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Effects of ketamine on glucose uptake by glucose transporter type 3 expressed in *Xenopus* oocytes: The role of protein kinase C

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ABSTRACT

We investigated the effects of ketamine on the type 3 facilitative glucose transporter (GLUT3), which plays a major role in glucose transport across the plasma membrane of neurons. Human-cloned GLUT3 was expressed in *Xenopus* oocytes by injection of GLUT3 mRNA. GLUT3-mediated glucose uptake was examined by measuring oocyte radioactivity following incubation with 2-deoxy-D-[1,2-^{3}H]glucose. While ketamine and *S*(+)-ketamine significantly increased GLUT3-mediated glucose uptake, this effect was biphasic such that higher concentrations of ketamine inhibited glucose uptake. Ketamine (10 μM) significantly increased V_{max} but not K_m of GLUT3 for 2-deoxy-D-glucose. Although staurosporine (a protein kinase C inhibitor) increased glucose uptake, no additive or synergistic interactions were observed between staurosporine and racemic ketamine or *S*(+)-ketamine. Treatment with ketamine or *S*(+)-ketamine partially prevented GLUT3 inhibition by the protein kinase C activator phorbol-12-myrisate-13-acetate. Our results indicate that ketamine increases GLUT3 activity at clinically relevant doses through a mechanism involving PKC inhibition.

Glucose provides the major source of energy within the mammalian brain. Glucose transport across the blood–brain barrier is mediated by a facilitative-diffusion type transport system [1,2]. Numerous subtypes of glucose transporter (GLUT) have been identified that are designated GLUT1 to GLUT12. Two of these isoforms – GLUT1 and GLUT3 – are expressed mainly within the brain and play key roles in cerebral glucose metabolism [1–4]. GLUT3 has a higher substrate affinity than GLUT1, as shown by its lower K_m value for 3-O-methylglucose transport [1]. GLUT3 expression is 5- to 10-fold higher than GLUT1 expression in cultured neurons [5]. Moreover, changes in cerebral glucose metabolism are associated more closely with alterations in GLUT3 levels than GLUT1 levels [4]. These findings suggest GLUT3 plays a greater role than GLUT1 in glucose uptake and utilization in the brain.

Cerebral ischemic injury (due to hypoxia or hypoglycemia) leads to elevated glucose transporter gene expression and increased GLUT functional activity within the brain [6–9]. Enhanced glucose uptake helps to protect neurons from lethal injury during hypoxic challenge. Cell death associated with hypoxia or ischemia occurs through an apoptotic mechanism. Acute brain ischemia results in altered protein kinase C (PKC) activity, leading to the subsequent impairment of Ca^{2+}-dependent phosphorylation systems (including protein kinase C itself) [10]. This contributes to irreversible neuronal damage and neuronal death. Ischemia-induced GLUT overexpression within the brain prevents hypoxia-induced apoptosis by inhibiting stress-activated protein kinase pathways [11]. Therefore, by maintaining glucose availability within the brain necessary for cellular energy metabolism enhanced GLUT expression delays apoptosis and cell necrosis.

Ketamine increases the cerebral metabolic rate for glucose [12–14], which is a major factor that limits its use as an anesthetic agent. Despite this effect, ketamine (which is a non-competitive N-methyl-D-aspartate [NMDA] receptor antagonist) has been reported to have neuroprotective properties [15–19]. Several studies have indicated neuroprotective effects for racemic or *S*(+)-ketamine, but not R(−)-ketamine [19]. The mechanisms that underlie these neuroprotective effects remain enigmatic.

We hypothesized that neuroprotective effects and increased cerebral glucose metabolism mediated by ketamine may be explained partly by GLUT3 upregulation. In the present study, we examined the effects of ketamine on [^{3}H]2-deoxy-D-glucose uptake by GLUT3 in *Xenopus* oocyte expression system. In addition, we examined whether PKC-mediated signaling processes, which modulate GLUT function, are involved in ketamine-induced effects upon [^{3}H]2-deoxy-D-glucose uptake by GLUT3.

