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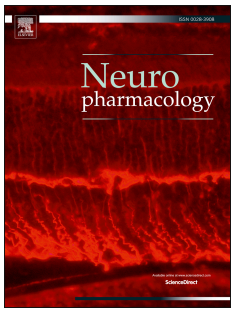

Two Cellular Hypotheses Explaining Ketamine's Antidepressant Actions: Direct Inhibition and Disinhibition

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A single, low dose of ketamine has antidepressant actions in depressed patients and in patients with treatment-resistant depression (TRD). Unlike classic antidepressants, which regulate monoamine neurotransmitter systems, ketamine is an antagonist of the N-methyl-D-aspartate (NMDA) family of glutamate receptors. The effectiveness of NMDAR antagonists in TRD unveils a new set of targets for therapeutic intervention in major depressive disorder (MDD) and TRD. However, a better understanding of the cellular mechanisms underlying these effects is required for guiding future therapeutic strategies, in order to minimize side effects and prolong duration of efficacy. Here we review the evidence for and against two hypotheses that have been proposed to explain how NMDAR antagonism initiates protein synthesis and increases excitatory synaptic drive in corticolimbic brain regions, either through selective antagonism of inhibitory interneurons via cortical disinhibition, or by direct inhibition of cortical pyramidal neurons.

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Chemical Compounds
Chemical compounds studied in this article:
Folimycin (PubChem CID: 6438151); Glutamate (PubChem CID: 33032); Ketamine (PubChem CID: 3821); Memantine (PubChem CID: 4054); MK-801 (PubChem CID: 180081); NBQX (PubChem CID: 3272524); Picrotoxin (PubChem CID: 5360688); Rapamycin (PubChem CID: 5284616); Riluzole (PubChem CID: 5070); Ro 25-6981 (PubChem CID: 6604887)

Abbreviations
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, **APs**; action potentials, **ECT**; electroconvulsive therapy, **EEG**; electroencephalography, **EPM**; elevated plus maze, **ERK**; extracellular receptor kinase pathway, **FS**; fast-spiking, **FST**; forced swim test, **$GABA_AR$** ; gamma-Aminobutyric acid A receptor, **$GABA_BR$**; gamma-Aminobutyric acid B receptor, **iINS**; inhibitory interneurons, **LH**; learned helplessness, **MDD**; major depressive disorder, **mEPSC**: miniature excitatory post-synaptic current, **mPFC**; medial prefrontal cortex, **NMDAR**; N-methyl-D-aspartate receptor, **PAM**; positive allosteric modulator, **PFC**; prefrontal cortex, **PNs**; pyramidal neurons, **PVs**; parvalbumin-expressing inhibitory interneurons, **RS**; regular spiking, **SPT**; sucrose preference test, **SSRIs**; selective serotonin reuptake inhibitors, **SSTs**; somatostatin-expressing inhibitory interneurons, **TRD**; treatment-resistant depression, **VIPs**; vasoactive intestinal peptide-expressing inhibitory interneurons

1. Introduction

A single, low dose of ketamine provides antidepressant action in depressed patients[1] and in patients with treatment-resistant depression (TRD)[2]. Unlike classic antidepressants, which act on monoamine neurotransmitter systems, ketamine is an antagonist of the N-methyl-D-aspartate (NMDA) family of glutamate receptors. While monoamine-based treatments such as selective serotonin reuptake inhibitors (SSRIs) show long latency to reach peak therapeutic efficacy, ketamine's effects occur with rapid onset. However, antagonism of NMDA receptors (NMDARs) can evoke psychotomimetic effects, and the antidepressant effects of a single infusion of ketamine are of limited duration. The effectiveness of NMDAR antagonists unveils a new set of targets for therapeutic intervention in major depressive disorder (MDD) and TRD. However, a better understanding of the cellular mechanisms underlying these effects is required for guiding future therapeutic strategies based upon modulation of NMDAR-mediated glutamatergic neurotransmission, in order to minimize side effects and prolong the duration of efficacy.

NMDARs are ionotropic, ligand-gated, glutamate-sensitive neurotransmitter receptors. Each NMDAR is a tetraheteromeric complex formed through assembly of two GluN1 and two GluN2 protein subunits. GluN1 is encoded by a single gene while GluN2 subunits are encoded by four different genes (GRIN2A-D). Ketamine is a non-competitive, voltage-dependent NMDAR channel blocker[3] and at membrane potentials near rest in the presence of physiological Mg^{2+}, ketamine blocks GluN2A- and GluN2B-containing NMDARs equally[4]. Ketamine works as an antidepressant only at low doses,

while with increasing doses it evokes psychotomimetic actions and eventually produces anesthesia. Data from preclinical models show that low-dose ketamine initiates protein synthesis and enhances excitatory synaptic drive in corticolimbic brain regions. This increase in excitatory drive is presumed to underlie the observed antidepressant-like behaviors[5–7]. Yet, the exact cellular mechanisms that initiate protein synthesis and increased excitatory synaptic drive remain unclear. In terms of mechanistic understanding, it is important to note that the initiation of a cellular action by an *antagonist* presupposes that the receptor is tonically activated in order for receptor blockade to induce a response. Here we describe and review the evidence for and against two hypotheses that have been proposed to explain how NMDAR antagonism initiates protein synthesis leading to increased excitatory synaptic drive in corticolimbic brain regions.

