

# NMDA Receptors Subserve Persistent Neuronal Firing during Working Memory in Dorsolateral Prefrontal Cortex

Min Wang,<sup>1</sup> Yang Yang,<sup>1</sup> Ching-Jung Wang,<sup>2</sup> Nao J. Gamo,<sup>1</sup> Lu E. Jin,<sup>1</sup> James A. Mazer,<sup>1</sup> John H. Morrison,<sup>2</sup> Xiao-Jing Wang,<sup>1,3</sup> and Amy F.T. Arnsten<sup>1,\*</sup>

<sup>1</sup>Department Neurobiology, Yale Medical School, New Haven, CT 06510, USA

<sup>2</sup>Fishberg Department of Neuroscience, The Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

<sup>3</sup>Present address: Center for Neural Science, New York University, 4 Washington Place, New York, NY 10003, USA

\*Correspondence: [amy.arnsten@yale.edu](mailto:amy.arnsten@yale.edu)

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## SUMMARY

Neurons in the primate dorsolateral prefrontal cortex (dlPFC) generate persistent firing in the absence of sensory stimulation, the foundation of mental representation. Persistent firing arises from recurrent excitation within a network of pyramidal Delay cells. Here, we examined glutamate receptor influences underlying persistent firing in primate dlPFC during a spatial working memory task. Computational models predicted dependence on NMDA receptor (NMDAR) NR2B stimulation, and Delay cell persistent firing was abolished by local NR2B NMDAR blockade or by systemic ketamine administration. AMPA receptors (AMPA) contributed background depolarization to sustain network firing. In contrast, many Response cells were sensitive to AMPAR blockade and increased firing after systemic ketamine, indicating that models of ketamine actions should be refined to reflect neuronal heterogeneity. The reliance of Delay cells on NMDAR may explain why insults to NMDARs in schizophrenia or Alzheimer's disease profoundly impair cognition.

## INTRODUCTION

Neurons in the highly evolved primate dorsolateral prefrontal cortex (dlPFC) have properties of mental representation, i.e., the ability to embody information in the absence of sensory stimulation (Arnsten et al., 2012). This capability is the foundation of abstract thought and a basic building block for more complex dlPFC cognitive operations. The higher cognitive functions of the dlPFC are devastated in disorders such as schizophrenia (Barch and Ceaser, 2012) and Alzheimer's disease (AD; Schroeter et al., 2012).

The neural basis of representational knowledge has been studied most extensively using visuospatial working memory paradigms in monkeys, in which dlPFC neurons generate persistent firing to maintain a remembered location over a brief delay period, so-called Delay cells. Delay cell persistent neuronal firing

arises from recurrent excitation within pyramidal cell microcircuits in deep layer III of primate dlPFC, maintaining neural excitation in the absence of "bottom-up" sensory inputs (Goldman-Rakic, 1995). Layer III dlPFC pyramidal cells excite each other through glutamatergic synapses on long, thin spines (Dumitriu et al., 2010; Paspalas et al., 2012). The spatial specificity of neuronal firing is refined by lateral inhibition from GABAergic interneurons, sculpting more precise representations of visual space (Goldman-Rakic, 1995). The numbers of layer III spines and synapses increase greatly in primate evolution and are thought to underlie the expansion of human cognition (Elston, 2003). However, these circuits are also heavily afflicted in schizophrenia (Glantz and Lewis, 2000) and in AD (Bussi ere et al., 2003). The dlPFC Delay cells appear to convey represented information to Response cells, which in turn project to the motor systems (Arnsten et al., 2012). Response cells are probably localized in layer V (Sawaguchi et al., 1989) and fire in anticipation of and/or during the motor response (perisaccadic Response cells), or during and/or after the motor response (post-saccadic Response cells), possibly reflecting feedback from sensory-motor systems regarding the response (Funahashi et al., 1991). Response-like cells appear to predominate in the rodent PFC (Caetano et al., 2012), and it is likely that the higher representational operations performed by Delay cells can only be studied in primate dlPFC (Preuss, 1995).