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Materials and methods

Materials. The pcDNA3.1/GS plasmid vector used to express full-length GLUT3 was purchased from Invitrogen Corporation (Carlsbad, CA); ketamine, *S*(+)-ketamine, 2-deoxy-D-glucose, and phloretin were purchased from Sigma Chemical Co. (St. Louis, MO); 2-deoxy-D-[1,2-^{3}H] glucose was obtained from NEN Life Science Products (Boston, MA) and American Radiolabeled Chemicals Inc. (St. Louis, MO). Ketamine and *S*(+)-ketamine were prepared as 100 mM stocks in modified Barths' saline (MBS). Phorbol-12-myristate-13-acetate (PMA) and 4α-phorbol 12,13-didecanoate (4αPDD) were prepared as 1000-folds stock in dimethyl sulfoxide (DMSO) and diluted in MBS before use. Staurosporine was diluted in MBS solution.

Synthesis of transcripts from cDNA. Plasmids containing the appropriate glucose transporter cDNA were linearized with the restriction enzyme *AgeI*. The cDNAs were recovered by phenol extraction followed by ethanol precipitation, and then transcribed in vitro into capped RNAs using Ambion's mMESSAGE mMACHINE in vitro transcription kit (Austin, TX). Complementary RNA was recovered by chloroform extraction and isopropanol precipitation, and then dissolved in sterile diethylpyrocarbonate-treated buffer to obtain 1 μg/μl samples. The yield of the in vitro transcription product was quantified by spectrophotometry at 260 nm.

Preparation of oocytes and RNA injection. Stage V–VI oocytes previously removed from adult female *Xenopus laevis* were isolated manually from their surrounding follicles in isolation media (108 mM NaCl, 1 mM EDTA, 2 mM KCl, 10 mM HEPES; pH 7.5), and then treated with 0.5 mg/ml collagenase in modified Barth's solution (MBS; composition: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4{\cdot}7H_2O$, 0.33 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.91 mM $CaCl_2$, 10 mM HEPES) for 10 min at room temperature. Oocytes were then washed six times with MBS and maintained in MBS at 15° for all subsequent procedures. One day after collagenase treatment, oocytes that appeared healthy visually were injected either with 30 nl of mRNA or sterile water using a Nanoject injector (Drummond Scientific, Broomall, PA) at the interface between the animal and vegetable poles. Before assays for glucose uptake were performed, injected individual oocytes were maintained at 18° for 3 days in 96-well microtiter plates containing MBS plus 2.5 mM sodium pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/L streptomycin, and 50 mg/L gentamycin. The incubation medium was changed daily and degenerating oocytes were discarded.

Glucose uptake measurement. Oocytes (3–10 per group) were incubated for 60 min in 1 ml MBS containing 16.5 μCi 2-deoxy-D-[1,2-^{3}H]glucose (final specific activity = 0.5 μCi/mmol) and 25 μM 2-deoxy-D-glucose. Preliminary studies have shown that transport of 2-deoxy-D-[1,2-^{3}H]glucose is linear with time for up to 120 min at room temperature (data not shown). Ketamine was added at concentrations ranging from 10 to 500 μM. Glucose uptake was rapidly terminated by three washes in 3 ml ice-cold MBS containing 0.1 mM phloretin. Pools of oocytes were dissolved in 500 μl of 2% sodium dodecyl sulfate (SDS) and then mixed with scintillation fluid. Internalized radioactivity was measured by a liquid scintillation spectrophotometer using an LSC-5100 liquid scintillation counter (ALOKA Co. Ltd., Tokyo, Japan). Background values obtained by averaging glucose uptake in 3–6 water-injected oocytes were subtracted from the total uptake observed in mRNA-injected oocytes to determine specific uptake. Specific 2-deoxy-D-glucose uptake was measured for kinetic studies using the same procedure at substrate concentrations of 25, 125, 250, and 1000 μM.

Data analysis. Data are presented as mean ± standard deviation (SD). Differences among groups were analyzed using either Student's *t* test or analysis of variance (ANOVA), followed by Bonferroni's or Dunnett's post hoc tests, as appropriate. A P value <0.05 was accepted as statistically significant. Transport kinetics were analyzed by best-fit analysis of data points (Prism version 4.0, GraphPad, San Diego, CA) and Eadie-Hofstee transformation.