The first hypothesis proposes that low dose ketamine selectively antagonizes NMDARs on cortical inhibitory interneurons (iINs) leading to *disinhibition* and indirect excitation of excitatory pyramidal neurons (PNs), which in turn initiates protein synthesis and activity-dependent synaptic plasticity resulting in an increase in excitatory synaptic drive. Under the second hypothesis, *direct* antagonism of NMDARs on PNs induces a protein synthesis-dependent and cell-autonomous form of homeostatic synaptic plasticity resulting in increased excitatory synaptic drive onto these neurons. Here we refer to these as the *disinhibition* and the *direct* hypothesis of ketamine's cellular action, respectively (Figure 1). In this review, we overview and contrast the data supporting each of these non-mutually exclusive mechanisms with the aim of providing a

framework for future experiments to test the cellular mechanisms of ketamine's actions and help define future therapeutic strategies.

2. A Disinhibition Hypothesis of Ketamine's Actions

One widely-cited hypothesis posits that ketamine's antidepressant effects require disruption of tonic GABA-mediated synaptic inhibition (disinhibition) resulting in increased activity in PNs and an increase in excitatory synapse number through an activity-dependent form of synaptic plasticity potentially similar to long-term potentiation (LTP)[5,8] (Figure 1B). According to this hypothesis, NMDARs on iINs are tonically active to maintain inhibitory synaptic tone and limit action potentials (APs) in PNs. Ketamine disrupts this balance by preferentially antagonizing NMDARs on iINs. Under this hypothesis, NMDAR activation must disproportionately support the excitability of iINs compared to PNs. Specifically, excitatory synapses onto iINs should be more dependent upon NMDAR-mediated neurotransmission than excitatory synapses onto PNs.

2.1 Contribution of NMDARs to iIN and PN Excitability: In Vitro Evidence

Three types of cells account for nearly 100% of iINs in cortex[9]. These are the parvalbumin-expressing iINs (PVs), somatostatin-expressing iINs (SSTs) and 5HT3a-expressing iINs, the last of which include all of the vasoactive intestinal peptide expressing iINs (VIPs). Of these three general classes, PVs have been the mostly highly studied. PV*s* are fast-spiking (high frequency of APs) and provide strong perisomatic inhibition to surrounding PNs[10]. Consistent with this hypothesis that

ketamine influences iIN excitabitily, some iINs in CA1 and CA3 regions of the hippocampus are highly sensitive to application of NMDA, and a significant NMDAR population on these cells contributes to their excitability as measured by bath application of NMDA in acute brain slices[11,12]. Additionally, the NMDAR antagonist, Ro 25-6981, which is selective to receptor complexes containing the GluN2B subunit, reduces synaptic NMDAR-mediated current and AP frequency more strongly in some iINs compared to PNs[13]. However, other reports have shown that current through NMDARs plays only a minor role in synaptic activation of fast-spiking (FS) iINs compared to regular spiking (RS) PNs in both hippocampal and cortical circuits *ex vivo*[14]. In cortical layer V of motor cortex for example, NMDARs contribute negligibly to post-synaptic currents in FS neurons at sub-threshold potentials[15]. In prefrontal cortex (PFC), synaptic activation leading to AP firing in iINs is significantly more dependent on AMPARs than on NMDARs, compared to PNs[16]. In light of these seemingly discrepant data it is important to note that differences in the contribution of NMDARs to postsynaptic currents depend upon the developmental age of the synapse[17] as well as the exact type of PN to iIN synapse under investigation.

In fact, some experimental evidence suggests that the NMDAR contribution to synaptic currents onto PNs is stronger than that onto PVs[14,16,17]. In ultrastructural studies of the hippocampus, lower levels of the obligatory NMDAR subunit GluN1 were found at glutamate synapses onto PVs compared with spiny synapses onto PNs[18]. Furthermore, excitatory synapses onto FS iINs show approximately threefold greater contribution of AMPARs than NMDARs[14,16,17,19,20], a ratio that is significantly higher than synapses onto

PNs. In addition to synaptic currents, tonic activation of NMDARs can also contribute to cell excitability. Persistent, low-level ambient glutamate in the extracellular space activates NMDARs to provide a constant, depolarizing current which brings the resting membrane potential closer to threshold, and thus renders cells more excitable[21]. Interestingly, blocking this tonic NMDAR-mediated current in either FS iINs or PNs in PFC results in equal changes in holding current in response to NMDAR antagonism, suggesting similar levels of basal activation between cell types[21]. It has also been shown that application of the use-dependent NMDAR antagonist MK-801 reduces inhibitory drive onto PNs in some, but not all areas of cortex[22], implying that the effects of NMDAR antagonism might result in brain region selective changes, based upon differences in basal levels of receptor activation.

2.2 Contribution of NMDARs to iIN and PN Excitability: In Vivo Evidence

The relative action of NMDAR antagonists on iINs and PNs has also been tested *in vivo*. Single unit recordings in PFC reveal that acute injection of the activity-dependent blocker MK-801 results in a reduction in AP frequency in FS cells (high frequency of APs = putative iINs), which is followed by a subsequent increase in the frequency of RS cells (low frequency of APs = putative PNs)[23]. However, in other studies, application of ketamine results in decreased firing of FS neurons but no change, on average, on RS neuron AP frequency[24]. While inconclusive, discrepancies between these results could, once again, be explained by distinct sub-populations of iINs being affected by treatment with the antagonist. Future studies will be needed to fully understand this complexity,

which could be due not only to the type of iIN but also the specific region of cortex under investigation.

