25* (2), 83-90.

16. Marcus, S. M.; Flynn, H. A.; Blow, F. C.; Barry, K. L., Depressive symptoms among pregnant women screened in obstetrics settings. *J Womens Health (Larchmt)* **2003,** *12* (4), 373-80.

17. Fisher, J.; Cabral de Mello, M.; Patel, V.; Rahman, A.; Tran, T.; Holton, S.; Holmes, W., Prevalence and determinants of common perinatal mental disorders in women in low- and lower-middle-income countries: a systematic review. *Bull World Health Organ* **2012,** *90* (2), 139G-149G.

18. Lopresti, A. L.; Drummond, P. D., Saffron (Crocus sativus) for depression: a systematic review of clinical studies and examination of underlying antidepressant mechanisms of action. *Hum Psychopharmacol* **2014,** *29* (6), 517-27.

19. Wu, R.; Tao, W.; Zhang, H.; Xue, W.; Zou, Z.; Wu, H.; Cai, B.; Doron, R.; Chen, G., Instant and Persistent Antidepressant Response of Gardenia Yellow Pigment Is Associated with Acute Protein Synthesis and Delayed Upregulation of BDNF Expression in the Hippocampus. *ACS Chem Neurosci* **2016,** *7* (8), 1068-76.

20. Zhang, L.; Previn, R.; Lu, L.; Liao, R. F.; Jin, Y.; Wang, R. K., Crocin, a natural product attenuates lipopolysaccharide-induced anxiety and depressive-like behaviors through suppressing NF-kB and NLRP3 signaling pathway. *Brain Res Bull* **2018,** *142*, 352-359.

21. Talaei, A.; Hassanpour Moghadam, M.; Sajadi Tabassi, S. A.; Mohajeri, S. A., Crocin, the main active saffron constituent, as an adjunctive treatment in major depressive disorder: a randomized, double-blind, placebo-controlled, pilot clinical trial. *J Affect Disord* **2015,** *174*, 51-6.

22. Amin, B.; Nakhsaz, A.; Hosseinzadeh, H., Evaluation of the antidepressant-like effects of acute and sub-acute administration of crocin and crocetin in mice. *Avicenna J Phytomed* **2015,** *5* (5), 458-68.

23. Zhang, H.; Xue, W.; Wu, R.; Gong, T.; Tao, W.; Zhou, X.; Jiang, J.; Zhang, Y.; Zhang, N.; Cui, Y.; Chen, C.; Chen, G., Rapid Antidepressant Activity of Ethanol Extract of Gardenia jasminoides Ellis Is Associated with Upregulation of BDNF Expression in the Hippocampus. *Evid Based Complement Alternat Med* **2015,** *2015*, 761238.

24. Howard, A. D.; Feighner, S. D.; Cully, D. F.; Arena, J. P.; Liberator, P. A.; Rosenblum, C. I.; Hamelin, M.; Hreniuk, D. L.; Palyha, O. C.; Anderson, J.; Paress, P. S.; Diaz, C.; Chou, M.; Liu, K. K.; McKee, K. K.; Pong, S. S.; Chaung, L. Y.; Elbrecht, A.; Dashkevicz, M.; Heavens, R.; Rigby, M.; Sirinathsinghji, D. J.; Dean, D. C.; Melillo, D. G.; Patchett, A. A.; Nargund, R.; Griffin, P. R.; DeMartino, J. A.; Gupta, S. K.; Schaeffer, J. M.; Smith, R. G.; Van der Ploeg, L. H., A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **1996,** *273* (5277), 974-7.

25. Conn, P. M.; Bowers, C. Y., A new receptor for growth hormone-release peptide. *Science* **1996,** *273* (5277), 923.

26. Schellekens, H.; Finger, B. C.; Dinan, T. G.; Cryan, J. F., Ghrelin signalling and obesity: at the interface of stress, mood and food reward. *Pharmacol Ther* **2012,** *135* (3), 316-26.

27. Steiger, A.; Dresler, M.; Schussler, P.; Kluge, M., Ghrelin in mental health, sleep, memory. *Mol Cell Endocrinol* **2011,** *340* (1), 88-96.

28. Zarouna, S.; Wozniak, G.; Papachristou, A. I., Mood disorders: A potential link between ghrelin and leptin on human body? *World J Exp Med* **2015,** *5* (2), 103-9.

29. Lutter, M.; Sakata, I.; Osborne-Lawrence, S.; Rovinsky, S. A.; Anderson, J. G.; Jung, S.; Birnbaum, S.; Yanagisawa, M.; Elmquist, J. K.; Nestler, E. J.; Zigman, J. M., The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nat Neurosci* **2008,** *11* (7), 752-3.

