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Ketamine regulates the presynaptic release machinery in the hippocampus

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Abstract

In the search for new drug targets, that may help point the way to develop fast-acting treatments for mood disorders, we have explored molecular pathways regulated by ketamine, an NMDA receptor antagonist, which has consistently shown antidepressant response within a few hours of administration. Using Sprague-Dawley rats we investigated the effects of ketamine on the presynaptic release machinery responsible for neurotransmitter release at 1, 2 and 4 h as well as 7 days after administration of a single subanesthetic dose of ketamine (15 mg/kg). A large reduction in the accumulation of SNARE complexes was observed in hippocampal synaptic membranes after 1, 2 and 4 h of ketamine administration. In parallel, we found a selective reduction in the expression of the synaptic vesicle protein synaptotagmin I and an increase in the levels of synapsin I in hippocampal synaptosomes suggesting a mechanism by which ketamine reduces SNARE complex formation, in part, by regulating the number of synaptic vesicles in the nerve terminals. Moreover, ketamine reduced Thr²⁸⁶-phosphorylated α CaMKII and its interaction with syntaxin 1A, which identifies CaMKII as a potential target for second messenger-mediated actions of ketamine. In addition, despite previous reports of ketamine-induced inhibition of GSK-3, we were unable to detect regulation of its activity after ketamine administration. Our findings demonstrate that ketamine rapidly induces changes in the hippocampal presynaptic machinery similar to those that are obtained only with chronic treatments with traditional antidepressants. This suggests that reduction of neurotransmitter release in the hippocampus has possible relevance for the rapid antidepressant effect of ketamine.

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Contributors

Authors Heidi K. Müller, Gregers Wegener, Carlos A. Zarate Jr, Maurizio Popoli, and Betina Elfving conceived and designed the study. Heidi K. Müller, Nico Liebenberg and Betina Elfving performed the experiments, assembled, analyzed and interpreted the data. Maurizio Popoli, Carlos A. Zarate Jr and Gregers Wegener contributed to the analysis and interpretation of data. All authors contributed to the writing of the article and have approved the final manuscript.

Conflict of interest

Dr. Zarate is listed as a co-inventor on a patent application for the use of ketamine and its metabolites in major depression. Dr. Zarate has assigned his rights in the patent to the U.S. government but will share a percentage of any royalties that may be received by the government. All other authors declare no financial or other conflict of interest relevant to the subject of this article.

Keywords

Ketamine; SNARE; Synapsin; Synaptotagmin; CaMKII; Antidepressant

1. Introduction

The monoamine theory of depression has contributed greatly to our understanding of mood disorders and has been essential for the development of effective antidepressants. However, the existing antidepressants usually have a delayed therapeutic effect of several weeks, which strongly argues for the need to develop new therapeutics with a more rapid onset of action. Agents targeting the glutamatergic system have shown great promise for the treatment of mood disorders (Hashimoto, 2009). Notably, diverse preclinical and clinical studies have demonstrated that NMDA (*N*-methyl-D-aspartate) receptor antagonists produce rapid antidepressant effects. Ketamine, a non-competitive NMDA receptor antagonist, has become a major focus of research in the search for molecular mechanisms associated with a rapid antidepressant response. In humans, a single low dose of ketamine produces a rapid antidepressant response within hours of administration. This response can last for up to at least 7 days and is effective in patients resistant to traditional anti-depressants (Berman et al., 2000; Zarate et al., 2006). Acute administration of ketamine also induces rapid and sustained antidepressant-like effects in animal behavioral models of depression (Autry et al., 2011; Garcia et al., 2008; Li et al., 2010, 2011; Maeng et al., 2008). Unfortunately, ketamine can cause symptoms that mimic psychosis and cognitive impairment, limiting its potential for widespread clinical use. However, the high efficacy and rapid action of ketamine in alleviating depressive symptoms have prompted researchers to explore the molecular and cellular mechanisms associated with its antidepressant effect. Increased AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) relative to NMDA glutamatergic throughput and increased synaptic plasticity and BDNF (brain-derived neurotrophic factor) signaling have been reported to constitute important mechanisms underlying the rapid antidepressant effect of ketamine (Autry et al., 2011; Maeng et al., 2008; Li et al., 2010). These mechanisms are also involved in the therapeutic effect after chronic treatment with monoamine-based antidepressants (Pittenger and Duman, 2008) and thus, could suggest a final common pathway for the action of ketamine and structurally unrelated antidepressants. Using microdialysis, ketamine has been reported to increase the extracellular levels of glutamate in the prefrontal cortex of rodents (Lorrain et al., 2003; Moghaddam et al., 1997) leading to the hypothesis that hyperglutamatergic mechanisms are involved in the antidepressant effect of ketamine. However, the mechanism underlying the increase in extracellular glutamate levels and whether this effect is restricted to certain brain regions is not known.

Neurotransmitter release requires the assembly of the core SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex, consisting of the plasma membrane associated proteins syntaxin 1A and SNAP25 (synaptosomal-associated protein 25 kDa) and the synaptic vesicle-associated protein VAMP2 (vesicle-associated membrane protein 2) (Südhof, 2004). A number of preclinical studies have demonstrated changes in gene and protein expression of SNAREs and their regulatory proteins after stress or conventional antidepressant treatment (Bonanno et al., 2005; Elfving et al., 2008; Gao et al., 2006; Musazzi et al., 2010; Rapp et al., 2004; Yamada et al., 2002). Postmortem studies also indicate abnormalities in the expression of individual SNARE proteins and regulatory proteins in the hippocampus and frontal cortex of subjects with depression and bipolar disorder (Fatemi et al., 2001; Scarr et al., 2006). This suggests that mechanisms associated with neurotransmitter release are involved in depressive disorders and may represent important molecular targets for antidepressant treatment.