2.3 Genetic Manipulations and the Disinhibition Hypothesis

An additional approach employed to test the role of NMDARs in specific subsets of neurons *in vivo* is directed gene deletion using conditional knockout alleles and cell type-specific promoters to drive expression of recombinase enzymes. According to the disinhibition hypothesis, removal of NMDARs from PVs using parvalbumin-promotor driven Cre-recombinase expression and a conditional GluN1 allele should disinhibit PNs and mimic ketamine's effects. However, mice lacking NMDARs specifically in PVs do not display alteration in basal levels of despair-like phenotype as measured either in the Forced Swim Test (FST) or Sucrose Preference Test (SPT)[25]. Furthermore, these animals retained their antidepressant-like behavioral response to ketamine despite lacking the putative substrate for NMDAR antagonism. In a second study, genetic removal of NMDARs from inhibitory interneurons using Ppp1r2-Cre failed to elicit an antidepressant response in FST, and Ro 25-6981's antidepressant effect remained intact in these mice[26]. In these studies, however, NMDARs were removed by genetic deletion of the obligatory GluN1 subunit during development and therefore compensatory alterations such as a decrease in PN firing rates may be responsible for the absence of a predicted effect. Interestingly, increased PN firing persisting into adulthood is observed in mice following developmental genetic knockout of NMDARs (GluN1) in cortical iINs. Moreover, these animals exhibit an increase in anxiety-like behavior measured in the Elevated Plus Maze (EPM)[27].

In contrast, recent work has shown that elimination of NMDARs in PNs *alone* is sufficient to induce antidepressant-like effects in mice. This was demonstrated by developmental, selective genetic deletion of the NMDAR subunit GluN2B from forebrain PNs[7]. In these animals, basal antidepressant- and anxiolytic-like effects were measured in FST, TST and EPM. GluN2B removal from PNs also occluded any further antidepressant-like action of ketamine. In addition to implicating NMDARs on PNs, rather than iINs, these data also support the idea of a critical role for GluN2B-containing NMDARs in ketamine's actions. Unfortunately, interpretation of these results is complicated by the fact that such genetic manipulations result in chronic and developmental removal of this receptor pool. Key experiments that remain to be performed are to test for antidepressant-like actions by acutely removing NMDAR subunits from iIN subclasses or PNs after development, to avoid compensatory confounds associated with chronic loss of NMDAR signaling. Therefore, understanding the cell-types and potential subtypes of NMDARs required for ketamine's actions will require more precise and acute manipulations, for example post-developmental injections of virally encoded Cre-recombinase into animals containing LoxP-flanked NMDAR alleles.

2.4 Pharmacological Manipulations and the Disinhibition Hypothesis

An additional prediction of the disinhibition hypothesis is that suppression of GABA receptor-mediated inhibition, for example by administration of GABA-receptor antagonists, should mimic ketamine's antidepressant effect through disinhibition of the

PN population. Surprisingly, in preclinical animal models, injection of the ionotropic $GABA_A$-receptor ($GABA_AR$) antagonist picrotoxin has no effect on depression-like behavior measured as immobility in FST[6]. Antagonism of the metabotropic GABAB-receptor ($GABA_BR$) also has inconsistent effects on behavioral depression tests, as the $GABA_BR$ antagonists CGP35348 and CGP56433A showed no effect on immobility in FST[28,29], whereas the antagonist CGP55845A caused a decrease in immobility in FST[29]. Conversely, *enhancing* inhibition either with a $GABA_AR$ agonist, or a $GABA_BR$ agonist or positive allosteric modulator (PAM) is sufficient to cause an antidepressant-like effect as measured in the FST[30,31]. However, a negative result after broad GABAR antagonism does not rule out a role for iIN suppression in providing antidepressant like actions. As discussed above, the effect of ketamine and other NMDAR antagonists on specific types of iINs *in vivo* remains to be elucidated.

Synchronized oscillations play important roles in brain circuit function and electroencephalography (EEG) measurements can reveal details about the state of excitatory and inhibitory balance in cortical circuits *in vivo*. Gamma frequency band (30-90Hz) oscillations reduce circuit noise, amplify signal gain, and enhance signal transmission in cortex[32,33]. These oscillations contribute to cognitive function and are disturbed in psychiatric disorders including schizophrenia[34] and depression[35]. NMDARs on PVs are known to regulate gamma oscillations[19,20,36,37]. In fact, elevated gamma band EEG power is considered a putative measure of cortical disinhibition[38,39]. In extracellular and EEG studies, acute application of NMDAR antagonists causes aberrant cortical gamma oscillations coincident with the time-

course of disinhibition[36,40–42]. However, while GluN2B antagonism alone recapitulates ketamine's antidepressant effects[5,7,43], GluN2B-specific antagonists do not alter gamma power *in vivo*[44,45].

The disinhibition hypothesis proposes that increased activity in PNs leads to glutamate release and induction of an LTP-like synaptic plasticity, in response to temporary removal of cortical inhibition. While elevated levels of glutamate can be measured in response to NMDAR antagonism, these do not appear to be causally related to the antidepressant effect. Specifically, while pan-NMDAR and GluN2A-specific antagonists elevate extracellular glutamate, GluN2B-selective NMDAR antagonists do not have this effect, despite their antidepressant actions[46]. What is clear, however, is that EEG and extracellular glutamate measurements support a role for GluN2B-containing NMDARs in the antidepressant actions of low-dose ketamine and together with the data discussed above implicate NMDARs on PNs rather than iINs as a critical target.