Results

[^{3}H]2-DOG uptake by oocytes expressing GLUT3

The uptake of 2-DOG by GLUT3 was first examined in *Xenopus* oocytes injected with mRNA encoding human GLUT3 protein. Uptake of [^{3}H]2-DOG correlated linearly with oocyte number and incubation time. We found that [^{3}H]2-DOG uptake into oocytes increased almost linearly for the first 120 min, after which saturation occurred (data not shown). Thus, we generally used an incubation time of 60 min that is within the linear range of [^{3}H]2-DOG uptake.

The uptake of [^{3}H]2-DOG in control oocytes (injected with water only) was 0.12 ± 0.1 pmol/oocyte/h. However, in oocytes injected with mRNA encoding GLUT3, the uptake of [^{3}H]2-DOG was approximately 10 times greater relative to control oocytes (1.3 ± 0.3 pmol/oocyte/h). This effect was inhibited almost completely by the selective GLUT inhibitor, phloretin (0.3 mM) (data not shown). These findings confirm that [^{3}H]2-DOG uptake is dependent on GLUT3 protein function.

Effects of ketamine or S(+)-ketamine on [^{3}H]2-DOG uptake by oocytes expressing GLUT3

The effects of ketamine on [^{3}H]2-DOG uptake by GLUT3 in oocytes were biphasic (Fig. 1A). Ketamine (10 μM or 25 μM) significantly enhanced [^{3}H]2-DOG uptake in oocytes. The uptake of [^{3}H]2-DOG was maximally enhanced by 25 μM ketamine, whereas uptake was significantly inhibited in a concentration-dependent manner by ketamine concentrations greater than 100 μM. The extent of inhibited [^{3}H]2-DOG uptake by ketamine (500 μM) was approximately 50% compared to controls cells. However, ketamine concentrations at or greater than 500 μM produced no further inhibition (data not shown).

Incubation of oocytes with increasing concentrations of [^{3}H]2-DOG (ranging from 25 to 1000 μM) showed that [^{3}H]2-DOG uptake is a saturable process (Fig. 2A). Using Eadie-Hofstee analysis, it was found that ketamine (10 μM) had a minimal effect on the apparent Michaelis constant (K_m) for GLUT3-mediated 2-DOG uptake (control: 327.4 ± 54.3 μM; 10 μM ketamine, 361.1 ± 41.8 μM). However, 10 μM ketamine treatment induced a significant increase in the maximal velocity of [^{3}H]2-DOG uptake (V_{max}; control: 110.4 ± 7.6 fmol/oocyte/h; 10 μM ketamine: 148.5 ± 7.3 fmol/oocyte/h). This finding suggests that 10 μM ketamine treatment increases the transport capacity of GLUT3 (Fig. 2B).

Treatment with 10 μM racemic ketamine resulted in a significant increase (40%) in [^{3}H]2-DOG uptake. An equimolar concentration of *S*(+)-ketamine also produced significant increase (41%) in [^{3}H]2-DOG uptake (which is a similar to that produced by racemic ketamine treatment). *S*(+)-ketamine (5 μM) also significantly increased [^{3}H]2-DOG uptake to a maximum of approximately 142% above control values. However, there was no statistical difference in GLUT3-mediated uptake of [^{3}H]2-DOG among oocytes treated with racemic ketamine, 5 μM *S*(+)-ketamine, and 10 μM *S*(+)-ketamine (Fig. 1B).

