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Materials and Methods

SOM Text

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References

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Ketamine-Induced Loss of Phenotype of Fast-Spiking Interneurons Is Mediated by NADPH-Oxidase

M. Margarita Behrens,* Sameh S. Ali, Diep N. Dao, Jacinta Lucero, Grigoriy Shekhtman, Kevin L. Quick, Laura L. Dugan*

Abuse of the dissociative anesthetic ketamine can lead to a syndrome indistinguishable from schizophrenia. In animals, repetitive exposure to this *N*-methyl-D-aspartate-receptor antagonist induces the dysfunction of a subset of cortical fast-spiking inhibitory interneurons, with loss of expression of parvalbumin and the γ -aminobutyric acid-producing enzyme GAD67. We show here that exposure of mice to ketamine induced a persistent increase in brain superoxide due to activation in neurons of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Decreasing superoxide production prevented the effects of ketamine on inhibitory interneurons in the prefrontal cortex. These results suggest that NADPH oxidase may represent a novel target for the treatment of ketamine-induced psychosis.

The *N*-methyl-D-aspartate (NMDA)-receptor (NMDA-R) hypofunction theory of schizophrenia proposes that the effects of NMDA-R antagonists, such as phencyclidine (PCP) and ketamine, produce symptoms of schizophrenia in healthy humans because of specific effects on inhibitory circuits that lead to disinhibition of neurotransmitter systems (1). Disinhibition of glutamatergic activity, resulting in increased excitatory transmission, was confirmed in the prefrontal cortex (PFC) of rodents and non-human primates (2). However, after prolonged exposure, the increased excitatory neurotransmission is followed by a depression of brain activity (3) that occurs through an unknown mechanism.

Derangements of γ -aminobutyric acid (GABA)-mediated systems in schizophrenia have been consistently observed in postmortem tissue (4).

Initial in situ hybridization studies showed reduced expression of GAD67, the main isoform synthesizing GABA in brain (5). Subsequent studies showed also that the expression of the calcium-binding protein parvalbumin (PV) was reduced in postmortem samples (6, 7). Finally, NMDA-R antagonists also induce a decrease in PV expression (8, 9). This apparent "loss of GABAergic phenotype" in PV-containing interneurons led to the suggestion that dysfunction of these fast-spiking inhibitory interneurons may be a core feature of the disease (10).

PV interneurons are involved in the generation of gamma oscillations responsible for temporal-encoding and storage or recall of information required for working memory (11). These interneurons receive the largest glutamatergic input among all GABA-releasing neurons in cortex (12) and are highly sensitive to NMDA-R antagonists (13), a feature that may be related to the role played by NMDA-Rs in the control of basal synaptic activation in these interneurons (14).

We previously showed that primary cortical neuronal cultures respond to NMDA-R antago-

nists with a reversible loss of GAD67 and PV in PV interneurons (15). These neuronal cultures contain about 10 to 20% GABAergic neurons, of which 50% are PV interneurons (15), and show spontaneous glutamatergic and GABAergic activity (16, 17). We hypothesized that if the initial disinhibition of excitatory transmission produced by NMDA-R antagonists observed in vivo also occurred in cultured cortical neurons, then bypassing the need for GABA production by adding a γ -aminobutyric acid type A GABA_A agonist should prevent NMDA-R antagonist-mediated effects (18). Exposure to the GABA agonist muscimol prevented ketamine-mediated decrease in PV and GAD67 in PV interneurons (Fig. 1 and fig. S1), which suggested that loss of an inhibitory input to excitatory neurons, the main neuronal subpopulation in these cultures, is involved in the subsequent loss of phenotype of PV interneurons.