2.5 Electroconvulsive Therapy and the Disinhibition Hypothesis

While evidence for a role of disinhibition in alleviating TRD remain somewhat contradictory, pre-clinical and clinical data do support a role for cortical over-excitation in providing antidepressant effects. Perhaps the strongest evidence of this is the effectiveness of electroconvulsive therapy (ECT), which is an effective treatment option in TRD. The principle function of ECT is to initiate an acute generalized seizure. The ECT-induced seizure progresses from low voltage fast activity, to high amplitude

polyspikes discharges, to 4-6Hz wave discharges[47]. Particularly important to ECTs efficaciousness is acutely increased prefrontal excitation in the theta and delta bands, the last of which is directly correlated with the magnitude of self-reported mood improvement in this treatment[48]. Similarly, ketamine treatment has been shown to acutely increase EEG power in these frequencies[42]. Furthermore, mechanisms underlying ECT-induced plasticity seem to be similar to ketamine in that they appear to depend on synaptogenesis and alter functional connectivity in limbic brain regions[49–51].

3. A Direct Hypothesis of Ketamine's Actions

On the other hand, evidence from preclinical mouse models strongly support the idea that *direct* targeting of NMDARs on PNs contribute to the initiation of ketamine's antidepressant actions through a cell-intrinsic mechanism (Figure 1C). According to this hypothesis, ketamine disrupts basal activation of NMDARs on PNs. Removing this activity engages a mechanism of homeostatic synaptic plasticity that results in a rapid compensatory *increase* in excitatory synaptic input onto these neurons in a protein-synthesis dependent manner. There is also strong evidence that this basal activation of NMDARs and ketamine's actions are mediated through a specific class of NMDARs, those containing the GluN2B subunit, which is consistent with the fact that GluN2B-selective antagonists are also effective antidepressants in patients and evoke antidepressant-like behaviors in preclinical animal models as mentioned above.

3.1 GluN2B-Containing NMDARs and the Direct Hypothesis

GluN2B-selective NMDAR antagonists mimic the antidepressant actions of ketamine in clinical populations and decrease immobility in preclinical models of depression including FST and TST[5,7,43]. Moreover, microinfusion of a GluN2B antagonist into medial prefrontal cortex (mPFC) is sufficient to elicit an antidepressant-like response in FST, yet genetic removal of NMDAR from iINs does not occlude this response[26]. GluN2B-containing NMDARs, distinct from GluN2A containing NMDARs, act via mTOR to limit protein synthesis and regulate excitatory synaptic plasticity in a homeostatic manner[7,52–54]. Predictive of a direct action on PNs, selective genetic removal of GluN2B from cortical PNs results in enhanced protein synthesis rates and increased excitatory drive measured in layer II/III pyramidal neurons of PFC[7]. In addition, this genetic manipulation both mimics and occludes the behavioral actions of ketamine measured in FST and TST[7]. Consistent with a role for mTOR, the behavioral effects of ketamine in rodents can be blocked with the mTOR antagonist rapamycin[2]. Furthermore, GluN2B-containing NMDARs specifically in PNs have been shown to be required for ketamine's actions and for regulation of protein-synthesis dependent homeostatic synaptic plasticity[52,53]. While these data clearly implicate the involvement of NMDAR antagonism on PNs directly, they also suggest a GluN2B-dependent mechanism, which raises the question of how a non-subunit selective antagonist like ketamine can provide a seemingly subunit specific antagonistic effect?

3.2 Ambient Glutamate and the Direct Hypothesis

One way in which this can be explained is by noting that GluN2B-containing NMDARs are uniquely activated under conditions of basal cortical activity by ambient glutamate[7].

In addition to being stimulated in a phasic manner by AP-coordinated transmitter release, NMDARs can also be activated by low-level ambient glutamate in the extracellular space (Figure 2). Levels of extracellular glutamate are tightly regulated by the glial glutamate transporter EAAT2 (GLT-1)[55,56]. While the absolute concentration and potential physiological roles of ambient glutamate are highly debated, ambient glutamate produces a tonic current that can be measured *in vitro* or *ex vivo*[21,57,58] and is mediated by GluN2B-containing NMDARs, as it is absent in GluN2B null cortical neurons and in neurons in which GluN2B has been genetically replaced with GluN2A[7]. Selective activation of GluN2B-containing NMDARs could be the result of receptor availability based on sub-cellular location, with GluN2B-containg NMDARS being more dominant component of the extrasynaptic receptor pool. Additionally, selective activation of GluN2B-containing NMDARs by low-level ambient glutamate is consistent with their higher sensitivity to agonist and decreased sensitivity to Mg2+ mediated block, compared to GluN2A-containing receptors. If levels of ambient glutamate are important for depression-like behaviors in rodents this could be tested, *in vivo*, using known regulators of glutamate reuptake. In support of this hypothesis, increasing or decreasing glutamate reuptake, and thereby decreasing and increasing tonic GluN2B activation, respectively, bidirectionally regulates excitatory synaptic drive measured as an increase or decrease in mEPSC frequency onto PNs both *in vitro* and *ex vivo* and increasing glutamate reuptake results in an antidepressant phenotype in the TST[7].

These data suggest that abnormally high ambient glutamate levels may act through a GluN2B-dependent signaling mechanism on PNs to limit excitatory synaptic drive and

lead to depression or depression-like behaviors in rodent models. A direct, PN-based model of ketamine's actions is therefore consistent with the association between decreased glial cell based glutamate transporter function and depression as observed in MDD and preclinical models of depression. This includes a large body of evidence from animal models; including, decreased expression of EAAT glutamate transporters following exposure to a learned-helplessness paradigm in rats[59], decreased glial cell density in the PFC of chronically stressed animals[60], retraction of glial cell coverage in response to stress[61], and the induction of depression-like behavior in animals following chemical ablation of glial cells in PFC[62]. There is also supporting data from humans where decreased density of glial cells has been documented in postmortem PFC of depressed patients[63].

