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Up-regulation of insulin-like growth factor 2 by ketamine requires glycogen synthase kinase-3 inhibition

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Abstract

An antidepressant dose of the rapidly-acting ketamine inhibits glycogen synthase kinase-3 (GSK3) in mouse hippocampus, and this inhibition is required for the antidepressant effect of ketamine in learned helplessness depression-like behavior. Here we report that treatment with an antidepressant dose of ketamine (10 mg/kg) increased expression of insulin-like growth factor 2 (IGF2) in mouse hippocampus, an effect that required ketamine-induced inhibition of GSK3. Ketamine also inhibited hippocampal GSK3 and increased expression of hippocampal IGF2 in mice when administered after the induction of learned helplessness. Treatment with the specific GSK3 inhibitor L803-mts was sufficient to up-regulate hippocampal IGF2 expression. Administration of IGF2 siRNA reduced ketamine's antidepressant effect in the learned helplessness paradigm. Mice subjected to the learned helplessness paradigm were separated into two groups, those that were resilient (non-depressed) and those that were susceptible (depressed). Non-depressed resilient mice displayed higher expression of IGF2 than susceptible mice. These results indicate that IGF2 contributes to ketamine's antidepressant effect and that IGF2 may confer resilience to depression-like behavior.

Keywords

ketamine; glycogen synthase kinase-3; insulin-like growth factor 2; depression; hippocampus; learned helplessness

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Disclosure of Interest

The authors report no conflict of interest.

1. Introduction

Ketamine treatment induces a rapid antidepressant effect in depressed patients with mood disorders (Newport et al., 2015; Niciu et al., 2014; Scheuing et al., 2015). The mechanism that underlies this action is currently unknown. In mice, ketamine's antidepressant effect in the learned helplessness model is dependent on its inhibition of glycogen synthase kinase-3 (GSK3) (Beurel et al., 2011). GSK3 refers to two isoforms that are primarily regulated by inhibitory phosphorylation on ser-21-GSK3 α and ser-9-GSK3 β , and this inhibitory phosphorylation of both GSK3 isoforms in mouse brain is increased following ketamine administration (Beurel et al., 2015). Ketamine also increased the serine-phosphorylation of GSK3 in lymphocytes of patients with depression (Yang et al., 2013). In GSK3 knockin mice the serines are mutated to alanines to prohibit this major mechanism of GSK3 inhibition, leaving GSK3 constitutively active (McManus et al., 2005). Ketamine treatment fails to induce an antidepressant effect in GSK3 knockin mice, demonstrating the necessity for ketamine-induced inhibitory serine-phosphorylation of GSK3 for this effect (Beurel et al., 2011). Further studies showed that administration of GSK3 inhibitors enhanced the antidepressant effects of ketamine (Chiu et al., 2014; Ghasemi et al., 2010; Liu et al., 2013). Thus, ketamine-induced inhibition of GSK3 is linked to its antidepressant action, but it remains unknown how this contributes to the antidepressant effects of ketamine (Zunszain et al., 2013).

Several reports have implicated insulin-like growth factor 2 (IGF2) in regulating depression and actions of antidepressants. IGF2 is one of the most abundant growth factors present in cerebrospinal fluid (Åberg et al., 2015; Tham et al., 1993), and IGF2 is expressed and binds receptors throughout the human hippocampus (Caracausi et al., 2016; Wilczak et al., 2000). Variability in the regulation of IGF2 expression has been associated with depression status in adult monozygotic twins (Córdova-Palomera et al., 2015). In rodents, antidepressant treatments up-regulate IGF2 in the hippocampus (Cline et al., 2012; Lisowski et al., 2013) and other brain regions (Lauterio et al., 1993), and IGF2 is down-regulated in the hippocampus in rodent models exhibiting depression-like behaviors (Andrus et al., 2012; Luo et al., 2015). Hippocampal overexpression of IGF2 ameliorated sucrose consumption and immobility time in the forced swim test displays of depression-like behaviors induced by chronic restraint stress in rats (Luo et al., 2015). Administration of IGF2 also increased neurogenesis in the hippocampus, a process that may contribute to the action of antidepressants (Bracko et al., 2012; Chen et al., 2007; Ferrón et al., 2015; Kita et al., 2014). In vivo administration of IGF2 in the hippocampus increased the expression of several neurotrophins and growth factors, such as brain-derived neurotrophic factor (BDNF) (Mellott et al., 2014). Thus, diminished IGF2 may contribute to susceptibility to depression and increased IGF2 is linked to antidepressant responses.