Involvement of PKC on [^{3}H]2-DOG uptake by oocytes expressing GLUT3

To investigate the possible regulation of GLUT3 by PKC, GLUT3-expressing oocytes were preincubated with the PKC activator PMA. Preincubation of oocytes with PMA (100 nM for 10 min) signifi-

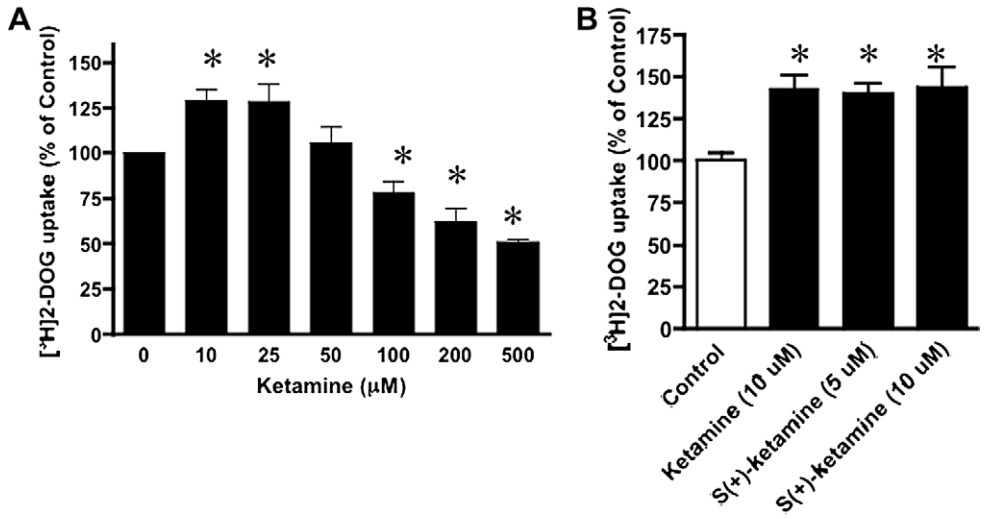

Fig. 1. (A) Effects of various concentrations of ketamine upon glucose uptake in *Xenopus* oocytes expressing GLUT3. Glucose uptake is expressed as a percentage relative to control values (0 μM ketamine treatment). Oocytes were incubated with various concentrations of ketamine for 1 h before glucose uptake was measured. Data are shown as means ± SD (three batches of oocytes; three oocytes per batch). (B) Effects of ketamine (10 μM) or *S*(+)-ketamine upon [^{3}H]2-DOG uptake in *Xenopus* oocytes. Oocytes were incubated with ketamine (10 μM), *S*(+)-ketamine (5 μM) or *S*(+)-ketamine (10 μM) for 1 h before glucose uptake was measured. Data are shown as means ± SD (for four separate experiments conducted in triplicate). $^*P < 0.05$ compared to control experiments.

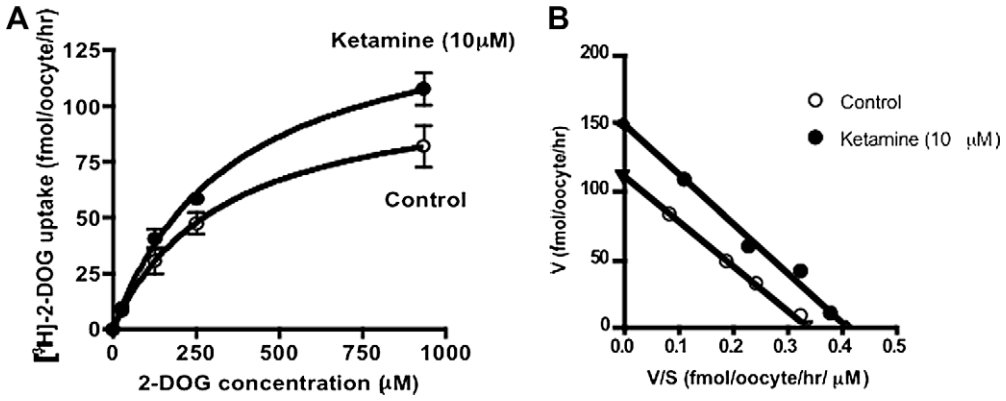

Fig. 2. (A) Saturation curve for [^{3}H]2-DOG uptake. Oocytes were incubated with or without 10 μM ketamine, or 10 μM ketamine in the presence of various concentrations of [^{3}H]2-DOG (ranging from 25 to 1000 μM) for 1 h. Data are shown as means ± SD (for four separate experiments conducted in triplicate). (B) Eadie-Hofstee analysis of [^{3}H]2-DOG uptake. Data used to perform this analysis were obtained from (A). *V*/*S* = velocity/substrate.