The working memory operations of the PFC are fundamentally different from classic synaptic neuroplasticity, involving the transient excitation of a specific subset of cortical circuits rather than enduring changes in synaptic strength. Although there have been extensive studies of the glutamate receptor mechanisms underlying classic neuroplasticity, the receptors mediating the recurrent excitatory circuits underlying working memory in the primate dlPFC are unknown. NMDA receptors (NMDARs) have been of particular interest, and alterations in NMDAR in cognitive disorders such as schizophrenia and Alzheimer's disease have focused research on these receptors (Kristiansen et al., 2010b; Krystal et al., 2003; Kurup et al., 2010; Lewis and Moghaddam, 2006; Ross et al., 2006; Weickert et al., 2012). In many non-PFC brain regions, NMDAR with NR2B subunits are enriched in the synapse during development but move to extrasynaptic locations in the adult, while NMDAR with NR2A subunits predominate in adult synapses (Dumas, 2005). The open state of NMDAR is regulated by nearby AMPA receptors

(AMPA), which depolarize the membrane and permit NMDAR actions.

Adult PFC working memory circuits are regulated differently from sensory cortex and subcortical structures. Computational theories have predicted that the persistent firing of dIPFC working memory networks requires stimulation of NMDAR rather than AMPAR (Compte et al., 2000; Lisman et al., 1998; Wang, 1999) and that the slow kinetics of NR2B-containing NMDAR are particularly well suited to maintaining dIPFC network firing in the absence of sensory stimulation (Wang, 2001) and may subserve decision computations as well as working memory (Wang, 2002). In contrast, the faster kinetics of AMPARs lead to dynamical instability and network collapse (Wang, 1999). Although rodents do not have dIPFC, studies of rodent medial PFC suggest that NMDARs are important for neuronal burst firing and cognitive functions (Dalton et al., 2011; Jackson et al., 2004; Murphy et al., 2005; Stefani et al., 2003), and in vitro slice recordings have found evidence of extensive NMDA NR2B signaling in adult rat PFC compared to primary visual cortex (Wang et al., 2008), consistent with computational predictions.

Here, we examined the role of NMDAR and AMPAR in the working memory circuits of the primate dIPFC. Immunoelectron microscopy (immuno-EM) showed that NMDA NR2B subunits are found exclusively within the postsynaptic densities of layer III dIPFC spinous synapses in the adult monkey. As recurrent network firing is the “weakest link” in cognitive operations, computational modeling was used to test the hypothesis that reduced NMDAR signaling in even a small subset of network synapses could induce network collapse. Finally, we examined the effects of blocking NMDAR versus AMPAR on dIPFC neuronal firing in monkeys performing a spatial working memory task. Antagonists were applied directly onto the neurons using iontophoresis and included agents that selectively blocked NMDAR with NR2A versus NR2B subunits. Neuronal firing was also examined after systemic administration of the noncompetitive NMDA antagonist ketamine, as this method is increasingly used to model schizophrenia. The results reveal that NMDA NR2B receptor actions are critical to working memory Delay cell persistent firing, in contrast to their relatively minor role in adult neuroplasticity in non-PFC circuits. The data also revealed a subset of Response cells that are sensitive to AMPAR blockade and excited by ketamine administration, similar to rodent PFC neurons after systemic administration of NMDAR antagonists (Jackson et al., 2004). In contrast, Delay cell firing in monkeys was reduced by systemic ketamine, reinforcing the finding that the more evolved circuits in the primate dIPFC require NMDAR actions and that strategies for cognitive remediation in patients should aim at strengthening, rather than weakening, NMDAR function.

## RESULTS

### Immunoelectron Microscopic Localization of NMDA NR2B Subunits in Primate dIPFC

Postembedding immunoelectron microscopy was used to localize NR2B subunits in layer III of the adult primate dIPFC. Separate antibodies were used to specifically target phosphorylated NR2B (Figure 2A) or NR2B in either a phosphorylated or