The idea that structurally non-related antidepressants may have converging mechanisms of actions with relevance for the time of their onset of action prompted us to investigate whether acute administration of ketamine targets the presynaptic molecular machinery similar to what has been shown after chronic but not acute treatment with conventional antidepressants.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 330–400 g (Taconic MB, Denmark) were pair-housed and maintained in standard conditions with a 12-h light/dark cycle and free access to food and water. All animal procedures were in accordance with protocols approved by the Danish National Committee for Ethics in Animal Experimentation (2007/561-1378). All possible measures to minimize suffering and stress were taken during the experiments.

2.2. Forced swim test

The modified forced swim test (FST) protocol was used as previously described (Cryan et al., 2002). On the first day of the experiment, rats were placed in acrylic plastic cylinders (60 cm in height, 24 cm in diameter) containing 40 cm of water (24 °C) for 15 min. Twenty-four hours later, the animals were reintroduced to the swim cylinders and their behavior recorded digitally for 7 min. Saline or ketamine (15 mg/kg; S-ketamine 25 mg/ml, Pfizer, CT, USA) were administered intraperitoneally 2 h prior to the final swim session. Animals were scored offline for displaying immobile behavior, defined as making only the necessary movements to keep their heads above the water. The FST was carried out 3 h following the start of the dark cycle under red light illumination.

2.3. Preparation of crude synaptosomes and synaptosomal membranes

During the light period of the cycle, groups of 5 animals received a single acute intraperitoneal injection of saline (control) or ketamine (15 mg/kg; S-ketamine 25 mg/ml, Pfizer, CT, USA) and were killed by decapitation at different time points after drug administration: control (1 h), ketamine (1 h, 2 h, 4 h and 7 days) (Fig. 2–4). In another batch of Sprague-Dawley rats three groups of 5 animals received a single acute intraperitoneal injection of saline, ketamine (15 mg/kg) or desipramine (10 mg/kg; Sigma, MO, USA) and were decapitated after 2 h (Fig. 5 and 6). To purify crude synaptosomes, hippocampi were dissected and homogenized in 10% (w/v) ice-cold buffer containing 0.32 M sucrose, 20 mM HEPES pH 7.4, 1 mM EDTA, 1× protease inhibitor cocktail (Roche, Mannheim, Germany), 5 mM NaF, 1 mM Na₃VO₄, and 5 mM Na₂PO₄. After centrifugation of the homogenate at 800g for 10 min at 4 °C, the supernatant was centrifuged at 12,000g for 10 min. The resultant pellet, designated the crude synaptosomal fraction, was either resuspended in synaptosomal lysis buffer containing 120 mM NaCl, 20 mM HEPES pH 7.4, 0.1 mM EGTA, 0.1 mM DTT, 1× protease inhibitor cocktail, 10 mM NaF, 1 mM Na₃VO₄, and 5 mM Na₂PO₄ to a final protein concentration of 2 µg/µl or lysed hypo-osmotically in 20 mM HEPES, 1× Proteinase Inhibitor cocktail, 20 mM NaF, 5 mM Na₂PO₄, 1 mM Na₃VO₄ and centrifuged at 29,000g for 30 min at 4 °C for further purification of the synaptosomal membrane fraction (LP1).

2.4. Immunoblotting

Aliquots of synaptosomal fractions were processed and analyzed by standard immunoblotting as previously described (Müller et al., 2011). The SDS-resistant SNARE complexes were detected in unboiled samples using an antibody against syntaxin 1A and normalized to the monomeric syntaxin 1A within the same lane. The following antibodies

were used: mouse anti-syntaxin 1A (1:1000) (Sigma, MO, USA), rabbit anti-SNAP25 (1:2000), mouse anti-synaptotagmin I (1:1000), and mouse anti-synapsin I (1:1000) (all from Synaptic System, Göttingen, Germany), mouse anti-Munc18 (BD Biosciences, NJ, USA), mouse anti-GAPDH (1:2000) (Covance, NJ, USA), mouse anti-phospho- α CaMKII (Thr286) (1:500), and mouse anti- α CaMKII (1:500) (Millipore, MA, USA), rabbit anti-phospho-GSK-3 β (Ser9) (1:500), rabbit anti-GSK-3 β (1:500) (Cell Signaling, MA, USA) and the horseradish peroxidase conjugated secondary antibodies: anti-rabbit antibody (1:20,000) and anti-mouse antibody (1:2000) (both obtained from Pierce, IL, USA). Immunoreactive bands were detected using ECL Advance Western Blotting Detection Reagent (GE Healthcare, UK) and captured on a KODAK Image Station 440. For the analysis of phospho- and total immunoreactivity within the same blot, the membranes were incubated in 0.1 M glycine solution (pH 2.0) preheated to boiling temperature for 7 min (according to the protocol Glycine Reprobing Protocol using the iBlot Dry Blotting System, Invitrogen). This step was repeated three times with fresh glycine solution. The membranes were washed three times in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) and blocked with 5% dry milk in TBS-T for 1 h before incubation with the primary antibody directed against total levels of the respective protein. Densitometric analysis of each protein band was conducted using the KODAK 1D3.6 Image Analysis Software. Relative intensities were normalized to the saline-treated control group and group means were analyzed for statistical significance using one-way ANOVA followed by Dunnett's test for multiple comparisons.