cantly decreased [^{3}H]2-DOG uptake. The uptake of [^{3}H]2-DOG was not affected by incubation with vehicle (0.1% DMSO) and 400 nM 4αPDD (an inactive analog of PMA) (Fig. 3A). Preincubation of oocytes with the nonselective PKC inhibitor staurosporine (1 μM) for 1 h significantly increased [^{3}H]2-DOG uptake. Staurosporine treatment (1 μM for 1 h) combined with PMA treatment (100 nM) for 10 min partially prevented PMA-induced inhibition of [^{3}H]2-DOG uptake (Fig. 3A). These results suggest that [^{3}H]2-DOG uptake by GLUT3 is mediated (at least partially) through PKC activation.

Effects of PKC activator or PKC inhibitor on [^{3}H]2-DOG uptake by oocytes expressing GLUT3 in the absence or presence of racemic ketamine or S(+)-ketamine

Preincubation of oocytes with PMA (100 nM) for 10 min significantly decreased [^{3}H]2-DOG uptake. To determine whether PMA interacts with racemic ketamine or *S*(+)-ketamine, PMA-treated oocytes were exposed separately to each of these compounds. Treatment with racemic ketamine (10 μM) or *S*(+)-ketamine (5 μM) of oocytes pretreated with PMA partially prevented PMA-induced inhibition of [^{3}H]2-DOG uptake. However, there was no statistical difference in [^{3}H]2-DOG uptake among oocytes treated with PMA, PMA plus racemic ketamine, or PMA plus *S*(+)-ketamine (Fig. 3B).

Preincubation of oocytes with staurosporine (1 μM) for 1 h significantly increased [^{3}H]2-DOG uptake relative to controls. To determine if staurosporine interacts with racemic ketamine or *S*(+)-ketamine, staurosporine-treated oocytes were exposed to racemic ketamine or *S*(+)-ketamine, and the effects upon [^{3}H]2-DOG uptake were compared with control oocytes. Oocytes treated with staurosporine (1 μM), staurosporine plus racemic ketamine (10 μM), or staurosporine plus *S*(+)-ketamine (5 μM) showed significant and similar increases in [^{3}H]2-DOG uptake compared to control oocytes. There were no statistical differences in [^{3}H]2-DOG uptake responses among oocytes treated with staurosporine, staurosporine plus racemic ketamine, or staurosporine plus *S*(+)-ketamine (Fig. 3B). Therefore, it appears that there is no additive or synergistic interaction between staurosporine and racemic ketamine or *S*(+)-ketamine that may affect [^{3}H]2-DOG uptake.