A rapid increase in reactive oxygen species (ROS) occurs in vitro (19), and in vivo (20) after exposure to NMDA-R antagonists, which indicates increased oxidative stress. However, what mechanism initiates this increase is not clear. The recent demonstration of expression of the superoxide-producing enzyme, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in hippocampus (21) led us to test the possibility that disinhibition of neurotransmission by NMDA-R antagonists leads to increased NADPH oxidase activity. We measured the oxidation product of dihydroethidium (DHE) by confocal microscopy and analyzed the levels of superoxide production in cultured neurons after prolonged exposure to low concentrations of ketamine. A significant increase in neuronal superoxide production was observed after 24 hours' exposure to 0.5 μ M ketamine, which was prevented by muscimol (Fig. 1). The increase in superoxide in response to ketamine was not restricted to PV interneurons (Fig. 1B), which suggested that activation of the enzyme(s) producing superoxide occurs throughout cortical neurons. We next determined whether the increase in superoxide was involved in the loss of GABAergic phe-

Department of Medicine, Division of Geriatric Medicine, University of California San Diego, La Jolla, CA 92093-0746, USA.

*To whom correspondence should be addressed. E-mail: mbehrens@ucsd.edu (M.M.B.); ladugan@ucsd.edu (L.L.D.)

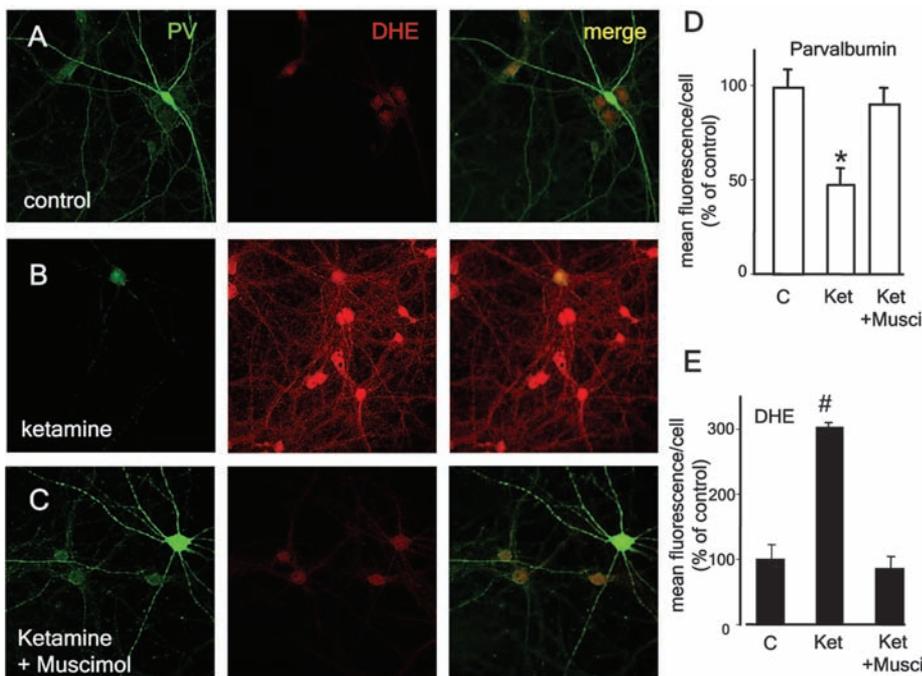


Fig. 1. Ketamine exposure in primary neuronal cultures increases superoxide production and induces the loss of PV immunoreactivity. Neuronal cultures were treated with ketamine (0.5 μ M) for 24 hours as described (15). DHE (1 μ g/ml) was added during the last hour of treatment. (A to C) Fluorescence confocal images of representative fields depicting a PV interneuron and surrounding neurons treated in the absence (control) or presence of ketamine, and in the presence of ketamine and muscimol (10 μ M). Quantification of (D) PV fluorescence, and (E) oxidized DHE. Significant when compared with control at $*P < 0.001$ by analysis of variance (ANOVA) followed by Tukey's test; $n = 5$ experiments per condition. Data are means \pm SEM.

notype of PV interneurons. Indeed, these effects of ketamine were prevented by cotreatment with a carboxyfullerene-based superoxide dismutase (SOD)-mimetic (C_3) (22) (Fig. 2, A and B).