2.5. Co-immunoprecipitation

Aliquots of 100 μ g synaptosomal fraction were incubated with antibody against either α CaMKII or Munc18 for 4 h at 4 °C with slow end-over-end rotation. Immunocomplexes were captured by incubating with protein G plus-agarose beads (Santa Cruz Biotechnology) at 4 °C overnight. Beads were washed three times in washing buffer (120 mM NaCl, 20 mM HEPES pH 7.4, 0.5% SDS) and proteins were eluted in SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, and 125 mM dithiothreitol). Precipitated proteins were fractionated by SDS-PAGE and analyzed by Immunoblotting.

3. Results

3.1. Ketamine reduces accumulation of assembled SNARE complexes in hippocampal synaptic membranes

The SDS-resistant ternary SNARE complex composed of syntaxin 1A, VAMP2, and SNAP25 can be visualized by SDS-PAGE and immunoblotting in unboiled samples with an antibody for syntaxin 1A (Hayashi et al., 1994; Musazzi et al., 2010). A single subanesthetic dose of ketamine (15 mg/kg), which significantly reduced total immobility time in the FST (Fig. 1) rapidly decreased the amount of the two major syntaxin 1A containing complexes migrating at ~80 kDa and ~100 kDa (Fig. 2A–C). The decreases were significant after 1 h (64% and 77% of control, respectively) and persisted up to at least 4 h after treatment (69% and 60% of control, respectively). After 7 days the amount of SNARE complexes had returned to control levels. Ketamine did not induce changes in the monomeric levels of the SNARE proteins syntaxin 1A and SNAP25 (Fig. 2D).

3.2. Ketamine differentially affects the levels of synapsin I and synaptotagmin I in hippocampal synaptosomes

The synaptic vesicle-associated protein synapsin I is involved in regulating SNARE complex assembly by limiting the mobilization of synaptic vesicles from the reserve pool to the active zone (Fdez and Hilfiker, 2006). Here, we found increased expression of synapsin I in hippocampal crude synaptosomes 2 h after ketamine administration (137% of control).

The increase in synapsin I levels persisted up to 7 days (160% of control) (Fig. 3A,B). The synaptic vesicle protein synaptotagmin I is a calcium binding protein that positively regulates membrane fusion and neurotransmitter release by promoting SNARE complex formation (Chapman, 2008; Verona et al., 2000). We found that synaptotagmin I levels were significantly reduced after 2 and 4 h of ketamine administration (51% and 49% of control, respectively) and had returned to control levels after 7 days (Fig. 3A,B).

3.3. Reduced Thr²⁸⁶ phosphorylation of α CaMKII in hippocampal synaptosomes after ketamine treatment

Activation of CaMKII (calcium/calmodulin-dependent protein kinase II) through autophosphorylation at Thr²⁸⁶ has been reported to positively modulate neurotransmitter release through regulation of SNARE protein interactions (Ohyama et al., 2002; Popoli et al., 2001; Wang, 2008). Here, we found that a single subanesthetic dose of ketamine markedly reduced Thr²⁸⁶ phosphorylation of α CaMKII in hippocampal synaptosomes. The effect was evident after 1 h of treatment, persisted up to 4 h, and returned to control levels after 7 days (Fig. 4A,B). Total α CaMKII protein levels were unchanged between treatment groups, compared to the control group. Therefore, the changes in α CaMKII Thr²⁸⁶ phosphorylation cannot be ascribed to changes in protein levels but must represent reduced phosphorylation of α CaMKII.

3.4. Ketamine decreases the interaction between syntaxin 1A and α CaMKII in hippocampal synaptosomes

α CaMKII phosphorylated at Thr²⁸⁶ has been shown to form an intermediate with syntaxin 1 which allows recruitment of SNAP25 and VAMP2. Consequently, the binding of phosphorylated CaMKII to syntaxin 1 has been proposed to promote SNARE complex formation (Nomura et al., 2003; Ohyama et al., 2002). Syntaxin 1 was shown to bind alternatively to either α CaMKII or Munc18, a presynaptic protein that was initially proposed to limit SNARE complex formation (Dulubova et al., 1999; Yang et al., 2000). However, recent findings have demonstrated that Munc18 can also bind to the ternary SNARE complex (Dulubova et al., 2007; Shen et al., 2007). Here, we investigated whether ketamine affects the interaction of syntaxin 1A with α CaMKII and Munc18 in hippocampal crude synaptosomes of saline (control), ketamine-treated, and desipramine-treated animals after 2 h of treatment. Using coimmunoprecipitation and immunoblotting, we measured the relative amount of syntaxin 1A co-immunoprecipitated with α CaMKII or Munc18 (Fig. 5A,B). The kinase-bound syntaxin 1A was significantly reduced by ketamine but not by the tricyclic antidepressant desipramine. Ketamine had no effect on the interaction between syntaxin 1A and Munc18, whereas desipramine significantly reduced the amount of syntaxin 1A bound to Munc18. These results suggest the decrease in α CaMKII Thr²⁸⁶ phosphorylation observed in response to ketamine (Fig. 4A,B) to lead to reduced interaction between α CaMKII and syntaxin 1A.