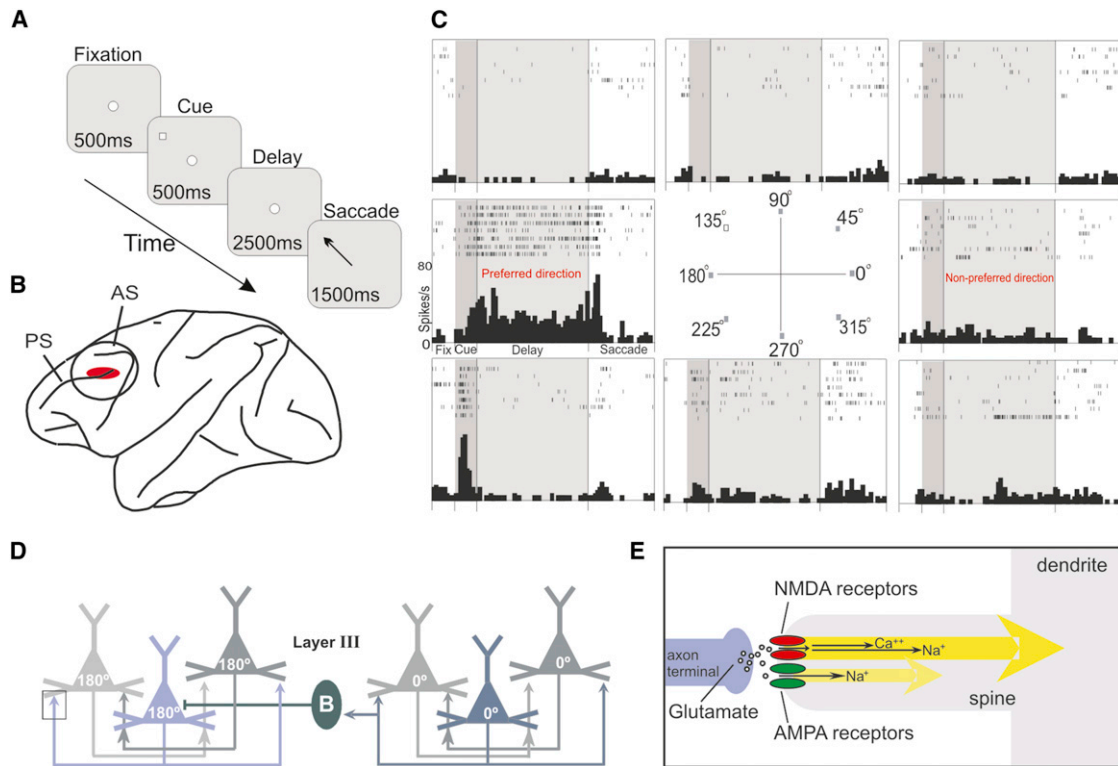
nonphosphorylated state (Figures 2B–2D). Both antibodies showed that NMDAR with NR2B subunits are localized exclusively within the postsynaptic density, with no evidence of extrasynaptic labeling (Figures 2A–2D). Thus, NR2B are synaptic receptors in layer III of the adult primate dIPFC.

### Computational Modeling of NMDA Actions in dIPFC Working Memory Circuits

Previous computations have shown that the slow kinetics of NMDAR with NR2B subunits are optimal for synaptic maintenance of dIPFC neuronal persistent firing (Wang, 1999, 2002). The current experiment examined the effects of blocking a small subset of NMDAR synapses within a larger, recurrent excitatory network, as likely occurs with the iontophoresis technique. During iontophoresis, a minute amount of drug alters the firing of only a small number of neurons; the vast majority of dIPFC neurons are unaffected and thus behavioral performance remains intact. The current experiment motivated new model simulations of this experiment, as well as offered a new test of this computational model. The model has 1,600 pyramidal cells and 400 interneurons; the pyramidal cells constitute a number of stimulus-selective populations; each of these populations has 240 spiking neurons. All neurons connect with each other through recurrent excitation, but the connection strength is stronger among neurons within a selective population. In model simulations, one particular neural population received a transient input (its preferred stimulus), triggering persistent activity that is self-sustained by virtue of NMDAR-dependent recurrent excitation within that neural population. In different simulation trials, we reduced the NMDA conductance in a subset of ten neurons out of the 240 neurons in the activated neural population. Figure 2E demonstrates the effects of reducing NMDAR actions from 100% (control conditions) to 90%, 80%, or 70% conductance in these ten affected neurons. Reducing NMDAR actions on ten neurons produced a “dose”-related reduction in task-related firing for all task epochs, with an almost complete loss of firing when NMDAR actions were reduced by only 30%, i.e., to 70% of control levels. On the other hand, the average firing rate of the 240 neuron population containing the ten neurons was only reduced from 42 Hz (control) to 34 Hz when there was a 30% NMDAR reduction in the ten cells (see Figure S1 available online). Therefore, the persistent activity of the overall population of neurons in the model is only mildly affected, and the network behavior remains intact, as expected in the iontophoresis experiment. These computational findings predict that dIPFC Delay cell networks would be particularly sensitive to reductions in NMDAR stimulation, with even small reductions in NMDAR conductance greatly diminishing task-related network firing.

### Physiological Recordings from Monkeys Performing Working Memory Tasks

The roles of ionotropic glutamate receptors on task-related neuronal firing were studied in monkeys performing an oculomotor delayed response (ODR) task (Figure 1A); patients with schizophrenia show deficits on this task (Keedy et al., 2006). In ODR, monkeys remember an ever-changing cued location over a brief delay and then make an eye movement to the remembered location to receive a juice reward (Figure 1A).