30. Walker, A. K.; Rivera, P. D.; Wang, Q.; Chuang, J. C.; Tran, S.; Osborne-Lawrence, S.; Estill, S. J.; Starwalt, R.; Huntington, P.; Morlock, L.; Naidoo, J.; Williams, N. S.; Ready, J. M.; Eisch, A. J.; Pieper, A. A.; Zigman, J. M., The P7C3 class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis. *Mol Psychiatry* **2015,** *20* (4), 500-8.
31. Moon, M.; Cha, M. Y.; Mook-Jung, I., Impaired hippocampal neurogenesis and its enhancement with ghrelin in 5XFAD mice. *J Alzheimers Dis* **2014,** *41* (1), 233-41.
32. Poretti, M. B.; Rask-Andersen, M.; Kumar, P.; Rubiales de Barioglio, S.; Fiol de Cuneo, M.; Schioth, H. B.; Carlini, V. P., Ghrelin effects expression of several genes associated with depression-like behavior. *Prog Neuropsychopharmacol Biol Psychiatry* **2015,** *56*, 227-34.
33. Ogaya, M.; Kim, J.; Sasaki, K., Ghrelin postsynaptically depolarizes dorsal raphe neurons in rats in vitro. *Peptides* **2011,** *32* (8), 1606-16.
34. Hansson, C.; Alvarez-Crespo, M.; Taube, M.; Skibicka, K. P.; Schmidt, L.; Karlsson-Lindahl, L.; Egecioglu, E.; Nissbrandt, H.; Dickson, S. L., Influence of ghrelin on the central serotonergic signaling system in mice. *Neuropharmacology* **2014,** *79*, 498-505.
35. Emanuel, A. J.; Ritter, S., Hindbrain catecholamine neurons modulate the growth hormone but not the feeding response to ghrelin. *Endocrinology* **2010,** *151* (7), 3237-46.
36. Carlini, V. P.; Machado, D. G.; Buteler, F.; Ghersi, M.; Ponzio, M. F.; Martini, A. C.; Schioth, H. B.; de Cuneo, M. F.; Rodrigues, A. L.; de Barioglio, S. R., Acute ghrelin administration reverses depressive-like behavior induced by bilateral olfactory bulbectomy in mice. *Peptides* **2012,** *35* (2), 160-5.
37. Xia, B.; Chen, C.; Zhang, H.; Xue, W.; Tang, J.; Tao, W.; Wu, R.; Ren, L.; Wang, W.; Chen, G., Chronic stress prior to pregnancy potentiated long-lasting postpartum depressive-like behavior, regulated by Akt-mTOR signaling in the hippocampus. *Sci Rep* **2016,** *6*, 35042.
38. Chan, S. W.; Harmer, C. J.; Norbury, R.; O'Sullivan, U.; Goodwin, G. M.; Portella, M. J., Hippocampal volume in vulnerability and resilience to depression. *J Affect Disord* **2016,** *189*, 199-202.
39. Duman, R. S.; Aghajanian, G. K., Synaptic dysfunction in depression: potential therapeutic targets. *Science* **2012,** *338* (6103), 68-72.
40. McEwen, B. S.; Eiland, L.; Hunter, R. G.; Miller, M. M., Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology* **2012,** *62* (1), 3-12.
41. McEwen, B. S.; Nasca, C.; Gray, J. D., Stress Effects on Neuronal Structure: Hippocampus, Amygdala, and Prefrontal Cortex. *Neuropsychopharmacology* **2016,** *41* (1), 3-23.
42. Ramaker, M. J.; Dulawa, S. C., Identifying fast-onset antidepressants using rodent models. *Mol Psychiatry* **2017,** *22* (5), 656-665.
43. Schaeffer, M.; Langlet, F.; Lafont, C.; Molino, F.; Hodson, D. J.; Roux, T.; Lamarque, L.; Verdie, P.; Bourrier, E.; Dehouck, B.; Baneres, J. L.; Martinez, J.; Mery, P. F.; Marie, J.; Trinquet, E.; Fehrentz, J. A.; Prevot, V.; Mollard, P., Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. *Proc Natl Acad Sci U S A* **2013,** *110* (4), 1512-7.
44. Flier, J. S., Obesity wars: molecular progress confronts an expanding epidemic. *Cell* **2004,** *116* (2), 337-50.
45. Al Massadi, O.; Lopez, M.; Tschop, M.; Dieguez, C.; Nogueiras, R., Current Understanding of the Hypothalamic Ghrelin Pathways Inducing Appetite and Adiposity. *Trends Neurosci* **2017,** *40* (3), 167-180.
46. Wortley, K. E.; del Rincon, J. P.; Murray, J. D.; Garcia, K.; Iida, K.; Thorner, M. O.; Sleeman, M. W., Absence of ghrelin protects against early-onset obesity. *J Clin Invest* **2005,** *115* (12), 3573-8.
47. Zigman, J. M.; Nakano, Y.; Coppari, R.; Balthasar, N.; Marcus, J. N.; Lee, C. E.; Jones, J. E.; Deysher, A. E.; Waxman, A. R.; White, R. D.; Williams, T. D.; Lachey, J. L.; Seeley, R. J.; Lowell, B. B.; Elmquist, J. K., Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest* **2005,** *115* (12), 3564-72.
48. Sun, Y.; Asnicar, M.; Saha, P. K.; Chan, L.; Smith, R. G., Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. *Cell Metab* **2006,** *3* (5), 379-86.