3.5. Ketamine has no effect on GSK-3 activity

Activation of GSK-3 (glycogen synthase kinase-3) has been shown to interfere negatively with the formation of the rat hippocampal SNARE complex through changes in specific SNARE-associated protein interactions (Zhu et al., 2010). Ketamine, on the other hand, has been shown to rapidly increase inhibitory phosphorylation of GSK-3 in mice hippocampal homogenate, a mechanism appearing to be required for its antidepressant effect (Beurel et al., 2011). Therefore, we sought to determine whether ketamine affects GSK-3 activity in rat hippocampal synaptosomes. We focused on GSK-3 β , which is the more abundant GSK-3 isoform. GSK-3 is constitutively active and primarily regulated through inhibitory serine phosphorylation at Ser⁹ in GSK-3 β (Frame and Cohen, 2001). We measured Ser⁹ phosphorylation of GSK-3 β in hippocampal synaptosomes of control, ketamine-treated, and

desipramine-treated animals at 2 h after treatment but found no changes in phosphorylation levels between control and treatment groups (Fig. 6A,B).

4. Discussion

4.1. Ketamine reduces the accumulation of SNARE complexes

Our results provide the first direct evidence that ketamine affects the presynaptic release machinery responsible for neurotransmitter release. The fusion of synaptic vesicles with the plasma membrane and neurotransmitter release requires the assembly of SNARE proteins anchored in the apposed membranes. Consequently, proteins regulating SNARE complex formation can significantly impact neurotransmitter release probability and synaptic strength. The main finding of this study is that a single low dose of ketamine, that displays antidepressant-like effects in the FST, reduces the amount of SNARE complexes in hippocampal synaptic membranes. Previous studies have shown the number of SNARE complexes to be correlated with the number of vesicles anchored to the presynaptic membrane ready for release (Lonart and Südhof, 2000). This raises the possibility that ketamine may reduce the number of vesicles to undergo exocytosis. A previous study found that chronic treatments with fluoxetine and reboxetine significantly reduced the levels of the three SNAREs in synaptic membranes, suggesting a reduction in SNARE complexes at the interface between docked vesicles and membrane (Bonanno et al., 2005). Therefore ketamine seems to rapidly induce a reduction of SNARE complexes similar to that observed after chronic treatment with traditional antidepressants. Further support for this notion comes from our finding of increased levels of the synaptic vesicle-associated protein synapsin I in the synaptosomal fraction. Synapsin I limits the mobilization of synaptic vesicles to the readily releasable pool (RRP) by clustering synaptic vesicles in the reserve pool thereby regulating the size of the RRP (Fdez and Hilfiker, 2006). Notably, the ketamine-induced increase in synapsin I levels remained elevated up to 7 days, whereas the amount of SNARE complexes had returned to control levels at that time point. This indicates that the sustained effect on synapsin I levels may also be involved in processes not related to SNARE complex formation. Synapsin I has multiple functions within the presynaptic terminal and has been shown to be an important regulator of synapse formation (Valtorta et al., 2011). Previous studies have shown a similar prolonged increase in synapsin I levels in the prefrontal cortex at 72 h after ketamine administration, accompanied by increased synaptogenesis (Li et al., 2010, 2011). The elevated synapsin I levels in the hippocampus are likely to be involved in similar delayed but possible long-lasting cellular processes.

The calcium binding protein synaptotagmin I triggers calcium sensitive neurotransmitter release by promoting interactions between SNARE proteins on synaptic vesicles and the plasma membrane (Chapman, 2008). Thus, our finding that ketamine transiently decreased the amount of synaptotagmin I is in line with our finding of reduced SNARE complex assembly.

4.2. CaMKII as a potential mediator of the rapid actions of ketamine

Activation and Thr²⁸⁶ autophosphorylation of presynaptic CaMKII has been reported to positively regulate neurotransmitter release, in part, through phosphorylation of downstream proteins and by promoting SNARE protein interactions (Ohshima et al., 2002; Popoli et al., 2001; Wang, 2008). A decrease in CaMKII Thr²⁸⁶ phosphorylation has previously been shown in total rat hippocampal lysates after treatment with anesthetic doses of ketamine (Cui et al., 2009). To explore a possible role of CaMKII in the actions of ketamine, we looked at Thr²⁸⁶ phosphorylation levels of α CaMKII. We found a significant decrease in the levels of phosphorylated α CaMKII in response to ketamine, suggesting reduced activation

of the kinase. Thr²⁸⁶ phosphorylation of α CaMKII has previously been found to be downregulated in hippocampal synaptosomes after chronic treatment with conventional antidepressants (Barbiero et al., 2007; Bonanno et al., 2005). Thus, reduced α CaMKII activity may represent a particular molecular change associated with the rapid antidepressant effect of ketamine. Since both synapsin I and synaptotagmin I are substrates for CaMKII, a change in α CaMKII activity is likely to affect their function. CaMKII phosphorylation of synapsin I promotes mobilization of synaptic vesicles from the reserve pool to the RRP (Fdez and Hilfiker, 2006). The increase in total synaptosomal levels of synapsin I combined with reduced CaMKII phosphorylation could therefore add further restraint to the size of the RRP by tethering the synaptic vesicles in the reserve pool. Similarly, phosphorylation by CaMKII is required to obtain maximal binding of synaptotagmin to SNARE proteins (Verona et al., 2000). A decrease in CaMKII activity is therefore likely to result in reduced interactions between synaptotagmin I and SNARE proteins, and hence possibly contributing to the reduced SNARE complex formation.