An interesting consideration in light of the preclinical successes of ambient glutamate modulators is the potential use of riluzole, a clinically approved ALS medication, in MDD. Riluzole has been shown to decrease extracellular glutamate via inhibition of presynaptic glutamate vesicle release and enhancement of EAAT-dependent glutamate reuptake *in vitro*[64–66], and based upon the direct hypothesis should elicit an antidepressant response. Preclinical data have been supportive of this hypothesis[60,67,68], however, clinical studies have produced mixed results[69–75]. Future investigations evaluating riluzole and other glutamate reuptake modulators will be paramount to understanding the role of ambient glutamate and its potential use for targeting depressive disorders.

3.3 Spontaneous Neurotransmitter Release and the Direct Hypothesis

In addition to ambient glutamate, basal activation of NMDARs via synaptic, vesicular, but AP-independent, glutamate release also regulates protein synthesis and synaptic strength in a homeostatic manner[76,77]. AP-independent glutamate release occurs by spontaneous fusion of single pre-synaptic vesicles[78,79] and each individual fusion event results in what is termed a miniature excitatory post-synaptic current (mEPSC). *In vitro*, these mEPSCs are sufficient to tonically suppress protein synthesis[80] to maintain synaptic strength. Applying ketamine to non-stimulated hippocampal slices reliably scales up AMPAR-mediated responses, as measured by field potential recording, and causes dephosphorylation eEF2[6,81], which is permissive for elongation of nascent polypeptides during protein synthesis (Figure 2). Moreover, selectively depleting vesicles from the spontaneously released vesicle pool[82] using folimycin in the presence of TTX mimics the effects of ketamine on hippocampal brain slices[81]. Folimycin inhibits re-acidification of transmitter vesicles and thereby disables VGLUT-mediated refilling of the spontaneously released vesicle pool. Unfortunately, it is challenging to test the role of miniature transmission *in vivo* and therefore its role in NMDAR antagonist-mediated antidepression remains untested. There is, however, convincing evidence suggesting that the vesicle pool and release machinery contributing to this spontaneously released vesicle pool are distinct from the readily-releasable pool of AP-driven vesicles[82–84] and that they may even be sensed by unique post-synaptic NMDARs[85]. Thus, *in vivo* manipulation of presynaptic proteins specifically associated with the spontaneously released vesicle pool, such as VAMP7/Vti1a, could be used to directly test the hypothesis that suppression of NMDAR activation by this release mechanism is involved in ketamine's effects[84,86]. Additionally, this could be tested by targeting the pool

of post-synaptic NMDARs that are sensitive to this spontaneous presynaptic release[85]. One clear and intriguing prediction from these data is that spontaneous activity is also sensed by GluN2B-containing NMDARs. Together, these data strongly support the direct hypothesis in which ketamine antagonizes the activity of NMDARs activated by spontaneously released and/or ambient glutamate to cause homeostatic increase in excitatory inputs in a protein-synthesis dependent manner.

4. A Role for AMPAR Activation

Studies in preclinical animal models have also shown that glutamate-sensitive, ionotropic AMPA receptors (AMPARs) are required for ketamine's effects, although the exact role they play has yet to be defined. Pretreatment with the AMPAR antagonist NBQX prevents the antidepressant-like actions of ketamine, as measured in FST, TST, and learned helplessness (LH)[5,8,87], and blocks the associated increase in protein synthesis[5]. While the effects of broad AMPAR antagonism would no doubt be wide-ranging, it is of interest to consider how AMPARs might play a role under the direct and disinhibition hypotheses. The disinhibition hypothesis posits that ketamine-induced increases in PN spiking drives synaptic release of glutamate resulting in elevated extracellular concentrations of transmitter, potentially via synaptic spillover. The resulting LTP-like synaptic plasticity proposed under this hypothesis would require AMPAR activation, consistent with the effects of AMPAR antagonists in blocking ketamine's actions. Interestingly, sub-anesthetic (which produce antidepressant actions) but not anesthetic doses of ketamine (which do not produce antidepressant actions)[6] significantly increase glutamate in the rat medial PFC (mPFC), and human anterior

cingulate cortex[88,89]. It is important to note, however, that strong, transient extracellular increases in glutamate can lead to glutamate-mediated cell toxicity, which needs to be considered under this hypothesis. Moreover, while the GluN2B antagonist Ro 25-6981 is a potent antidepressant in both preclinical models and patients, it does not result in elevated extracellular glutamate[46], suggesting that elevated extracellular glutamate is not causal for NMDAR antagonist-mediated antidepression. An unexpected increase in levels of the inhibitory transmitter GABA in mPFC of rats in response to low-dose ketamine has also been observed. Although it is unclear what the functional consequence of this potentially compensatory enhancement of inhibition might be[88], it should also be noted that a study in humans revealed no change in the amount of cortical GABA in response to ketamine[89]. Nonetheless, if increased extracellular glutamate is associated with low-dose ketamine there is a prediction for the requirement for AMPAR activation under the disinhibition hypothesis in supporting LTP-like synaptic plasticity.