49. Wang, Q.; Liu, C.; Uchida, A.; Chuang, J. C.; Walker, A.; Liu, T.; Osborne-Lawrence, S.; Mason, B. L.; Mosher, C.; Berglund, E. D.; Elmquist, J. K.; Zigman, J. M., Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. *Mol Metab* **2014,** *3* (1), 64-72.
50. Dorri, S. A.; Hosseinzadeh, H.; Abnous, K.; Hasani, F. V.; Robati, R. Y.; Razavi, B. M., Involvement of brain-derived neurotrophic factor (BDNF) on malathion induced depressive-like behavior in subacute exposure and protective effects of crocin. *Iran J Basic Med Sci* **2015,** *18* (10), 958-66.
51. Asai, A.; Nakano, T.; Takahashi, M.; Nagao, A., Orally administered crocetin and crocins are absorbed into blood plasma as crocetin and its glucuronide conjugates in mice. *J Agr Food Chem* **2005,** *53* (18), 7302-7306.
52. Marco, F. D.; Romeo, S.; Nandasena, C.; Purushothuman, S.; Adams, C.; Bisti, S.; Stone, J., The time course of action of two neuroprotectants, dietary saffron and photobiomodulation, assessed in the rat retina. *Am J Neurodegener Dis* **2013,** *2* (3), 208-20.
53. Fukumoto, K.; Toki, H.; Iijima, M.; Hashihayata, T.; Yamaguchi, J. I.; Hashimoto, K.; Chaki, S., Antidepressant Potential of (R)-Ketamine in Rodent Models: Comparison with (S)-Ketamine. *J Pharmacol Exp Ther* **2017,** *361* (1), 9-16.
54. Berton, O.; Nestler, E. J., New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* **2006,** *7* (2), 137-51.
55. Castagne, V.; Moser, P.; Porsolt, R. D., *Behavioral Assessment of Antidepressant Activity in Rodents*. 2009; p 103-117.
56. Hosang, G. M.; Shiles, C.; Tansey, K. E.; McGuffin, P.; Uher, R., Interaction between stress and the BDNF Val66Met polymorphism in depression: a systematic review and meta-analysis. *BMC Med* **2014,** *12*, 7.
57. Vollmayr, B.; Henn, F. A., Learned helplessness in the rat: improvements in validity and reliability. *Brain Res Brain Res Protoc* **2001,** *8* (1), 1-7.
58. Petit-Demouliere, B.; Chenu, F.; Bourin, M., Forced swimming test in mice: a review of antidepressant activity. *Psychopharmacology* **2005,** *177* (3), 245-255.
59. Cryan, J. F.; Slattery, D. A., Animal models of mood disorders: Recent developments. *Curr Opin Psychiatry* **2007,** *20* (1), 1-7.
60. Fitzgerald, P. J.; Yen, J. Y.; Watson, B. O., Stress-sensitive antidepressant-like effects of ketamine in the mouse forced swim test. *PLoS One* **2019,** *14* (4), e0215554.
61. Abedimanesh, N.; Ostadrahimi, A.; Bathaie, S. Z.; Abedimanesh, S.; Motlagh, B.; Jafarabadi, M. A.; Sadeghi, M. T., Effects of Saffron Aqueous Extract and Its Main Constituent, Crocin, on Health-Related Quality of Life, Depression, and Sexual Desire in Coronary Artery Disease Patients: A Double-Blind, Placebo-Controlled, Randomized Clinical Trial. *Iran Red Crescent Me* **2017,** *19* (9), e13676.
62. Guan, X. M.; Yu, H.; Palyha, O. C.; McKee, K. K.; Feighner, S. D.; Sirinathsinghji, D. J.; Smith, R. G.; Van der Ploeg, L. H.; Howard, A. D., Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* **1997,** *48* (1), 23-9.
63. Perello, M.; Scott, M. M.; Sakata, I.; Lee, C. E.; Chuang, J. C.; Osborne-Lawrence, S.; Rovinsky, S. A.; Elmquist, J. K.; Zigman, J. M., Functional implications of limited leptin receptor and ghrelin receptor coexpression in the brain. *J Comp Neurol* **2012,** *520* (2), 281-94.
64. Zigman, J. M.; Jones, J. E.; Lee, C. E.; Saper, C. B.; Elmquist, J. K., Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol* **2006,** *494* (3), 528-48.
65. Huang, H. J.; Zhu, X. C.; Han, Q. Q.; Wang, Y. L.; Yue, N.; Wang, J.; Yu, R.; Li, B.; Wu, G. C.; Liu, Q.; Yu, J., Ghrelin alleviates anxiety- and depression-like behaviors induced by chronic unpredictable mild stress in rodents. *Behav Brain Res* **2017,** *326*, 33-43.
66. Han, Q. Q.; Huang, H. J.; Wang, Y. L.; Yang, L.; Pilot, A.; Zhu, X. C.; Yu, R.; Wang, J.; Chen, X. R.; Liu, Q.; Li, B.; Wu, G. C.; Yu, J., Ghrelin exhibited antidepressant and anxiolytic effect via the p38-MAPK signaling pathway in hippocampus. *Prog Neuropsychopharmacol Biol Psychiatry* **2019,** *93*, 11-20.