Thr²⁸⁶ phosphorylation of CaMKII increases its interaction with syntaxin 1A and forms an intermediate complex that is thought to promote SNARE complex formation (Ohyama et al., 2002). The reduction in the phosphorylation state of α CaMKII is therefore likely to account for the decrease in the α CaMKII/syntaxin 1A complex observed by co-immunoprecipitation, thus possibly reducing the availability of syntaxin 1A for SNARE complex assembly. Acute treatment with the antidepressant desipramine had no effect on the α CaMKII/syntaxin 1A complex, which is in line with previous studies showing that chronic but not acute treatment with desipramine decreased α CaMKII Thr²⁸⁶ autophosphorylation in hippocampal synaptosomes. Therefore, acute ketamine treatment induces reduction of α CaMKII phosphorylation and α CaMKII/syntaxin 1A interaction, both of which are also outcomes of chronic treatments with traditional antidepressants (Barbiero et al., 2007; Bonanno et al., 2005).

Using co-immunoprecipitation, we also looked at the interaction between syntaxin 1A and Munc18. Binding of Munc18 to syntaxin 1A was initially proposed to restrain interactions of syntaxin 1A with its cognate SNARE proteins and thus believed to serve a negative modulatory role in SNARE complex formation and neurotransmitter release (Dulubova et al., 1999; Yang et al., 2000). However, the role of Munc18 has been rather controversial and recent studies show that Munc18 is continuously associated with syntaxin during SNARE complex formation and that Munc18 in fact can play a stabilizing role in SNARE complex assembly (Dulubova et al., 2007; Shen et al., 2007). However, we found the interaction between syntaxin 1A and Munc18 to be unaffected by ketamine, suggesting that Munc18 does not play a role in the ketamine-induced regulation of SNARE complex formation.

4.3. GSK-3 is not regulated by ketamine

The signaling pathways and mechanisms by which ketamine exerts its antidepressant action are largely unknown, but a recent study has demonstrated that ketamine increases inhibitory serine phosphorylation of GSK-3, and that GSK-3 knock-in mice, in which the phosphorylation of GSK-3 is blocked, did not respond to ketamine in a behavioral model of depression (Beurel et al., 2011). Therefore, we investigated whether ketamine affected the inhibitory serine phosphorylation of the abundant GSK-3 β isoform in hippocampal synaptosomes. However, we did not detect regulation of phosphorylated GSK-3 β at 2 h after acute ketamine administration. Several important technical differences exist between our study and the study by Beurel and colleagues. Firstly, in the study by Beurel GSK-3 phosphorylation was measured in crude homogenates of hippocampus and cerebral cortex, not in synaptosomal fractions. GSK-3 is expressed throughout the cell but concentrated in the soma and dendrites (Leroy and Brion, 1999). Therefore, it is likely that ketamine regulates GSK-3 phosphorylation levels in these compartments and not in the smaller

synaptosomal compartment, which could explain the apparent discrepancy between our study and the findings by Beurel. Secondly, experiments were performed in mice and conducted at earlier time points (30 and 60 min) after ketamine administration. It is important to emphasize that our results do not rule out the possibility that levels of phosphorylated GSK-3 β in hippocampal synaptosomes are altered after different doses or at earlier time points after ketamine administration, or that GSK-3 inhibition plays an important role in the antidepressant effect of ketamine. However, changes in GSK-3 β activity do not appear to be involved in the ketamine-mediated regulation of SNARE complex assembly that we observe in rat hippocampal synaptic membranes.

4.4. Summary

Our molecular results demonstrate that ketamine rapidly induces changes in the presynaptic machinery similar to those that are obtained with chronic treatments with traditional antidepressants. Ketamine not only binds to the NMDA receptor but also to a number of neurotransmitter receptors and monoamine re-uptake transporters with an affinity that is comparable to its affinity for the NMDA receptor (Kapur and Seeman, 2002; Nishimura et al., 1998). The mechanism by which ketamine causes its effects on presynaptic proteins could therefore include both retrograde signaling and non-NMDA receptor mediated presynaptic pathways.

Overall, the observed changes in the presynaptic machinery could represent a molecular correlate of reduced neurotransmitter release. Both stress and glucocorticoids have been shown by microdialysis to increase extracellular glutamate levels in the hippocampus (Bagley and Moghaddam, 1997; Gilad et al., 1990; Lowy et al., 1995; Moghaddam et al., 1994) and enhancement of presynaptic glutamate release by stress/glucocorticoids has been shown by different methodologies (Hascup et al., 2010; Karst et al., 2005; Musazzi et al., 2010; Hascup et al., 2011). Dendritic atrophy/remodeling observed in chronic stress models has been suggested to be due to excessive glutamate release/transmission (Sanacora et al., 2012), and hippocampal glutamate-mediated excitotoxicity has been implicated in the pathophysiology of mood disorders (Sapolsky, 2000). Thus, we speculate that ketamine may exert its antidepressant action, at least in part, by limiting excessive release of glutamate in the hippocampus via dynamic molecular changes in the presynaptic release machinery. A similar mechanism has been suggested for the effect of conventional antidepressants after chronic, but not acute treatment (Bonanno et al., 2005; Musazzi et al., 2010). In addition, clinical studies have shown antidepressant properties for the glutamate release inhibitor riluzole (Banasr et al., 2010; Machado-Vieira et al., 2009). These findings suggest that dampening of glutamate release in the hippocampus may be a converging mechanism of structurally diverse agents.