An alternative hypothesis we propose is that AMPAR activation might be required at newly formed, or recently potentiated, excitatory synapses for the *maintenance* of these contacts following induction by ketamine. Functional AMPARs are expressed on the surface of nascent spines[90] and their activation is required for their preservation[91–93]. Under both the disinhibition and direct hypotheses, NMDAR antagonism rapidly induces, or strengthens existing AMPAR-competent spines, which then would require activation by glutamate in order to be maintained. It also follows then that variability in the duration of ketamine's antidepressant actions could be causally-related to the

maintenance of the antagonist-induced increase in excitatory synapses. Alternatively, AMPAR activation may be necessary during NMDAR blockade for the initiation of ketamine's effects by providing basal activation of additional signaling pathways including those involved in supporting protein synthesis, for example, activation of the extracellular receptor kinase (ERK) pathway. However, at this point, other than a clearly predicted role for AMPAR activation, these mechanisms are inferred and a better understanding of the requirement for AMPAR activation in ketamine's antidepressant actions will require additional experiments. These investigations should include assessing the temporal requirement of AMPAR antagonism in blocking the antidepressant-like effects of ketamine in animal models, to dissociate possible roles in induction and/or maintenance of these excitatory synapses.

5. Requirement for Low Dose NMDAR Antagonism

Why does NMDAR antagonism result in such different actions based upon dose? Ketamine is an antidepressant at low dose, but evokes psychomimetic actions and eventually anesthesia at increasing doses. The direct hypothesis predicts that low levels of ketamine selectively block a pool of receptors dominated by GluN2B-containing receptors that are tonically activated by spontaneous release and/or ambient glutamate. As mentioned above, the higher sensitivity of GluN2B-containing NMDARs to ambient glutamate and therefore their tonic activation would make them more sensitive to low-dose antagonism. Under higher concentrations of ketamine, synaptic NMDARs might be gradually blocked leading to dissociative effects and eventually anesthesia. Although the location of GluN2B- and GluN2A-containing NMDARs relative to the post-synaptic

density remains highly debated, there is evidence from many studies that GluN2B-containing NMDARs dominate the peri- or extra-synaptic population of NMDARs, potentially making them more accessible to exogenous antagonism. While differential NMDAR subtype antagonist sensitivities provide a potential explanation for the diverging effects of ketamine across concentrations, it is important to note that off-target, non-NMDAR effects may come into play at higher doses[94–96]. For example, at high concentration, ketamine binds to opioid receptors and enhances phosphorylation of ERK1/2. Across labs, in vitro studies to date have utilized inconsistent and potentially non-clinically relevant concentrations to dissect the basic mechanisms underlying ketamine's antidepressant effect. Future *in vitro* testing of these hypotheses should prioritize the use of appropriate doses. To allow for this, a more rigorous analysis of ketamine's circulating CNS concentration following a clinically relevant peripheral dose needs to be determined for specific experimental mouse and rat strains. These studies must be guided by detailed knowledge of ketamine pharmacology including properties such as non-specific protein-binding, transporter substrate information, and therefore calculation of the free concentration of ketamine available to the NMDAR at doses correlated with its antidepressant actions *in vivo*.

6. Future directions

Further studies are required to clearly delineate the cellular mechanisms underlying the rapid antidepressant actions of ketamine and define future intervention in MDD and TRD. The two, non-mutually exclusive, cellular hypotheses described here provide a framework for these experiments. Many major questions remain, for example: what are

the brain circuits and cell types effected by NMDAR antagonism and how might changes in these individual brain regions underlie behavioral endophenotypes associated with depression and depression-like behaviors in preclinical models? For example, optogenetic experiments have shown that decreased immobility in FST can be driven by activation of a subset of PFC PN neurons[97] while measures of anhedonia but not despair-like behavior are associated with synaptic changes in nucleus accumbens[98]. This effort will be critically aided by widely available genetic techniques for manipulation of NMDARs and cell signaling pathways using conditional genetic knockout and recombinase-encoding viruses, as well as techniques in optogenetics and chemogenetics to probe cell types and brain regions involved by direct manipulation of their activity. The relative expression and contribution of NMDARs of specific subunit subtype also needs to be clearly delineated in terms of their contribution to synaptic and extrasynaptic events and in different iIN and PN synapses.

Going forward it is also important to remember that NMDAR function is fundamental to normal brain function. As such, NMDAR antagonism not surprisingly produces side effects that could render ketamine or any other pan-NMDAR antagonist unsuitable for long-term treatment. However, despite this and the current lack of knowledge regarding the exact synaptic and cellular mechanisms, the ability of ketamine to evoke antidepressant action in severe TRD patients with just a single low-dose infusion is a remarkable effect and one that exposes the field to a diverse new set of targets associated with the post-synaptic NMDAR complex[99]. The potential of NMDAR modulation as an effective treatment in MDD is also supported by three recent phase II

clinical trials in which the NMDAR modulators GLYX-13 (NCT01234558), NRX-1074 (NCT02067793), and AZD6765[100] (NCT01482221) demonstrated significant improvement in depression scores. Positive results like these have been extremely rare in target-based therapy development in neuropsychiatric disorders in general, and provide great hope for developing new and effective drugs for TRD and MDD.