**Figure 1. The Experimental Paradigm and dlPFC Neural Circuitry Underlying Spatial Working Memory**

(A) The ODR spatial working memory task. Trials began when the monkey fixated on a central point for 0.5 s. A cue was present in one of eight possible locations for 0.5 s and was followed by a delay period of 2.5 s. When the fixation point was extinguished, the monkey made a saccade to the location of the remembered cue. The position of the cue changed on each trial in a quasirandom manner, thus requiring the constant updating of working memory stores.

(B) The region of monkey dlPFC where recordings occurred. PS, principal sulcus; AS, arcuate sulcus.

(C) An example of a Delay cell with spatially tuned, persistent firing during the delay period. Rasters and histograms are arranged to indicate the location of the corresponding cue. The neuron's preferred direction and the opposing, nonpreferred direction are indicated; subsequent figures will show neuronal responses to only these two directions. This cell exhibited significant delay-related activity for the 180° location but not other directions.

(D) An illustration of the deep layer III microcircuits subserving spatially tuned, persistent firing during the delay period, based on Goldman-Rakic (1995). B, GABAergic Basket cell.

(E) Working model of glutamate actions at NMDARs and AMPARs on long, thin dendritic spines of layer III pyramidal cells in monkey dlPFC.

Single-unit recordings were made from the principal sulcal dlPFC subregion essential for spatial working memory (Goldman-Rakic, 1995) (Figure 1B). We classified cells into one of three types based on their patterns of task-related firing: (1) Cue cells that briefly fire during the visuospatial cue, (2) Delay cells that maintain persistent firing through the delay period, and often fire to the cue and/or response as well, and (3) Response cells (likely layer V; Sawaguchi et al., 1989) that fire during or after the saccadic response to the remembered location (Goldman-Rakic, 1995). The persistent firing of Delay cells is often spatially tuned to a "preferred direction," (Figure 1C), arising from recurrent excitation within a microcircuit of layer III pyramidal cells with similar tuning (Figure 1D; Goldman-Rakic, 1995), which interconnect on dendritic spines (Figure 1E). The spatial tuning of the network is sculpted by GABA and dopamine (Goldman-Rakic, 1995; Vijayraghavan et al., 2007), e.g., the basket cell (B) shown in Figure 1D.

Drugs were applied using iontophoresis; the iontophoresis electrode consisted of a central carbon fiber for recording, sur-

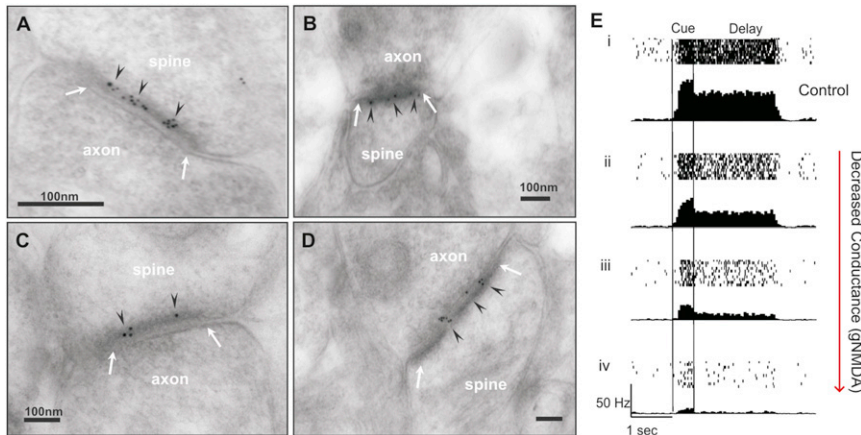
rounded by six glass pipettes that deliver drug by applying a small electrical current. A minute amount of drug is released that affects cells on the spatial scale of a cortical column (Rao et al., 2000) but does not alter behavior; iontophoresis of saline with low pH similar to the drug solutions used in this study has no effect on neuronal firing (Vijayraghavan et al., 2007; Figure S2).

#### **Iontophoresis of NMDA Receptor Antagonists**

The role of NMDARs was probed using three different NMDAR antagonists: the noncompetitive, general NMDA antagonist MK801; the selective NR2A NMDA subunit antagonist PPPA ((2R\*,4S\*)-4-(3-Phosphonopropyl)-2-piperidinecarboxylic acid); and the selective NR2B NMDA subunit antagonist Ro25-6981. A brief pilot study also examined the effects of stimulating NMDAR by iontophoresis of NMDA.