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Abbreviations

ANOVA	analysis of variance
CaMKII	calcium/calmodulin-dependent protein kinase II
FST	forced swim test
GSK	glycogen synthase kinase
NMDA	<i>N</i> -methyl-D-aspartate
RRP	readily releasable pool
RT	room-temperature
SNAP	synaptosomal-associated protein
SNARE	soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
VAMP	vesicle-associated membrane protein

References

- Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF, et al. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature*. 2011; 475:91–5. [PubMed: 21677641]
- Bagley J, Moghaddam B. Temporal dynamics of glutamate efflux in the prefrontal cortex and in the hippocampus following repeated stress: effects of pretreatment with saline or diazepam. *Neuroscience*. 1997; 77:65–73. [PubMed: 9044375]
- Banasr M, Chowdhury GM, Terwilliger R, Newton SS, Duman RS, Behar KL, et al. Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. *Molecular Psychiatry*. 2010; 15:501–11. [PubMed: 18825147]
- Barbiero VS, Giambelli R, Musazzi L, Tiraboschi E, Tardito D, Perez J, et al. Chronic antidepressants induce redistribution and differential activation of alphaCaM kinase II between presynaptic compartments. *Neuropsychopharmacology*. 2007; 32:2511–9. [PubMed: 17356571]
- Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, et al. Antidepressant effects of ketamine in depressed patients. *Biological Psychiatry*. 2000; 47:351–4. [PubMed: 10686270]
- Beurel E, Song L, Joje RS. Inhibition of glycogen synthase kinase-3 is necessary for the rapid antidepressant effect of ketamine in mice. *Molecular Psychiatry*. 2011; 16:1068–70. [PubMed: 21502951]
- Bonanno G, Giambelli R, Raiteri L, Tiraboschi E, Zappettini S, Musazzi L, et al. Chronic antidepressants reduce depolarization-evoked glutamate release and protein interactions favoring formation of SNARE complex in hippocampus. *Journal of Neuroscience*. 2005; 25:3270–9. [PubMed: 15800181]
- Chapman ER. How does synaptotagmin trigger neurotransmitter release? *Annual Review of Biochemistry*. 2008; 77:615–41.
- Cryan JF, Markou A, Lucki I. Assessing antidepressant activity in rodents: recent developments and future needs. *Trends in Pharmacological Sciences*. 2002; 23:238–45. [PubMed: 12008002]
- Cui X, Li J, Li T, Ji F, Bu X, Zhang N, et al. Propofol and ketamine-induced anesthetic depth-dependent decrease of CaMKII phosphorylation levels in rat hippocampus and cortex. *Journal of Neurosurgical Anesthesiology*. 2009; 21:145–54. [PubMed: 19295394]
- Dulubova I, Khvotchev M, Liu S, Huryeva I, Südhof TC, Rizo J. Munc18-1 binds directly to the neuronal SNARE complex. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:2697–702. [PubMed: 17301226]
- Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Südhof TC, et al. A conformational switch in syntaxin during exocytosis: role of munc18. *The EMBO Journal*. 1999; 18:4372–82. [PubMed: 10449403]

- Elfving B, Bonefeld BE, Rosenberg R, Wegener G. Differential expression of synaptic vesicle proteins after repeated electroconvulsive seizures in rat frontal cortex and hippocampus. *Synapse*. 2008; 62:662–70. [PubMed: 18563845]
- Fatemi SH, Earle JA, Stary JM, Lee S, Sedgewick J. Altered levels of the synaptosomal associated protein SNAP-25 in hippocampus of subjects with mood disorders and schizophrenia. *Neuroreport*. 2001; 12:3257–62. [PubMed: 11711867]
- Fdez E, Hilfiker S. Vesicle pools and synapsins: new insights into old enigmas. *Brain Cell Biology*. 2006; 35:107–15. [PubMed: 17957477]
- Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochemical Journal*. 2001; 359:1–16. [PubMed: 11563964]
- Gao Y, Bezchlibnyk YB, Sun X, Wang JF, McEwen BS, Young LT. Effects of restraint stress on the expression of proteins involved in synaptic vesicle exocytosis in the hippocampus. *Neuroscience*. 2006; 141:1139–48. [PubMed: 16757120]
- Garcia LS, Comim CM, Valvassori SS, Reus GZ, Barbosa LM, Andreazza AC, et al. Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 2008; 32:140–4. [PubMed: 17884272]
- Gilad GM, Gilad VH, Wyatt RJ, Tizabi Y. Region-selective stress-induced increase of glutamate uptake and release in rat forebrain. *Brain Research*. 1990; 525:335–8. [PubMed: 1979236]
- Hascup ER, Hascup KN, Stephens M, Pomerleau F, Huettl P, Gratton A, et al. Rapid microelectrode measurements and the origin and regulation of extracellular glutamate in rat prefrontal cortex. *Journal of Neurochemistry*. 2010; 115:1608–20. [PubMed: 20969570]
- Hascup KN, Hascup ER, Stephens ML, Glaser PE, Yoshitake T, Mathe AA, et al. Resting glutamate levels and rapid glutamate transients in the prefrontal cortex of the flinders sensitive line rat: a genetic rodent model of depression. *Neuropsychopharmacology*. 2011; 36:1769–77. [PubMed: 21525860]
- Hashimoto K. Emerging role of glutamate in the pathophysiology of major depressive disorder. *Brain Research Reviews*. 2009; 61:105–23. [PubMed: 19481572]
- Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, Südhof TC, et al. Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *The EMBO Journal*. 1994; 13:5051–61. [PubMed: 7957071]
- Kapur S, Seeman P. NMDA receptor antagonists ketamine and PCP have direct effects on the dopamine D(2) and serotonin 5-HT(2) receptors-implications for models of schizophrenia. *Molecular Psychiatry*. 2002; 7:837–44. [PubMed: 12232776]
- Karst H, Berger S, Turiault M, Tronche F, Schutz G, Joels M. Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:19204–7. [PubMed: 16361444]
- Leroy K, Brion JP. Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *Journal of Chemical Neuroanatomy*. 1999; 16:279–93. [PubMed: 10450875]
- Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, et al. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science*. 2010; 329:959–64. [PubMed: 20724638]
- Li N, Liu RJ, Dwyer JM, Banasr M, Lee B, Son H, et al. Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biological Psychiatry*. 2011; 69:754–61. [PubMed: 21292242]
- Lonart G, Südhof TC. Assembly of SNARE core complexes prior to neurotransmitter release sets the readily releasable pool of synaptic vesicles. *The Journal of Biological Chemistry*. 2000; 275:27703–7. [PubMed: 10970903]
- Lorrain DS, Baccei CS, Bristow LJ, Anderson JJ, Varney MA. Effects of ketamine and N-methyl-D-aspartate on glutamate and dopamine release in the rat prefrontal cortex: modulation by a group II selective metabotropic glutamate receptor agonist LY379268. *Neuroscience*. 2003; 117:697–706. [PubMed: 12617973]