To determine whether the activity of NADPH oxidase is involved in the ketamine-mediated increase in superoxide, we used the inhibitor apocynin (4-hydroxy-3-methoxy-acetophenone) (23). When cultures were exposed to ketamine in the presence of apocynin (at 0.5 mM), superoxide production was significantly reduced (Fig. 2A), and the loss of PV and GAD67 immunoreactivity in PV interneurons was prevented (Fig. 2B). Furthermore, this ketamine treatment increased significantly the expression of the NADPH oxidase subunit Nox2 in neurons (fig. S2).

NADPH oxidase subunits Nox2 and Nox4 are the main core-subunits expressed in forebrain (21). Nox2 is the isoform expressed in phagocytes and requires the membrane protein p22^{phox}, as well as a series of cytosolic proteins involved in its priming and activation. Bacterial infection and inflammation are known activators of Nox2. Nox4 is also dependent on p22^{phox} for activity, but seems to be a constitutive enzyme not requiring activation by cytosolic components. To determine whether ketamine induces NADPH oxidase in vivo, we used a subchronic regimen that consisted of intraperitoneal injections of ketamine (30 mg/kg on two consecutive days) to male C57BL/6 mice, followed by brain dissection 18 hours later. Although the acute effects of ketamine are not detected by this regimen, such

treatment permits the analysis of events that follow the initial disinhibition of the circuitry. We observed a significant increase in the expression of Nox2 and p22^{phox} (Fig. 3A), but not Nox4 (fig. S3C), in membrane preparations from cortex after ketamine treatment. This increase in protein levels was accompanied by an increase in NADPH oxidase activity in synaptosomes isolated from cortex of ketamine-treated animals (Fig. 3B), which suggested that the active enzyme was present at synapses. The increased oxidase activity in synaptosomes was inhibited in vitro by apocynin (Fig. 3B), which confirmed that the main oxidase isoform induced by ketamine in brain is Nox2. Metabolic activities of synaptosomal mitochondria were not affected by the treatment (fig. S3, A and B).

To assess the role of NADPH oxidase activation and superoxide production on PV interneurons, we characterized these interneurons in mouse PFC and analyzed the effects of the 2-day ketamine regimen on PV and GAD67 immunoreactivity. Ketamine induced a significant reduction in immunoreactivity for both proteins in PV interneurons (Fig. 4, A and C), which confirmed that GAD67 decreases in the same subset of interneurons. Moreover, this treatment produced a widespread increase in superoxide (Fig. 4, B and D), which was prevented when animals were pretreated with the NADPH oxidase inhibitor apocynin (5 mg/kg per day) for 1 week in the drinking water, or with the SOD-mimetic C_3 for 1 month (1.0 mg/kg per day, Alzet minipumps). Both treat-

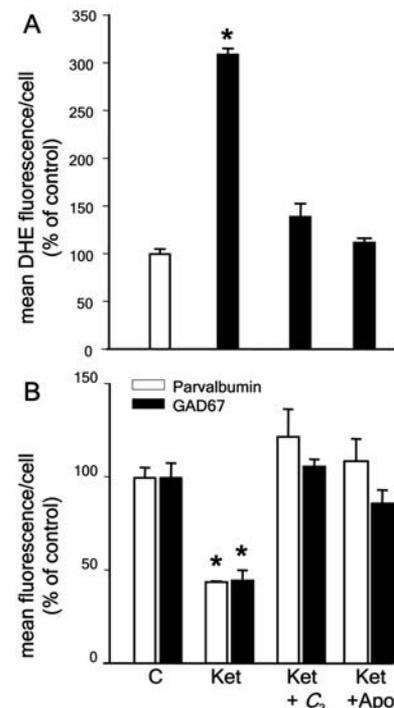


Fig. 2. Removal of superoxide or inhibition of NADPH oxidase activation prevents superoxide increase and reduction of PV and GAD67 in PV interneurons in culture. Cultures were treated with ketamine as in Fig. 1 in the absence or presence of the carboxyfullerene-based SOD-mimetic C_3 (20 μ M) or the NADPH oxidase inhibitor apocynin (0.5 mM). Quantification (A) of oxidized DHE fluorescence, and (B) of PV and GAD67 fluorescence in PV interneurons. Significant when compared with control at $*P < 0.05$ by ANOVA followed by Tukey's test; $n = 4$ experiments per condition. Data are means \pm SEM.

ments completely prevented the loss of PV immunoreactivity in PV interneurons (Fig. 4, B and E). Apocynin also prevented the decrease in GAD67 in PV interneurons in the PFC region (fig. S4).