67. Chuang, J. C.; Perello, M.; Sakata, I.; Osborne-Lawrence, S.; Savitt, J. M.; Lutter, M.; Zigman, J. M., Ghrelin mediates stress-induced food-reward behavior in mice. *J Clin Invest* **2011,** *121* (7), 2684-92.
68. Wittekind, D. A.; Kluge, M., Ghrelin in psychiatric disorders - A review. *Psychoneuroendocrinology* **2015,** *52*, 176-94.
69. Guo, L.; Niu, M.; Yang, J.; Li, L.; Liu, S.; Sun, Y.; Zhou, Z.; Zhou, Y., GHS-R1a Deficiency Alleviates Depression-Related Behaviors After Chronic Social Defeat Stress. *Front Neurosci* **2019,** *13*, 364.
70. Kanehisa, M.; Akiyoshi, J.; Kitaichi, T.; Matsushita, H.; Tanaka, E.; Kodama, K.; Hanada, H.; Isogawa, K., Administration of antisense DNA for ghrelin causes an antidepressant and anxiolytic response in rats. *Prog Neuropsychopharmacol Biol Psychiatry* **2006,** *30* (8), 1403-7.
71. Furness, J. B.; Hunne, B.; Matsuda, N.; Yin, L.; Russo, D.; Kato, I.; Fujimiya, M.; Patterson, M.; McLeod, J.; Andrews, Z. B.; Bron, R., Investigation of the presence of ghrelin in the central nervous system of the rat and mouse. *Neuroscience* **2011,** *193*, 1-9.
72. Boersma, G. J.; Tamashiro, K. L., Individual differences in the effects of prenatal stress exposure in rodents. *Neurobiol Stress* **2015,** *1*, 100-8.
73. Murgatroyd, C. A.; Pena, C. J.; Podda, G.; Nestler, E. J.; Nephew, B. C., Early life social stress induced changes in depression and anxiety associated neural pathways which are correlated with impaired maternal care. *Neuropeptides* **2015,** *52*, 103-11.
74. Barim, A. O.; Aydin, S.; Colak, R.; Dag, E.; Deniz, O.; Sahin, I., Ghrelin, paraoxonase and arylesterase levels in depressive patients before and after citalopram treatment. *Clin Biochem* **2009,** *42* (10-11), 1076-81.
75. Kluge, M.; Schussler, P.; Schmid, D.; Uhr, M.; Kleyer, S.; Yassouridis, A.; Steiger, A., Ghrelin plasma levels are not altered in major depression. *Neuropsychobiology* **2009,** *59* (4), 199-204.
76. Duman, R. S.; Aghajanian, G. K.; Sanacora, G.; Krystal, J. H., Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants. *Nat Med* **2016,** *22* (3), 238-49.
77. Liu, W.; Ge, T.; Leng, Y.; Pan, Z.; Fan, J.; Yang, W.; Cui, R., The Role of Neural Plasticity in Depression: From Hippocampus to Prefrontal Cortex. *Neural Plast* **2017,** *2017*, 6871089.
78. Hallahan, B.; Newell, J.; Soares, J. C.; Brambilla, P.; Strakowski, S. M.; Fleck, D. E.; Kieseppa, T.; Altshuler, L. L.; Fornito, A.; Malhi, G. S.; McIntosh, A. M.; Yurgelun-Todd, D. A.; Labar, K. S.; Sharma, V.; MacQueen, G. M.; Murray, R. M.; McDonald, C., Structural magnetic resonance imaging in bipolar disorder: an international collaborative mega-analysis of individual adult patient data. *Biol Psychiatry* **2011,** *69* (4), 326-35.
79. Li, N.; Lee, B.; Liu, R. J.; Banasr, M.; Dwyer, J. M.; Iwata, M.; Li, X. Y.; Aghajanian, G.; Duman, R. S., mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* **2010,** *329* (5994), 959-64.
80. Duman, R. S.; Aghajanian, G. K., Neurobiology of rapid acting antidepressants: role of BDNF and GSK-3beta. *Neuropsychopharmacology* **2014,** *39* (1), 233.
81. Chung, H.; Seo, S.; Moon, M.; Park, S., Phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase-3 beta and ERK1/2 pathways mediate protective effects of acylated and unacylated ghrelin against oxygen-glucose deprivation-induced apoptosis in primary rat cortical neuronal cells. *J Endocrinol* **2008,** *198* (3), 511-21.
82. Chen, L.; Xing, T.; Wang, M.; Miao, Y.; Tang, M.; Chen, J.; Li, G.; Ruan, D. Y., Local infusion of ghrelin enhanced hippocampal synaptic plasticity and spatial memory through activation of phosphoinositide 3-kinase in the dentate gyrus of adult rats. *Eur J Neurosci* **2011,** *33* (2), 266-75.
83. Ferri, S. L.; Kreibich, A. S.; Torre, M.; Piccoli, C. T.; Dow, H.; Pallathra, A. A.; Li, H.; Bilker, W. B.; Gur, R. C.; Abel, T.; Brodkin, E. S., Activation of basolateral amygdala in juvenile C57BL/6J mice during social approach behavior. *Neuroscience* **2016,** *335*, 184-94.
84. Arenas, M. C.; Mateos-Garcia, A.; Manzanedo, C.; Rodriguez-Arias, M.; Aguilar, M. A.; Navarrete, F.; Gutierrez, M. S.; Manzanares, J.; Minarro, J., Topiramate increases the rewarding

properties of cocaine in young-adult mice limiting its clinical usefulness. *Psychopharmacology (Berl)* **2016,** *233* (23-24), 3849-3859.

85. Porsolt, R. D.; Bertin, A.; Jalfre, M., Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther* **1977,** *229* (2), 327-36.

86. Opal, M. D.; Klenotich, S. C.; Morais, M.; Bessa, J.; Winkle, J.; Doukas, D.; Kay, L. J.; Sousa, N.; Dulawa, S. M., Serotonin 2C receptor antagonists induce fast-onset antidepressant effects. *Mol Psychiatry* **2014,** *19* (10), 1106-14.

87. Tang, J.; Xue, W.; Xia, B.; Ren, L.; Tao, W.; Chen, C.; Zhang, H.; Wu, R.; Wang, Q.; Wu, H.; Duan, J.; Chen, G., Involvement of normalized NMDA receptor and mTOR-related signaling in rapid antidepressant effects of Yueju and ketamine on chronically stressed mice. *Sci Rep* **2015,** *5*, 13573.