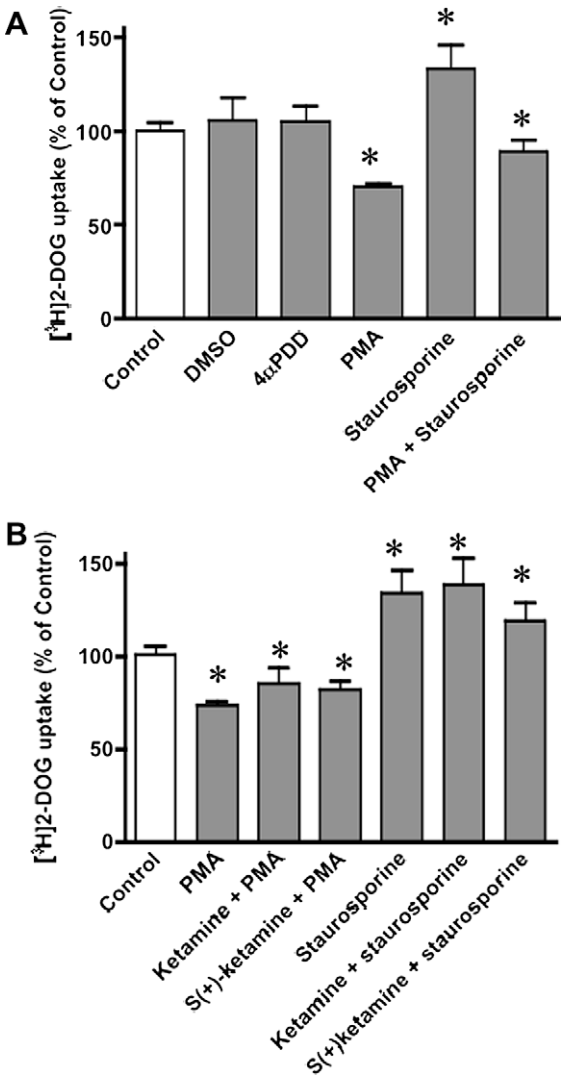

Fig. 3. (A) Specificity of PMA upon inhibition of GLUT3-mediated [^{3}H]2-DOG uptake. Oocytes expressing GLUT3 were preincubated in the bath-medium with the indicated agents, and [^{3}H]2-DOG uptake assays were then performed. Oocytes were preincubated with DMSO (0.1% for 1 h), 4αPDD (400 nM for 1 h), PMA (100 nM for 10 min), staurosporine (1 μM for 1 h), and PMA (100 nM for 10 min) in the presence of staurosporine (1 μM for 1 h). Data are shown as means ± SD (for three separate experiments performed in triplicate). *$P < 0.05$ compared to control experiments (no drug). (B) Effects of PKC activation or PKC inhibition upon GLUT3-mediated [^{3}H]2-DOG uptake in the presence or absence of ketamine or *S*(+)-ketamine. Oocytes were exposed to PMA (100 nM for 10 min), PMA and ketamine (10 μM for 1 h), or PMA (100 nM for 10 min) in the presence of *S*(+)-ketamine (5 μM for 1 h), staurosporine (1 μM for 1 h), staurosporine (1 μM for 1 h) in the presence of ketamine (10 μM for 1 h), or staurosporine (1 μM for 1 h) in the presence of *S*(+)-ketamine (5 μM for 1 h). Data are shown as means ± SD (for three separate experiments performed in triplicate). *$P < 0.05$ compared to control experiments (no drug).

Discussion

Effects of ketamine on glucose uptake by GLUT3

Our findings indicate that low concentrations of ketamine (⩽25 μM) enhanced [^{3}H]glucose uptake by GLUT3. Because ketamine-induced anesthesia is accompanied by an increase in cerebral rate of glucose metabolism (CMR_{Glc}), it is possible that upregulated cerebral metabolism is due (at least in part) to enhanced glucose uptake induced by ketamine. Findings from various studies provide support for upregulated cerebral glucose metabolism due to enhanced glucose uptake by GLUT3. Brain regions where CMR_{Glc} is stimulated by ketamine correlate strongly with the regions that display significant GLUT3 expression. Despite conflicting *in vivo* studies regarding the brain regions stimulated by ketamine, there is general agreement that ketamine stimulates CMR_{Glc} in the hippocampus [12–14]. GLUT3 protein expression has been demonstrated within limbic structures such as the hippocampus, dentate gyrus, and several amygdala nuclei [1–3,20]. In addition, treatment with 10 μM ketamine (which significantly enhanced glucose uptake by GLUT3), is close to the clinical dose, since the plasma ketamine concentration that corresponds to a IV dose of 2 mg/kg in humans is between 4 and 12 μM [21]. Furthermore, the extent of the ketamine-induced increase in CMR_{Glc} found *in vivo* is almost identical to the enhancement in glucose uptake by GLUT3 described in the present study. At a ketamine dose sufficient to induce anesthesia in humans, glucose utilization was shown to be increased in the hippocampus by 33% [12]. In the present study, 10 μM ketamine treatment enhanced average glucose uptake by GLUT3 by 35%. Finally, GLUT3 may closely be associated with increased metabolic activity in the hippocampus in hippocampal-induced seizures triggered by ketamine. Upregulated GLUT3 expression in localized regions is accompanied by similar increases in regional cerebral glucose utilization [3,4]. Rates of glucose utilization correlate most closely with electrical activity in synaptic terminals [2,22]. Thus, upregulated GLUT3 function may reflect increased cerebral metabolic activity.