Functional deficits in brain regions other than the PFC, such as hippocampus and thalamus, are known to contribute to schizophrenia symptoms (24, 25). We observed substantial increases in superoxide in several brain regions in addition to the PFC, including CA3 in the hippocampus and the reticular nucleus of the thalamus (Fig. 4, F and G) which suggested that increased NADPH oxidase activity occurs throughout the brain after drug exposure.

Regulatory redox sites have been found in many proteins that are involved in glutamatergic neurotransmission. These include the NMDA receptor itself, in which the oxidation status of a specific redox site on NR2A subunits (from the second class of NMDA receptor subunits) regulates the physiological activity of the receptor (26–29).

Although it is not clear whether the dysfunction of PV interneurons is a cause or consequence of the disease, and extrapolation from the NMDA-R antagonist model to schizophrenia is highly speculative, it is possible that prolonged inactivation of

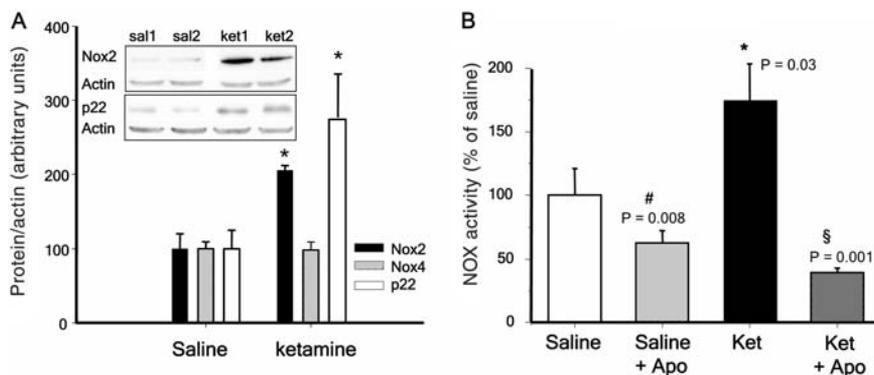


Fig. 3. In vivo ketamine treatment increased NADPH oxidase and p22^{phox} protein expression in brain membranes and increased the percentage of apocynin-inhibitable NADPH oxidase (Nox) activity in synaptosomes. Mice were treated with ketamine (30 mg/kg) on two consecutive days followed by 18 hours without the drug. (A) Membrane fractions were analyzed for the expression of the indicated proteins by Western blots (inset) and for Nox4 (fig. S3C). Bars graphically represent the quantification of Western blots normalized for actin content. Blots shown are for two saline-treated (sal1 and 2) and two ketamine-treated (ket1 and 2) animals. Significant compared with saline at **P* < 0.001 by ANOVA followed by Tukey's test; *n* = four animals per condition. (B) Increased NADPH oxidase activity was observed in

synaptosomal preparations from animals treated with ketamine as in (A). This activity was inhibited by apocynin. Values of NADPH-induced oxygen consumption (nmol O₂/mg protein per min) were 4.67 ± 0.98, control; 7.9 ± 1.8, ketamine (*n* = four animals per condition). Data are means ± SEM.