Figure 1: PNS mice showed depression-like behaviors at juvenile and adulthood.

a. At postnatal day 28, PNS mice had significant lower body weight than control group, there was no difference in the body weight between these two groups at postnatal day 60. **b**.PNS mice also demonstrated increased immobility in TST test compared with control group, and this abnormal behavior lasted to postnatal day 60. **c**. PNS group also showed a significant increase in immobility time in FST test both at postnatal day 28 and day 60. **d**. There was no difference in the latency to feed between PNS and control group either at postnatal day 28 or day 60 between two groups in NSF test. **e**. PNS mice had a higher food consumption than control group at postnatal day 28, with this difference disappeared at day 60. Data were presented as mean ± SEM. *$p < 0.05$, n= 13-16.

Figure 2: Crocin alleviated the depression-like behaviors in PNS mice at a dose-dependent manner.

a&b. No difference was found in total distance traveled and time spent in central zone among all groups in the OFT test. **c**. PNS mice treated with vehicle showed increased immobility time compare with control group in TST test, high dose of crocin (40mg/kg) significantly decreased the immobility time in PNS mice, and this effect is similar with ketamine. **d**, In FST test, PNS mice treated with vehicle still maintained increased immobility time, high dose of crocin (40mg/kg) as well as ketamine significantly decreased the elevated immobility time in PNS mice. **e**. Vehicle group showed a decreased preference while 40mg/kg crocin and ketamine significantly reversed the decreased sucrose preference in PNS mice. Data were presented as mean ± SEM. *compared with control, $p < 0.05$; #compared with vehicle group, $p < 0.05$, n= 7-10.

Figure 3: Acute treatment of crocin induced a rapid and long-lasting antidepressant-like effect in PNS mice.

a. PNS mice treated with vehicle still showed an increased immobility time in TST compared with control group, while crocin remarkably reduced the immobility time in PNS mice. **b**. Two hours after treatment, crocin also blocked the elevated immobility time in PNS mice. **c**. 24 hours after crocin treatment, vehicle group still maintained a depressive-like behaviors as shown by the increased immobility time in FST, crocin significantly attenuated the immobility time. **d**. vehicle group had decreased preference for sucrose, crocin increased sucrose preference in PNS mice. Data were presented as mean ± SEM. **e**. 3 days after the acute treatment, PNS mice treated with vehicle showed increased immobility time in FST test, crocin reversed the increased immobility time in PNS mice 3 days after the treatment. **f**. Crocin reversed the decreased sucrose preference in PNS mice 3 days after the treatment. Data were presented as mean ± SEM. *compared with control, $p < 0.05$; #compared with vehicle group, $p < 0.05$, n= 7-10.

Figure 4: Crocin increased the hippocampal expression of synaptic plasticity-associated proteins through the restoration of GHSR-PI3K signaling in PNS mice.

a. PNS mice treated with vehicle showed significantly reduced GHSR levels while crocin significantly increased the GHSR levels in hippocampus of PNS mice. **b**. Crocin also restored the phosphorylation level of PI3K, this effect was similar with ketamine. **c&d**. The phosphorylation levels of AKT and mTOR were also decreased in the vehicle group, crocin and ketamine significantly increased the phosphorylation levels of these two effectors. **e**. Crocin and ketamine significantly reversed the decreased BDNF expression level in PNS mice. **f&g.** Crocin also restored the reduced expression levels of GluR1 and PSD95. **h**. The expression level of

synapsin1 remained unchanged. Data were presented as mean ± SEM. *compared with control, $p < 0.05$; #compared with vehicle group, $p < 0.05$, n= 3-5.

Figure 5: Blockade of GHSR and PI3K abolished the antidepressant-like effect of crocin in PNS mice.

a. PNS mice showed increased immobility time in TST test, with crocin significantly reversed the higher immobility time in PNS mice. JMV2959 alone did not change the increased immobility in PNS mice. However, pretreatment of JMV2959 abolished the antidepressant-like effect of crocin. **b**. Crocin significantly reduced the increased immobility time in PNS mice in FST test and pretreatment of JMV2959 blocked the antidepressant-like effect of crocin while JMV2959 alone did not change this abnormal behavior. **c**. Mice treated with crocin showed a decrease immobility time in FST. PI3K antagonist LY294002 alone did not change the increased immobility time in PNS mice. However, pretreatment of LY294002 attenuated the reduced immobility time induced by crocin. **d**. In FST test, crocin significantly decresed the immobility time in PNS mice ($p<0.05$), and pretreatment of LY294002 abolished the antidepressant-like effect of crocin in FST test ($p<0.05$). Data were presented as mean ± SEM. *compared with vehicle group, $p < 0.05$; #compared with crocin alone, $p < 0.05$. Dotted lines showed the mean value of control group, n= 8-10.

Figure 6: Blockade of GHSR abolished the enhanced the hippocampal expression of synaptic plasticity-associated proteins induced by crocin in PNS mice.

a. Crocin restored the lower phosphorylation level of PI3K in PNS mice. GHSR antagonist JMV2959 reversed the enhanced effect of crocin on phosphorylation level of PI3K. **b&c**. Crocin increased the phosphorylation levels of AKT and mTOR while the inhibition of GHSR

significantly abolished this effect. **d**. Inhibition of GHSR decreased the enhanced expression level of BDNF in PNS mice treated with crocin, suggesting a decrease expression of synaptic plasticity-associated proteins in the hippocampus. **e**. The expression level of PSD95 was increased after the crocin treatement, while GHSR inhibitor reversed the enhanced the expression of synaptic plasticity-associated proteins of crocin treatment. Data were presented as mean ± SEM. *compared with vehicle group, $p < 0.05$; #compared with crocin alone, $p < 0.05$. Dotted lines showed the mean value of control group, n= 3-5.