Here we report that administration of an antidepressant dose of ketamine up-regulated IGF2 expression in mouse hippocampus, and that this requires inhibition of GSK3. Furthermore, administration of a specific GSK3 inhibitor was sufficient to up-regulate hippocampal IGF2 expression. In mice with GSK3 activation induced by the learned helplessness paradigm, ketamine restored GSK3 inhibition and increased expression of IGF2. Administration of IGF2 siRNA reduced ketamine's antidepressant effect in the learned helplessness paradigm,

and mice that were resilient to learned helplessness displayed higher hippocampal expression of IGF2 than control mice. Thus, increased hippocampal IGF2 contributes to ketamine's antidepressant effect and may contribute to resilience or susceptibility of mice to depression-like behavior.

2. Methods

2.1. Mice

Adult (8-12 weeks of age) male C57BL/6 wild-type or homozygous GSK3 α / β ^{21A/21A/9A/9A} knockin mice (McManus et al., 2005) were used. Adult female C57BL/6 mice (8-12 weeks of age) were used where indicated. GSK3 knockin mice reproduce and develop normally with no overt phenotypes (Eom and Jope, 2009; McManus et al., 2005; Polter et al., 2010). Mice were housed in standard cages in a temperature and light controlled room. Mice were treated in accordance with the regulations of the National Institutes of Health and the University of Miami Institutional Animal Care and Use Committee. Mice were injected intraperitoneally (i.p.) with saline or ketamine (10 mg/kg; Vedco Inc.). Mice were treated intranasally with L803-mts, a substrate-competitive peptide GSK3 inhibitor (Plotkin et al., 2003), (60 μ g/mouse; 24 hr pretreatment; produced in the Eldar-Finkelman laboratory) diluted in DDX1 vehicle (128 mM NaCl, 8 mM citric acid, 17 mM Na₂HPO₄, 0.0005% benzalkonium chloride) as described previously (Beurel et al., 2013; Kaidanovich-Beilin et al., 2004). Mice were treated with IGF2 siRNA 2 hr after ketamine treatment (10 mg/kg; i.p.) and again 2 hr before exposure to escapable foot shocks (10 μ g/mouse/treatment in DDX1; intranasal; 5 μ L/nostril; #J-043709-09, GE Healthcare Dharmacon, Inc). Scrambled siRNA (AM4642; Ambion) was used as control.

2.2. Western Blotting

The hippocampus was rapidly dissected in ice-cold phosphate-buffered saline and stored at -80°C after being snap-frozen. Brain regions were homogenized in Triton lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-100, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 nM okadaic acid. Tissue lysates were centrifuged at 14000 rpm for 10 min at 4°C to remove the insoluble fraction. The Bradford protein assay was used to determine the concentration of protein in the supernatants. Hippocampal proteins (5-20 μ g) were resolved with SDS-PAGE, transferred to nitrocellulose membranes and then immunoblotted. Antibodies used were to phospho-Ser9-GSK3 β (#9336, Cell Signaling Technology), total GSK3 β (#610202, BD Transduction Laboratories), phospho-Ser21-GSK3 α (#9316, Cell Signaling Technology) and total GSK3 α / β (clone 4G-1E, #05-412, Millipore). The membranes were reblotted with β -actin (#A5441, Sigma Aldrich) to ensure equal protein loading.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Proteins from hippocampal extracts were prepared as described above, and IGF2 ELISAs were performed according to the manufacturer's instructions (RayBiotech) using 100 μ g protein.

2.4. Quantitative Real-Time Polymerase Chain Reaction

Total RNA from mouse hippocampus or prefrontal cortex was isolated using TRIzol extraction according to the manufacturer's instructions (Invitrogen). RNA was converted to cDNA using the ImProvII reverse transcriptase (Promega) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green master mix, according to the manufacturer's instructions (Applied Biosystems), and the primers described below. Quantification of cDNAs was made using the 2^{-C_t} method. The primers for IGF2 were: (Forward-TGTGCTGCATCGCTGCTTAC; Reverse-CGGTCCGAACAGACAAACTGA). The primers used for GAPDH, which was used as housekeeping gene, were: (Forward-AGGTCGGTGTGAACGGATTTG; Reverse-TGTAGACCATGTAGTTGAGGTCA). Experiments were performed on a 7900 HT Fast instrument (Applied Biosystems).