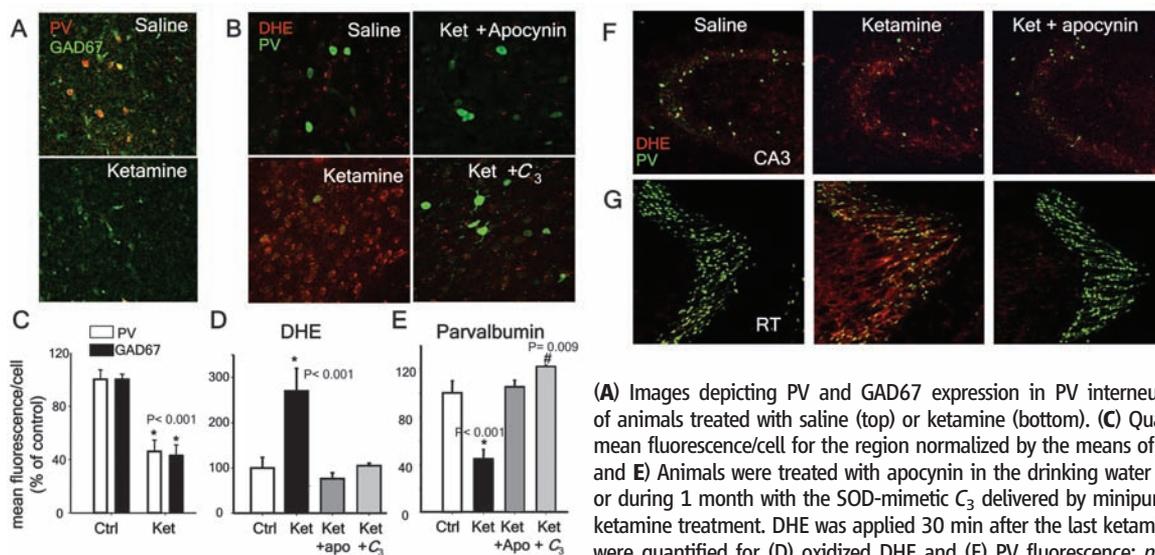


Fig. 4. Pretreatment of animals with the NADPH oxidase inhibitor apocynin, or with the SOD-mimetic C₃ reduces superoxide production and prevents the loss of PV immunoreactivity induced by ketamine. Animals were treated with ketamine as in Fig. 3, and coronal sections comprising the prelimbic and infralimbic regions were analyzed.

(A) Images depicting PV and GAD67 expression in PV interneurons in the prelimbic region of animals treated with saline (top) or ketamine (bottom). (C) Quantification of PV and GAD67 mean fluorescence/cell for the region normalized by the means of saline-treated animals. (B, D, and E) Animals were treated with apocynin in the drinking water for 1 week (5mg/kg per day), or during 1 month with the SOD-mimetic C₃ delivered by minipumps (1 mg/kg per day) before ketamine treatment. DHE was applied 30 min after the last ketamine injection. Coronal sections were quantified for (D) oxidized DHE and (E) PV fluorescence; *n* = six animals per condition. Significance with respect to saline at the indicated *P* values by ANOVA followed by Tukey's test is indicated by asterisk and #. (F and G) Confocal images of PV-stained sections depicting the increase in DHE oxidation in (F) hippocampal CA3 region and (G) the reticular nucleus of the thalamus induced by the 2-day ketamine treatment, and its prevention by pretreatment of animals with apocynin in the drinking water.

NMDA-Rs in PV interneurons, due to blockade by antagonists, or more physiologically, by NADPH oxidase-dependent oxidation, leads to a “misinterpretation” of the lack of signal through these receptors as decreased glutamatergic transmission. This, in turn, could be the signal that initiates processes resulting in reduced expression of GABAergic markers and loss of inhibitory capacity in PV interneurons, finally leading to a chronically decreased inhibitory tone in cortex. Recently, a specific decrease in GAD67 and NR2A was observed in a subpopulation of GABAergic interneurons in schizophrenic postmortem tissue (30).

In summary, we hypothesize that NADPH oxidase may be a contributor to oxidative mechanisms involved not only in the psychotomimetic effects of NMDA-R antagonists, but also in schizophrenia and other processes involving increased oxidative stress in the brain. Further understanding of mechanisms underlying activation or induction of brain NADPH oxidase may provide a completely new avenue for drug discovery aimed at the treatment of psychosis and cognitive deficits.

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Figs. S1 to S4
References

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