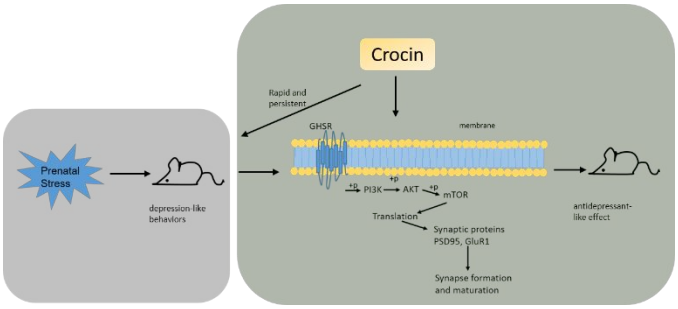
Rapid and prolonged antidepressant-like effect of crocin is associated with GHSR mediated hippocampal plasticity-related proteins in mice exposed to prenatal stress

Ruyan Wu, Ph.D.[1,2]*, Dong Xiao, B.A[3], Xin Shan, B.A[3], Yu Dong, B.A[3], Weiwei Tao, Ph.D.[3]*

[1] School of Medicine, Yangzhou University, Yangzhou, 225000, China

[2] Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, 14203, USA

[3] Key Laboratory of Integrative Biomedicine for Brain Diseases, School of Basic Biomedical Science, Nanjing University of Chinese Medicine, Nanjing, 210023, China



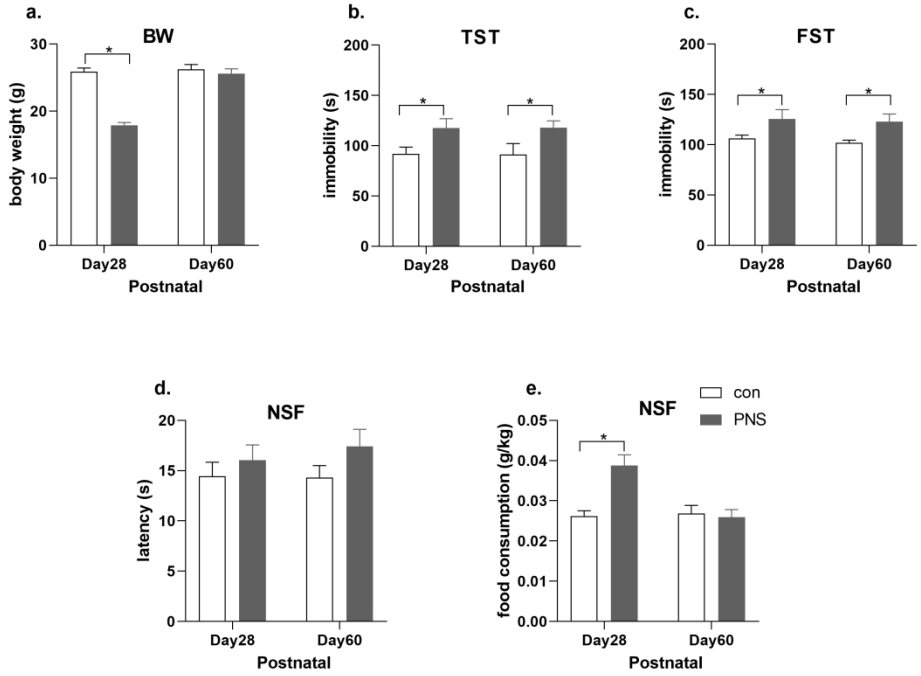


Figure1

252x183mm (300 x 300 DPI)

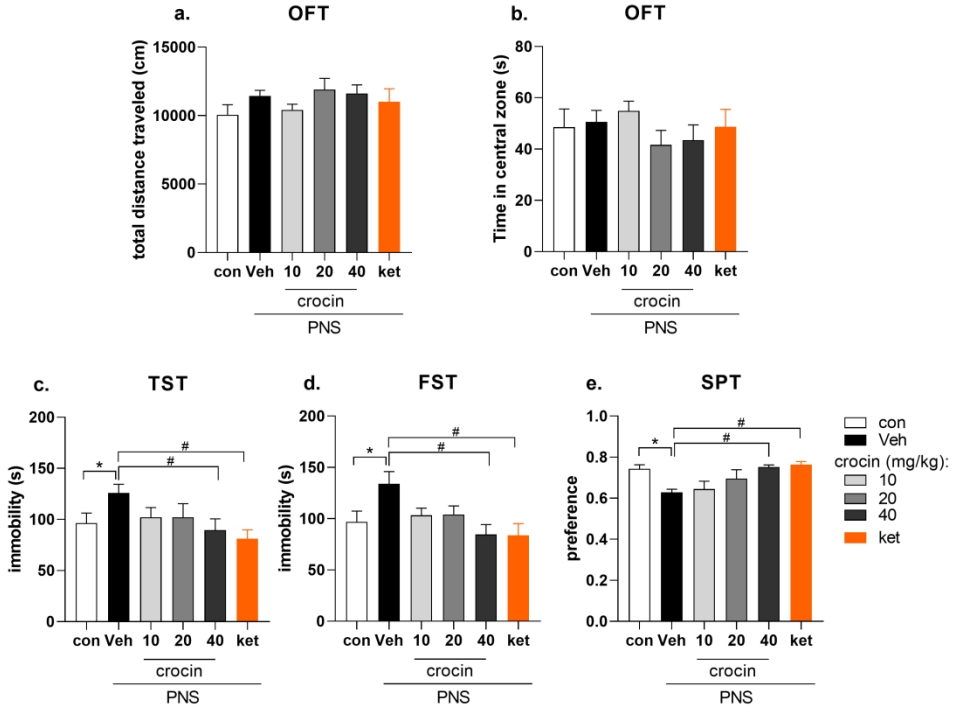


Figure2

277x211mm (300 x 300 DPI)