2.5. Learned Helplessness

Learned helplessness depression-like behavior was measured as described previously (Beurel et al., 2013; Polter et al., 2010). Mice were placed inside the Modular Shuttle Box (Med Associates Inc., St. Albans, VT, USA), with the gate between the chambers in the closed position. A total of 180 inescapable foot shocks were given at an amplitude of 0.3 mA at random durations of 6-10 sec. Random inter-shock intervals were 5-45 sec. Learned helplessness was tested 24 hr later by exposing mice to 30 trials of escapable 0.3 mA foot shocks that lasted for a maximum of 24 sec if the mouse did not escape, and inter-shock intervals of 30 s. The latency to escape from these shocks was acquired using MED-PC® Data Acquisition Software. Escape failures were tallied when mice did not escape the shock within 24 sec, and greater than 15 escape failures out of the 30 trials was defined as learned helpless behavior.

2.6. Statistical Analysis

Statistical significance was analyzed with the Student's *t* test, one- or two-way ANOVA with a Bonferroni post-hoc test as indicated or Kruskal-Wallis Test with a Dunn's post hoc test, or a Chi-square test, and $p < 0.05$ was considered significant.

3. Results

3.1. Ketamine treatment increases expression of IGF2 in hippocampus

IGF2 mRNA expression was measured in the hippocampus of male mice 0.5, 12, 24, and 48 hr after administration of a sub-anesthetic, antidepressant dose of ketamine (10 mg/kg; i.p.). There was an up-regulation of IGF2 mRNA 24 hr after ketamine treatment (2.75 ± 0.2 -fold of control levels), which then returned to control levels by 48 hr, indicating that ketamine induces a transient increase in IGF2 (one-way ANOVA; $F_{(4,32)}=3.070$; Bonferroni post-hoc test, $*p < 0.05$, compared to saline-treated mice; Figure 1A). Female mice had higher basal levels of hippocampal IGF2 mRNA than male mice (Student's *t* test; $t_{(22)}=2.772$, $*p < 0.05$; Figure 1B), but also exhibited an increase in IGF2 mRNA levels 24 hr after ketamine treatment (Student's *t* test; $t_{(12)}=2.658$, $*p < 0.05$; Figure 1C). IGF2 protein levels were also significantly increased in the hippocampus of male mice 24 hr after ketamine administration (Student's *t* test; $t_{(8)}=2.622$, $*p < 0.05$; Figure 1D). Basal hippocampal IGF2 mRNA levels

were equivalent in male wild-type and GSK3 knockin mice. However, the increase level of IGF2 mRNA in response to ketamine was absent 24 hr after ketamine administration in GSK3 knockin mice (two-way ANOVA (genotype X treatment); $F_{\text{interaction}(1,24)}=6.659$; Bonferroni post-hoc test, $*p<0.05$, compared to saline-treated wild-type mice; Figure 1E). This demonstrates the necessity for ketamine-induced inhibitory serine-phosphorylation of GSK3 for the up-regulation of IGF2 expression. Furthermore, inhibition of GSK3 was sufficient to up-regulate IGF2 expression, as administration of the selective GSK3 inhibitor L803-mts (60 μg ; intranasal; 24 hr) increased IGF2 levels in the hippocampus of male wild-type mice (Student's t test; $t_{(5)}=2.728$, $*p<0.05$; Figure 1F). Ketamine did not increase IGF2 mRNA expression in prefrontal cortex (Figure 1G), which had similar basal IGF2 mRNA expression as the hippocampus (Figure 1H).