Effects of S(+)-ketamine isomer on glucose uptake by GLUT3

Ketamine, which usually is administered as a racemate, occurs in 2 isomeric forms, *S*(+)-ketamine and R(−)-ketamine. The *S*(+) isomer shows approximately 2 to 3 times greater analgesic and hypnotic potency than the *R*(-)isomer. Furthermore, it has been shown that certain locomotor and psychomimetic side effects associated with ketamine treatment are less intense for the *S*(+) form, and that the therapeutic index of the *S*(+) form is greater relative to its racemate. In addition, *S*(+)-ketamine was found to have higher neuroprotective potency in vitro against NMDA-induced injury compared with the racemate and the *R*(-)form [23]. Although psychotomimetic doses of *S*(+)-ketamine were found to increase cerebral glucose metabolism, PET (positron emission tomography) scans showed a tendency for equimolar doses of *R*(−)-ketamine to decrease cerebral glucose metabolic rate [24]. Although it was not possible to examine the effects of R(−)-ketamine on glucose uptake by GLUT3 in the present study, the extent of the increase of 2-DOG uptake induced by racemic ketamine was similar to increases observed by equimolar and half-molar *S*(+)-ketamine. This finding suggests that increased 2-DOG uptake by racemic ketamine may be dependent on the effects of *S*(+)-ketamine.

Involvement of protein kinase C in the effects of ketamine on glucose uptake by GLUT3

GLUT trafficking between intracellular compartments and plasma membrane of cell surface has been suggested to be a major regulatory mechanism of GLUT activity [25]. To determine whether functional activation of GLUT3 induced by ketamine is accompanied by significant changes in the kinetic constant for glucose uptake, the dependence of 2-DOG uptake upon 2-DOG concentration was investigated using Eadie-Hofstee analysis. This showed that treatment with 10 μM ketamine significantly increased V_{max}, but not K_m, for 2-DOG uptake. These findings suggest that ketamine affects glucose uptake by increasing the number or turnover rate of GLUT3 molecules, rather than through an effect upon the affinity of GLUT3 for 2-DOG.

PKC regulates the activity of numerous neurotransmitter transporters through alterations in cell surface trafficking. When *Xenopus* oocytes expressing GLUT3 were pretreated with bath-applied PMA (a PKC activator), [^{3}H]2-DOG uptake was found to be significantly decreased in the present study. This inhibition appeared to be PKC-specific because incubation with the inactive form of the phorbol ester, 4αPDD, did not affect [^{3}H]2-DOG uptake by GLUT3. Moreover, PMA inhibition could be blocked partially by the nonselective PKC inhibitor staurosporine, which is consistent with results reported for human dopamine transporters [26] and serotonin transporters [27]. The inhibition of the protein kinase $C\beta_{II}$ increases glucose uptake in 3T3-L1 adipocytes through elevated expression of GLUT1 at the plasma membrane [28]. In our experiments, preincubation with staurosporine significantly increased [^{3}H]2-DOG uptake relative to controls, and addition of racemic ketamine or *S(+)*-ketamine to oocytes pretreated with staurosporine did not affect [^{3}H]2-DOG uptake. Therefore, it appears that there is no additive or synergistic interaction between staurosporine and ketamine upon GLUT3 activity. This finding suggests both these agents increase GLUT3 activity through a common pathway.

The PKC family consists of at least 11 isoforms that can be divided into three classes: the classical or conventional PKC isoforms (α, β_I, β_{II}, and γ); the novel isoforms (δ, ε, η and θ); and the atypical isoforms (λ, ζ and ι). It is known that at least several PKC isoforms (PKCα, β_I, β_{II}, γ, δ and ζ) are present in *Xenopus* oocytes [29]. The mechanism through which PKC isoforms mediate increased glucose uptake by GLUT3 is unclear. Thus, further study is required to determine the PKC isoforms that are important in GLUT3-mediated glucose transport.

In conclusion, our data suggest that racemic ketamine and *S(+)*-ketamine increase GLUT3 activity *via* a mechanism involving PKC inhibition. This mechanism may be important in mediating neuroprotective effects and increased cerebral glucose metabolism by ketamine.

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