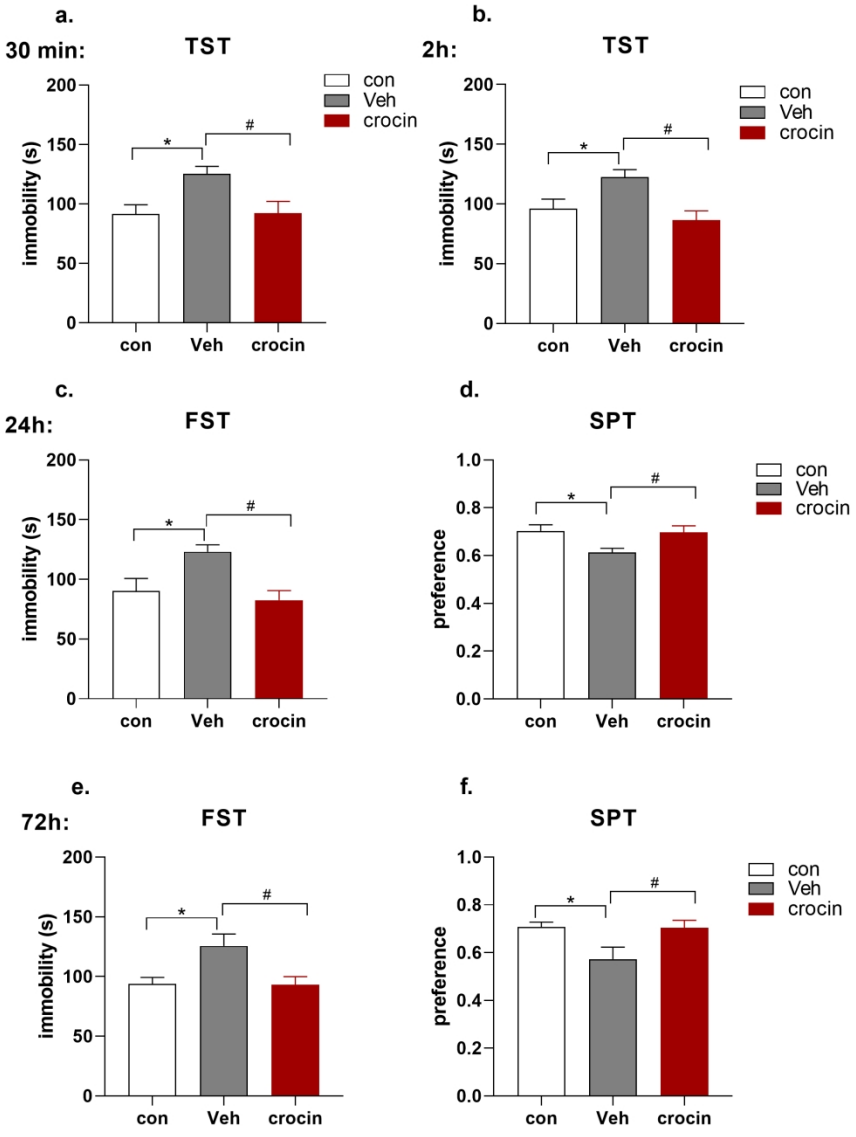


Figure 3

189x248mm (300 x 300 DPI)

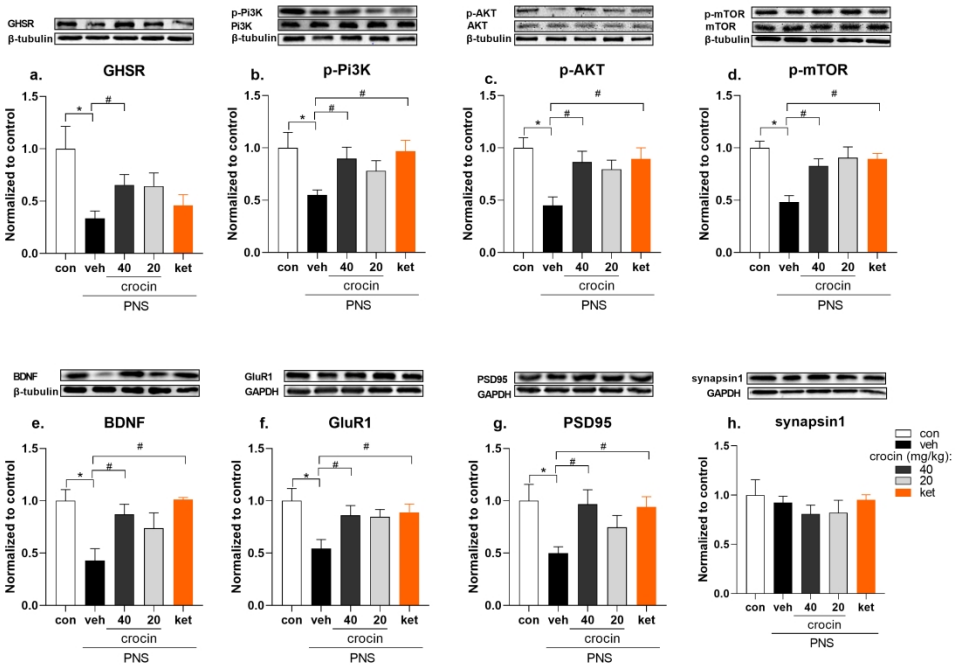


Figure4

274x191mm (300 x 300 DPI)