3.2. Ketamine, IGF2 and learned helplessness depression-like behavior

We also tested if administration of ketamine inhibits GSK3 and up-regulates IGF2 mRNA in mice after induction of learned helplessness. Mice were exposed to inescapable foot shocks and 24 hr later learned helplessness was tested by determining the number of escape failures. If mice failed to escape from >15 out of the 30 trials, they are considered learned helpless. Two days after escapable shock treatment, to allow normalization of acute effects of the foot shocks, learned helpless mice were treated with ketamine (10 mg/kg; i.p.), and sacrificed 24 hr later. Hippocampal serine-phosphorylated GSK3 α and GSK3 β levels were lower in learned helpless mice compared to control mice that did not receive the foot shocks, and the serine-phosphorylation of both GSK3 α and GSK3 β were restored to control levels by ketamine treatment (one-way ANOVA; $F_{(2,21)}=4.694$, Bonferroni post-hoc test, $*p<0.05$, compared to naïve control mice or depressed saline-treated mice; Figure 2A; and one-way ANOVA; $F_{(2,21)}=7.287$, Bonferroni post-hoc test, $*p<0.05$, compared to control mice or depressed saline-treated mice; Figure 2B). Ketamine treatment also up-regulated IGF2 mRNA (2.70 ± 0.5 -fold of control levels) in learned helpless mice to **the same extent (2.7-fold)** as was observed in mice not exposed to the learned helplessness paradigm (one-way ANOVA; $F_{(2,23)}=4.706$, Bonferroni post-hoc test, $*p<0.05$, compared to control non-shocked mice; Figure 2C). To determine if the antidepressant effect of ketamine is dependent on IGF2 up-regulation, we tested if knocking down IGF2 using IGF2 siRNA reduced ketamine's antidepressant effect in the learned helplessness paradigm. 100% of vehicle-treated mice developed learned helplessness, whereas only 36% of the mice exhibited learned helplessness after ketamine-treatment. In contrast, pretreatment with IGF2 siRNA blocked the antidepressant effect of ketamine, resulting in 69% of mice developing learned helplessness, demonstrating that ketamine's antidepressant effect was blocked by IGF2 siRNA administration (Chi-square; $X^2_{(2)}=10.34$; $*p<0.05$; Figure 2D).

The learned helplessness paradigm was used to separate mice into two groups, those that were resilient (non-depressed) and those that were susceptible (depressed) to learned helplessness (Figure 3A). Mice were exposed to inescapable shocks, 24 hr later they were tested with escapable shocks, and mice were sacrificed 1 hr after the last escapable shock. IGF2 mRNA levels were significantly higher in the hippocampus of non-depressed resilient mice compared to control non-shocked mice or depressed mice (Kruskal-Wallis test, Dunn's post-hoc test, $*p<0.05$, compared to untreated control mice or depressed mice; Figure 3),

whereas the level of IGF2 in the depressed mice was similar to the level of IGF2 in the control non-shocked mice. This raises the possibility that IGF2 contributes to resilience of mice to learned helplessness.

4. Discussion

Ketamine can induce rapid antidepressant effects in depressed patients with mood disorders (Newport et al., 2015; Niciu et al., 2014; Scheuing et al., 2015) and the antidepressant mechanism of action of ketamine is linked to GSK3 inhibition (Beurel et al., 2011; Chiu et al., 2014; Ghasemi et al., 2010; Liu et al., 2013; Yang et al., 2013; Zhou et al., 2014). Here we found that up-regulation of IGF2 expression in mouse hippocampus appears to contribute to the GSK3 inhibition-dependent antidepressant effect of ketamine in mice. Ketamine-induced up-regulation of hippocampal IGF2 expression was evident in both male and female mice, but female mice had higher basal IGF2 expression in the hippocampus than male mice, which may be related to the reported up-regulation of IGF2 expression by estrogen (Takeo et al., 2008; Sarvari et al., 2015; Sarvari et al., 2016). Furthermore, inhibition of GSK3 is sufficient to up-regulate IGF2 expression, and increased IGF2 may be associated with mice that are resilient to learned helplessness depression-like behavior.

In response to ketamine, the up-regulated expression of IGF2 was linked to GSK3-inhibition by two findings. First, up-regulation of IGF2 by ketamine did not occur in GSK3 knockin mice, in which GSK3 cannot be inhibited (McManus et al., 2005). Second, administration of the specific GSK3 inhibitor L803-mts was sufficient to increase IGF2 expression in the hippocampus to a similar extent as ketamine treatment. These results demonstrate that inhibition of GSK3 is both necessary and sufficient to up-regulate the expression of IGF2 in the hippocampus.

Several approaches were used to determine if up-regulation of IGF2 contributes to the antidepressant response to ketamine. Ketamine induced up-regulation of IGF2 expression in the hippocampus coincides with (24 hr) ketamine's antidepressant actions in mice (Autry et al., 2011; Beurel et al., 2011; Li et al., 2010; Maeng et al., 2008; Zanos et al., 2016). In mice that were first rendered learned helpless and subsequently treated with ketamine, an experimental condition that models the clinical situation, IGF2 expression was increased by ketamine treatment similarly to that occurring in mice treated with ketamine alone without being subjected to learned helplessness. Finally, IGF2 siRNA administration significantly reduced ketamine's antidepressant efficacy. Taken together these results demonstrate that IGF2 expression is up-regulated by ketamine and contributes to ketamine's antidepressant effect in mice.