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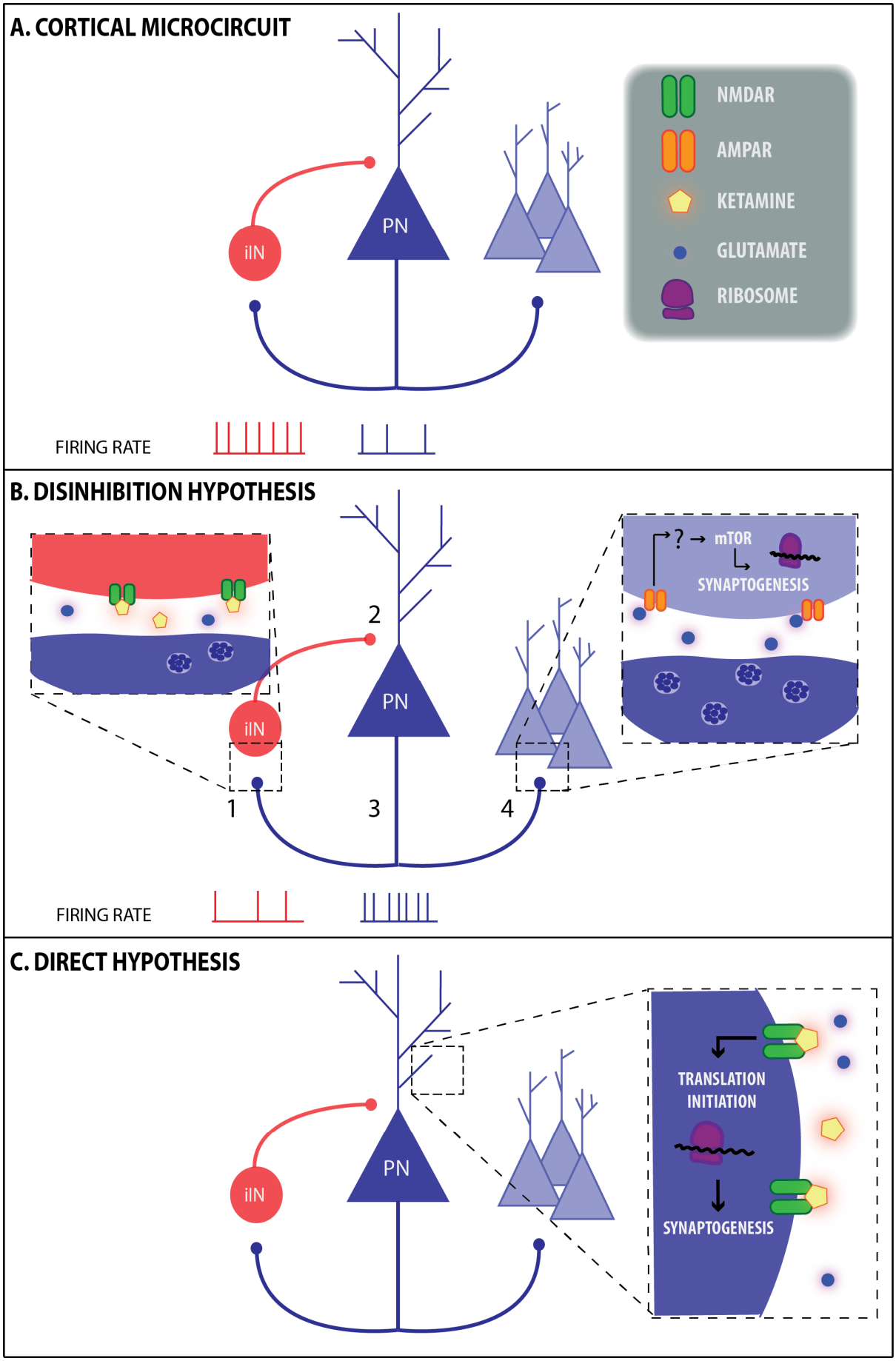
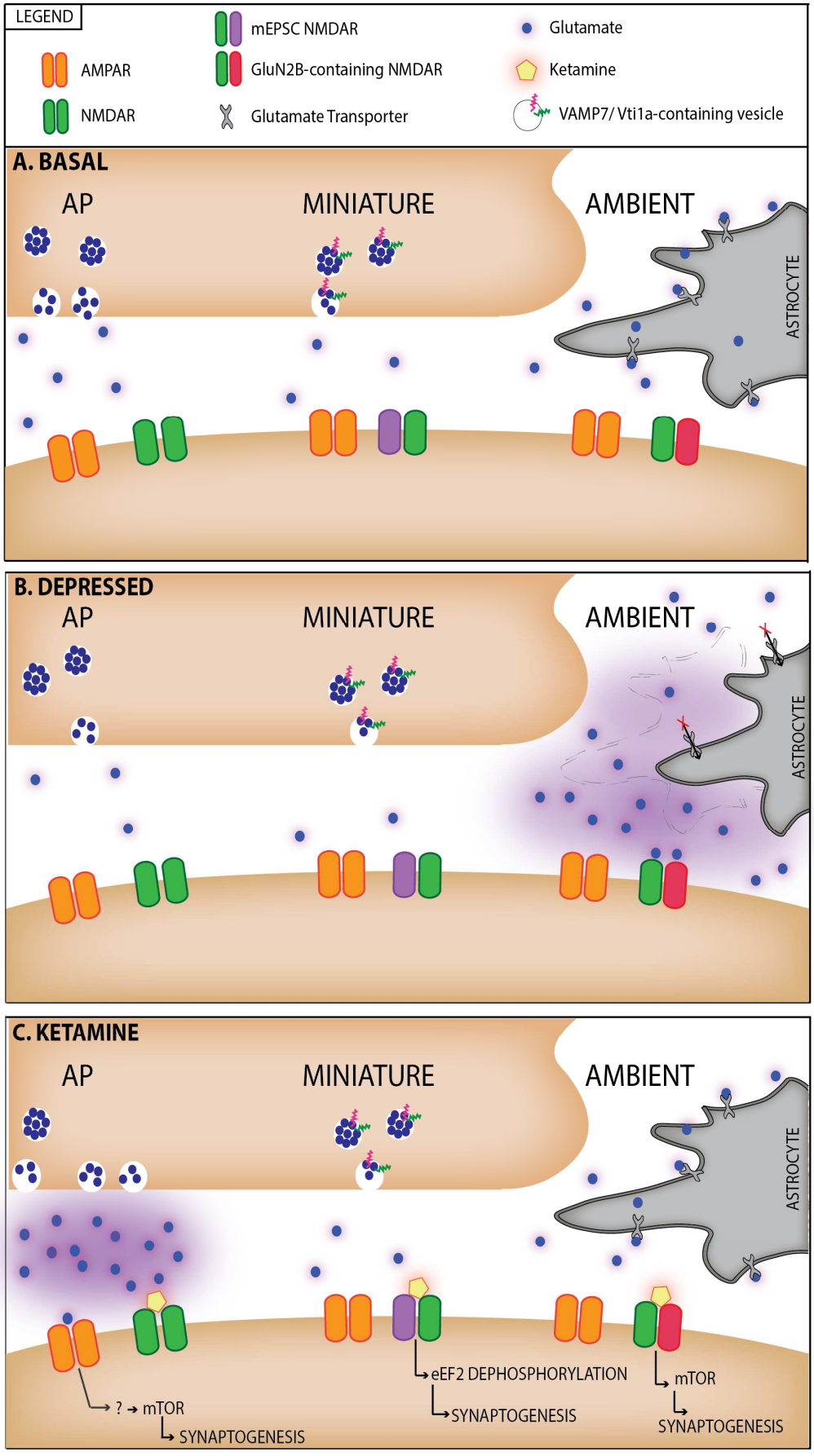
Figure Legends:

Figure 1: Disinhibition and Direct Inhibition: Two Hypothesis Explaining the Initiation of Protein Synthesis and Increase in Excitatory Synapses by Ketamine. A) A simplified cortical microcircuit is shown to demonstrate the two potential sites of action for ketamine, which leads to increased protein synthesis and excitatory synapses. Local interneurons (iIN), including fast spiking parvalbumin-expressing neurons, provide inhibition of principal pyramidal neurons (PN) that have low basal firing rates. B) Under the disinhibition hypothesis ketamine selectively antagonizes the PN to iIN excitatory synapses leading to loss of tonic inhibition and increased firing followed by LTP-like synaptic plasticity and an increase in excitatory synapses. C) According to the direct hypothesis ketamine antagonizes NMDARs at excitatory synapses onto PNs that are tonically activated by ambient glutamate and/or spontaneous transmitter release. This results in homeostatic increase in excitatory synaptic drive in a protein-synthesis dependent manner.

Figure 2: Tonic Activation of NMDARs by Glutamate and Blockade by Ketamine Under Basal and Depressed Conditions. Initiation of protein synthesis by an NMDAR antagonist – ketamine - suggests that NMDARs are tonically active. A) Three mechanisms of basal NMDAR activation are shown: AP-dependent vesicular release of glutamate, spontaneous (miniature) vesicular release of glutamate, and NMDAR activation by ambient glutamate. B) Changes in levels of activity or the concentration of ambient glutamate result in changes in excitatory transmission in the depressed state, which may include retraction or decreased functioning of glutamate buffering astrocytes. C) Under the disinhibition hypothesis ketamine blocks synapses onto iINs leading to increased AP-mediated glutamate release, increased extracellular levels of transmitter and synaptogenesis through an mTOR mediated increase in protein synthesis. Under the direct hypothesis alterations in spontaneous, miniature vesicular release are blocked by ketamine to drive eEF2 dephosphorylation and synaptogenesis. Alternatively, ketamine may block an increase in activation of GluN2B-containing NMDARs by increased levels of ambient glutamate, which evokes mTOR resulting in increased synaptogenesis.


A. CORTICAL MICROCIRCUIT
PN
iIN
NMDAR
AMPAR
KETAMINE
GLUTAMATE
RIBOSOME
FIRING RATE
B. DISINHIBITION HYPOTHESIS
2
PN
iIN
1
3
4
? → mTOR
SYNAPTOGENESIS
FIRING RATE
C. DIRECT HYPOTHESIS
PN
iIN
TRANSLATION INITIATION
SYNAPTOGENESIS


LEGEND
AMPAR
NMDAR
mEPSC NMDAR
GluN2B-containing NMDAR
Glutamate Transporter
Glutamate
Ketamine
VAMP7/ Vti1a-containing vesicle
A. BASAL
AP
MINIATURE
AMBIENT
ASTROCYTE
B. DEPRESSED
AP
MINIATURE
AMBIENT
ASTROCYTE
C. KETAMINE
AP
MINIATURE
AMBIENT
ASTROCYTE
? → mTOR
SYNAPTOGENESIS
eEF2 DEPHOSPHORYLATION
SYNAPTOGENESIS
mTOR
SYNAPTOGENESIS

Title: Two Cellular Hypotheses Explaining Ketamine's Antidepressant Effects: Direct Inhibition and Disinhibition

Authors: Oliver H. Miller, Jacqueline T. Moran and Benjamin J. Hall

Highlights

- Low dose ketamine produces antidepressant actions in MDD and TRD patients
- The cellular mechanisms underlying ketamine's antidepressant actions remain unclear
- Two mechanistic hypotheses to explain how ketamine's actions are initiated are reviewed here
- These two hypotheses offer a framework for studying ketamine's effects as an anti-depressant