- Lowy MT, Wittenberg L, Yamamoto BK. Effect of acute stress on hippocampal glutamate levels and spectrin proteolysis in young and aged rats. *Journal of Neurochemistry*. 1995; 65:268–74. [PubMed: 7790870]
- Machado-Vieira R, Salvatore G, Ibrahim LA, Diaz-Granados N, Zarate CA Jr. Targeting glutamatergic signaling for the development of novel therapeutics for mood disorders. *Current Pharmaceutical Design*. 2009; 15:1595–611. [PubMed: 19442176]
- Maeng S, Zarate CA Jr, Du J, Schloesser RJ, McCammon J, Chen G, et al. Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biological Psychiatry*. 2008; 63:349–52. [PubMed: 17643398]
- Moghaddam B, Adams B, Verma A, Daly D. Activation of glutamatergic neuro-transmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the pre-frontal cortex. *The Journal of Neuroscience*. 1997; 17:2921–7. [PubMed: 9092613]
- Moghaddam B, Bolinao ML, Stein-Behrens B, Sapolsky R. Glucocorticoids mediate the stress-induced extracellular accumulation of glutamate. *Brain Research*. 1994; 655:251–4. [PubMed: 7812782]
- Müller HK, Wegener G, Popoli M, Elfving B. Differential expression of synaptic proteins after chronic restraint stress in rat prefrontal cortex and hippocampus. *Brain Research*. 2011; 1385:26–37. [PubMed: 21354112]
- Musazzi L, Milanese M, Farisello P, Zappettini S, Tardito D, Barbiero VS, et al. Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: the dampening action of antidepressants. *PLoS One*. 2010; 5:e8566. [PubMed: 20052403]
- Nishimura M, Sato K, Okada T, Yoshiya I, Schloss P, Shimada S, et al. Ketamine inhibits monoamine transporters expressed in human embryonic kidney 293 cells. *Anesthesiology*. 1998; 88:768–74. [PubMed: 9523822]
- Nomura K, Ohyama A, Komiya Y, Igarashi M. Minimal residues in linker domain of syntaxin 1A required for binding affinity to Ca²⁺/calmodulin-dependent protein kinase II. *Journal of Neuroscience Research*. 2003; 72:198–202. [PubMed: 12671994]
- Ohyama A, Hosaka K, Komiya Y, Akagawa K, Yamauchi E, Taniguchi H, et al. Regulation of exocytosis through Ca²⁺/ATP-dependent binding of autophosphorylated Ca²⁺/calmodulin-activated protein kinase II to syntaxin 1A. *The Journal of Neuroscience*. 2002; 22:3342–51. [PubMed: 11978810]
- Pittenger C, Duman RS. Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*. 2008; 33:88–109. [PubMed: 17851537]
- Popoli M, Mori S, Brunello N, Perez J, Gennarelli M, Racagni G. Serine/threonine kinases as molecular targets of antidepressants: implications for pharmacological treatment and pathophysiology of affective disorders. *Pharmacology & Therapeutics*. 2001; 89:149–70. [PubMed: 11316518]
- Rapp S, Baader M, Hu M, Jennen-Steinmetz C, Henn FA, Thome J. Differential regulation of synaptic vesicle proteins by antidepressant drugs. *The Pharmacogenomics Journal*. 2004; 4:110–3. [PubMed: 14708000]
- Sanacora G, Treccani G, Popoli M. Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology*. 2012; 62:63–77. [PubMed: 21827775]
- Sapolsky RM. The possibility of neurotoxicity in the hippocampus in major depression: a primer on neuron death. *Biological Psychiatry*. 2000; 48:755–65. [PubMed: 11063972]
- Scarr E, Gray L, Keriakous D, Robinson PJ, Dean B. Increased levels of SNAP-25 and synaptophysin in the dorsolateral prefrontal cortex in bipolar I disorder. *Bipolar Disorders*. 2006; 8:133–43. [PubMed: 16542183]
- Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ. Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell*. 2007; 128:183–95. [PubMed: 17218264]
- Südhof TC. The synaptic vesicle cycle. *Annual Review of Neuroscience*. 2004; 27:509–47.
- Valtorta F, Pozzi D, Benfenati F, Fornasiero EF. The synapsins: multitask modulators of neuronal development. *Seminars in Cell & Developmental Biology*. 2011; 22:378–86. [PubMed: 21798361]