In response to foot shocks, some mice, but not all, develop learned helpless behavior, although they are from the same litter, with some mice being resilient and others susceptible to developing depression-like behaviors (McEwen et al., 2015). Comparison of IGF2 expression in these two cohorts of mice exposed to the learned helplessness paradigm revealed that hippocampal IGF2 expression was highest in mice that were resilient to learned helplessness, suggesting that the level of IGF2 expression may predict resilience.

5. Conclusion

This study found that ketamine up-regulates IGF2 in mouse hippocampus, this was dependent on GSK3-inhibition and was matched by administration of a specific GSK3 inhibitor. Ketamine also induced inhibition of GSK3 and up-regulated IGF2 expression in learned helpless mice. Administration of IGF2 siRNA significantly reduced ketamine's antidepressant effects, altogether indicating that up-regulation of IGF2 contributes to ketamine's antidepressant effect. Furthermore, increased IGF2 expression was linked to resilience in mice. Altogether these findings indicate that IGF2 contributes to ketamine's antidepressant effects in mice.

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Highlights

1. An antidepressant dose of ketamine up-regulates IGF2 in mouse hippocampus
2. GSK3 inhibition is necessary and sufficient for up-regulation of IGF2
3. Ketamine inhibits activated GSK3 and increases IGF2 expression in depressed mice
4. IGF siRNA blocks ketamine's antidepressant efficacy in learned helplessness
5. IGF2 levels may differentiate resilient and susceptible mice to learned helplessness

Figure 1. Ketamine treatment up-regulates hippocampal IGF2 expression

(A) IGF2 mRNA levels in hippocampus 30 min (n=4), 12 hr (n=6), 24 hr (n=10), and 48 hr (n=5) after ketamine treatment (open bars; 10 mg/kg; i.p.) compared with saline (Sal) treatment (n=12) in male wild-type mice. (B) IGF2 mRNA levels in hippocampus from untreated male and female wild-type mice (n=12). (C) IGF2 mRNA levels in hippocampus 24 hr after administration of ketamine (Ket) in female wild-type mice (n=6-8). (D) IGF2 protein levels in hippocampus 24 hr after administration of ketamine in male wild-type mice (n=5). (E) IGF2 mRNA levels in hippocampus 24 hr after administration of ketamine or saline in male wild-type and GSK3 knockin (KI) mice (n=6-8). (F) IGF2 protein levels in hippocampus 24 hr after intranasal administration of L803-mts (60 μ g) in male wild-type mice (n=3-4). (G) IGF2 mRNA levels in prefrontal cortex (PFC) 24 hr after administration of ketamine or saline in male wild-type mice (n=4). (H) IGF2 mRNA levels in hippocampus (HC) and PFC in male wild-type mice (n=4). Data represent Means $\pm$ SEM, *p<0.05.

Figure 2. In learned helpless mice ketamine restores GSK3 inhibition and up-regulates IGF2 mRNA

Learned helplessness was induced in male wild-type mice (Depressed) and two days later saline (Sal) or ketamine (Ket; 10 mg/kg; i.p.) were administered followed 24 hr later by measurements in the hippocampus of (A) serine9-phosphorylated GSK3 β and total GSK3 β (n=6 controls, n=6 saline-treated, n=12 ketamine-treated), (B) serine21-phosphorylated GSK3 α and total GSK3 α (n=6 controls, n=6 saline-treated, n=12 ketamine-treated), and (C) IGF2 mRNA levels (n=7 controls, n=7 saline-treated, n=12 ketamine-treated). Non-shocked mice were used as control (Ctl). (D) Escape failures of mice treated with vehicle plus a scrambled siRNA (n=11), ketamine plus a scrambled siRNA (n=11), or ketamine plus an IGF2 siRNA (n=13). Each symbol represents an individual mouse. The dashed bar at 15 escape failures indicates the demarcation between mice that are defined as learned helpless (depressed) or not. Data represent Means $\pm$ SEM, *p<0.05.

Figure 3. Elevated hippocampal IGF2 mRNA in male mice is associated with resistance to learned helplessness

(A) Escape failures in the learned helplessness paradigm. Each point represents the number of failures to escape for a single mouse. The dashed bar at 15 escape failures indicates the demarcation between mice that are defined as learned helpless (depressed; **n=7**) or not (**n=10**). Mice were divided into two groups, those that were resistant to learned helplessness (non-depressed; ND) and those that developed learned helplessness (depressed; D). (B) IGF2 mRNA levels in the hippocampus 1 hr after the last escapable foot shocks (**n=6-9**). Data represent Means $\pm$ SEM, * $p<0.05$. Non-shocked mice were used as control (Ctl).