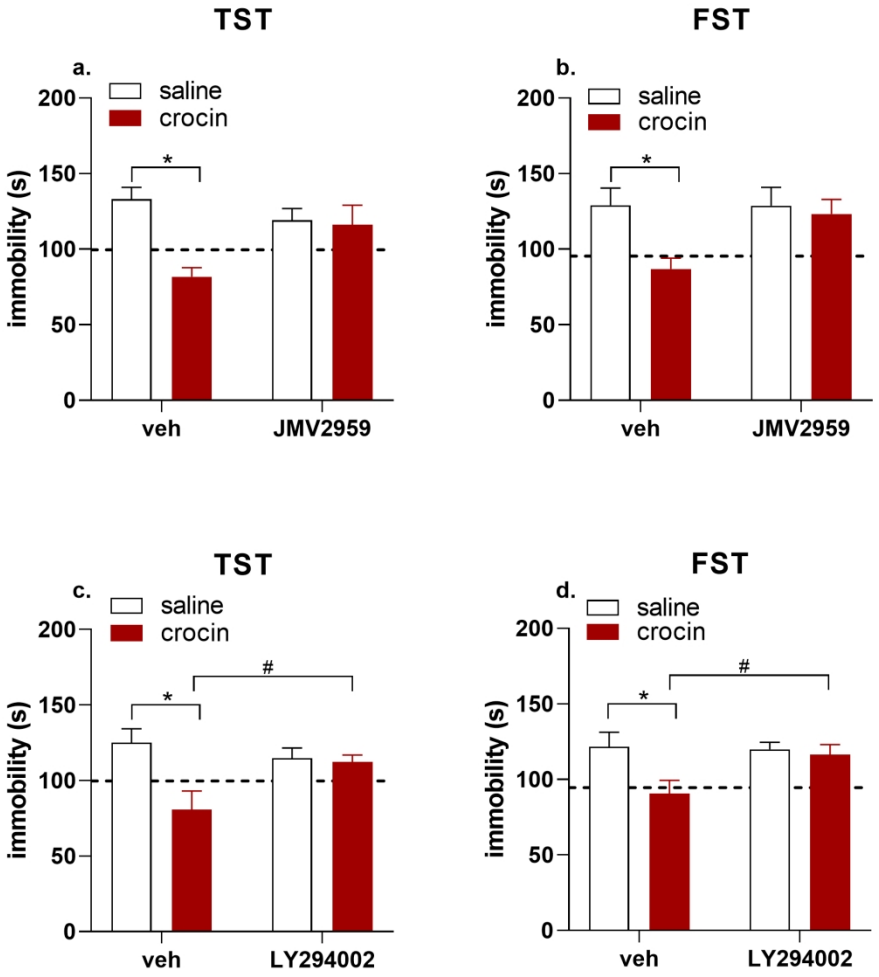


Figure5

185x195mm (300 x 300 DPI)

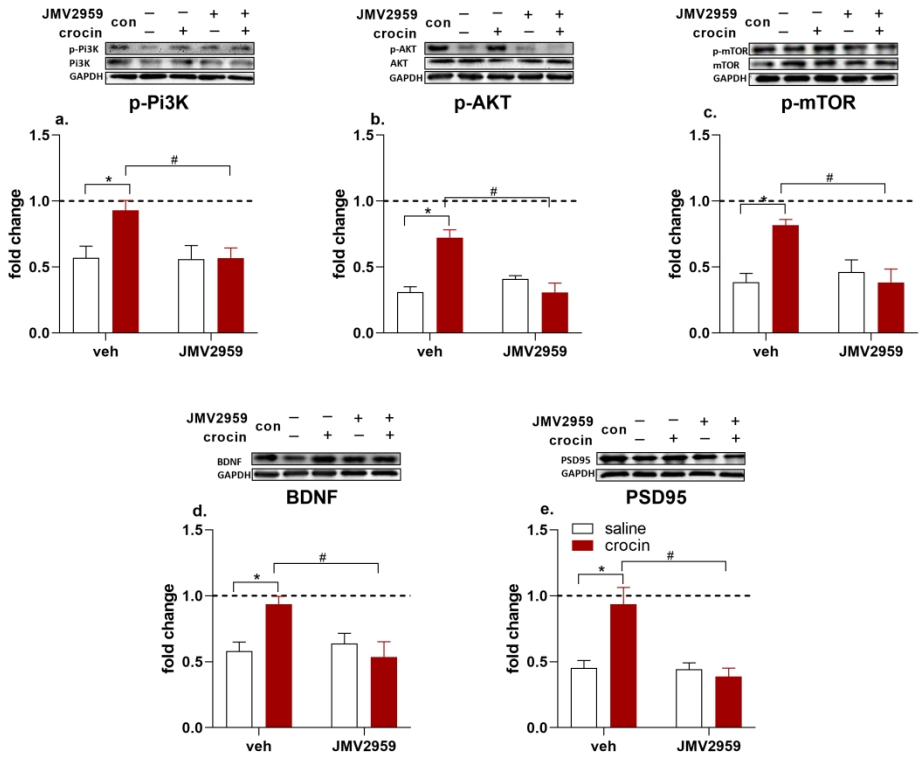


Figure6

256x206mm (300 x 300 DPI)