- Verona M, Zanotti S, Schafer T, Racagni G, Popoli M. Changes of synaptotagmin interaction with t-SNARE proteins in vitro after calcium/calmodulin-dependent phosphorylation. *Journal of Neurochemistry*. 2000; 74:209–21. [PubMed: 10617122]
- Wang ZW. Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Molecular Neurobiology*. 2008; 38:153–66. [PubMed: 18759010]
- Yamada M, Takahashi K, Tsunoda M, Nishioka G, Kudo K, Ohata H, et al. Differential expression of VAMP2/synaptobrevin-2 after antidepressant and electroconvulsive treatment in rat frontal cortex. *Pharmacogenomics Journal*. 2002; 2:377–82. [PubMed: 12629503]
- Yang B, Steegmaier M, Gonzalez LC Jr, Scheller RH. nSec1 binds a closed conformation of syntaxin1A. *The Journal of Cell Biology*. 2000; 148:247–52. [PubMed: 10648557]
- Zarate CA Jr, Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA, et al. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Archives of General Psychiatry*. 2006; 63:856–64. [PubMed: 16894061]
- Zhu LQ, Liu D, Hu J, Cheng J, Wang SH, Wang Q, et al. GSK-3 beta inhibits presynaptic vesicle exocytosis by phosphorylating P/Q-type calcium channel and interrupting SNARE complex formation. *The Journal of Neuroscience*. 2010; 30:3624–33. [PubMed: 20219996]

Fig. 1. Forced swim test. Compared with the saline group, ketamine administration significantly decreased the immobility time of rats during the FST ($n = 10$). Data are expressed as mean $\pm$ SEM of control. (* $p < 0.05$).

Fig. 2.

Ketamine decreases the accumulation of SNARE complexes in hippocampal synaptic membranes. (A) Representative immunoblots of time course for ketamine-induced changes in the accumulation of SNARE complexes and the levels of the monomeric proteins syntaxin 1A and SNAP25 in hippocampal synaptic membranes (LP1 fraction). (B) Quantification of the 80 kDa SNARE complex normalized to monomeric syntaxin 1A in the same lane ($n = 5$ rats/group). (C) Quantification of the 100 kDa SNARE complex normalized to monomeric syntaxin 1A in the same lane ($n = 5$ rats/group). (D) Quantification of monomeric syntaxin 1A and SNAP25 normalized to GAPDH in the same lane ($n = 5$ rats/group). Data are expressed as mean percentage SEM of control mean values (***) $p < 0.001$ compared to control, one-way ANOVA followed by Dunnett's test for multiple comparisons).

Fig. 3. Ketamine increases synapsin I and transiently decreases synaptotagmin I levels in hippocampal crude synaptosomes. (A) Representative immunoblots of time course for the expression levels of synapsin I and synaptotagmin I in response to ketamine in hippocampal crude synaptosomes. (B) Quantification of synapsin I and synaptotagmin I normalized to GAPDH in the same lane ($n = 5$ rats/group). Data are expressed as mean percentage $\pm$ SEM of control mean values (** $p < 0.001$ compared to control, one-way ANOVA followed by Dunnett's test for multiple comparisons).

Fig. 4.

Autophosphorylation of αCaMKII at Thr²⁸⁶ is reduced by ketamine. (A) Representative immunoblots of time course for the expression level and Thr²⁸⁶ phosphorylation of αCaMKII in hippocampal crude synaptosomes. (B) Quantitative analysis of phosphorylated αCaMKII normalized to total αCaMKII in the same lane ($n = 5$ rats/group). Data are expressed as mean percentage $\pm$ SEM of control mean values (*** $p < 0.001$ compared to control, one-way ANOVA followed by Dunnett's test for multiple comparisons).

Fig. 5.

Ketamine decreases the binding of syntaxin 1A to α CaMKII in hippocampal crude synaptosomes. (A) Immunoblots of immunoprecipitated α CaMKII and Munc18 and the corresponding co-immunoprecipitated syntaxin 1A in hippocampal crude synaptosomes from saline-, ketamine-, and desipramine-treated rats 2 h after treatment. (B) Quantitative analysis of co-immunoprecipitated syntaxin 1A normalized to the corresponding immunoprecipitated α CaMKII or Munc18 in the same lane ($n = 3$ rats/group). Data are expressed as mean percentage $\pm$ SEM of control mean values (** $p < 0.01$ compared to control, one-way ANOVA followed by Dunnett's test for multiple comparisons).

Fig. 6. GSK-3β activity is not regulated by ketamine. Representative immunoblots of the expression level and inhibitory Ser⁹ phosphorylation of GSK-3β in hippocampal crude synaptosomes from saline-, ketamine-, and desipramine-treated rats 2 h after treatment. (B) Quantitative analysis of Ser⁹ phosphorylated GSK-3β normalized to total GSK-3β in the same lane ($n = 5$ rats/group). Data are expressed as mean percentage $\pm$ SEM of control mean values (one-way ANOVA followed by Dunnett's test for multiple comparisons).