#### **Effects of MK801 on Delay Cells**

Iontophoresis of the NMDA antagonist MK801 produced a marked, dose-dependent suppression of neuronal firing (Figures 3A–3C, Figure S3; one-way ANOVA with repeated measures [1-ANOVA-R],  $p < 0.05$  for 14 out of 15 individual cells;



**Figure 2. NMDAR in Primate dlPFC: Immunogold Labeling and Computational Theory**

(A–D) Localization of NMDA NR2B subunits using immunogold labeling in layer III of the rhesus monkey dlPFC. Four typical synapses are shown, including a perforated synapse in (D): (A) shows pNR2B labeling, while (B)–(D) show total NR2B label. Both pNR2B and NR2B labeling was found exclusively within the postsynaptic density; no labeling was observed outside the synapse. Black arrowheads indicate pNR2B or NR2B labeling; white arrows delineate the synapse.

(E) The effects of iontophoretic NMDA blockade on working memory activity in a computational model of dlPFC neuronal persistent firing. Under control conditions, a stimulus cue selectively activates a group of neurons, leading to persistent activity sustained by NMDAR-dependent recurrent excitation. NMDA conductance is reduced

from control (i) to 90% (ii), 80% (iii), and 70% (iv) of a reference level in ten pyramidal neurons in the network model. Stimulus-selective persistent activity gradually decreases with more NMDAR blockade and eventually disappears in these affected cells; model based on Brunel and Wang (2001) and Wang (2002). See text for more details.

Tdep for the average,  $p < 10^{-5}$ ). Firing was reduced for all task epochs, with higher doses producing an almost complete suppression of network firing in some neurons (Figures 3A and 3B). Firing was preferentially reduced on preferred direction trials, thus leading to a significant decrease in the neuron's spatial tuning index (TI) (Figure 2C; Tdep,  $p < 0.01$ ; Wilcoxon,  $p = 0.012$ ). Thus, neurons no longer maintained information regarding spatial position of the cue. Firing slowly returned to normal firing patterns when drug application was stopped (Figure 3A; 1-ANOVA-R,  $p < 0.05$ ; drug versus recovery). In contrast to task-related firing, iontophoresis of MK801 produced only a small, nonsignificant reduction in spontaneous neuronal firing when the monkey rested (average spontaneous firing rate control:  $9.28 \pm 3.93$ ; MK801:  $7.12 \pm 3.29$ ;  $p = 0.12$ ).

In contrast to blockade of NMDAR, stimulation of NMDAR through iontophoresis of NMDA increased Delay cell firing (Figure S4). A very low dose of NMDA (5 nA) produced a specific enhancement of firing for the neurons' preferred direction; however, higher doses (10–40 nA) produced nonspecific increases in neuronal firing (Figure S4). The generalized increases in firing at higher doses probably arose from the widespread effects of exogenous drug application and emphasizes that blockade of endogenous glutamate actions is the more effective strategy for illuminating innate glutamate actions in primate dlPFC.

#### Effects of NR2A or NR2B NMDA Subunit Blockade on Delay Cells

Iontophoresis of either PPPA (Figure S5) or Ro25-6981 (Figures 3D and 3E) markedly reduced Delay cell firing. As computational models predicted an important role for NR2B receptors, we focused on this subtype. Extended studies of Ro25-6981 revealed dose-related reductions in task-related firing (Figures 3D and 3E; 1-ANOVA-R,  $p < 0.05$  for 26 out of 31 individual cells; Tdep for the average,  $p < 10^9$ ). Reduced firing was particularly evident for the neurons' preferred direction, leading to a significant decrease in the spatial tuning index (Figure 3F; Tdep,  $p < 10^{-5}$ ; Wilcoxon,  $p < 0.0001$ ). Firing patterns recovered when

drug delivery was stopped (Figure 3D; 1-ANOVA-R,  $p < 0.05$ ; drug versus recovery). Taken together, these data suggest that both NR2A and NR2B NMDA subunits contribute to task-related firing in Delay cells, and loss of both leads to an almost complete loss of PFC network firing.

#### Effects of NMDA Receptor Blockade on Cue and Response Cells

The effects of NMDAR blockade were also examined on Cue cells and Response cells. Iontophoresis of the NMDA NR2B antagonist Ro25-6981 significantly decreased the firing of both Cue cells (an example in Figure 4A, 1-ANOVA-R,  $p < 0.05$  for 4 out of 4 cells) and Response cells (an example in Figure 4B, 1-ANOVA-R,  $p < 0.05$  for 7 out of 7 cells).

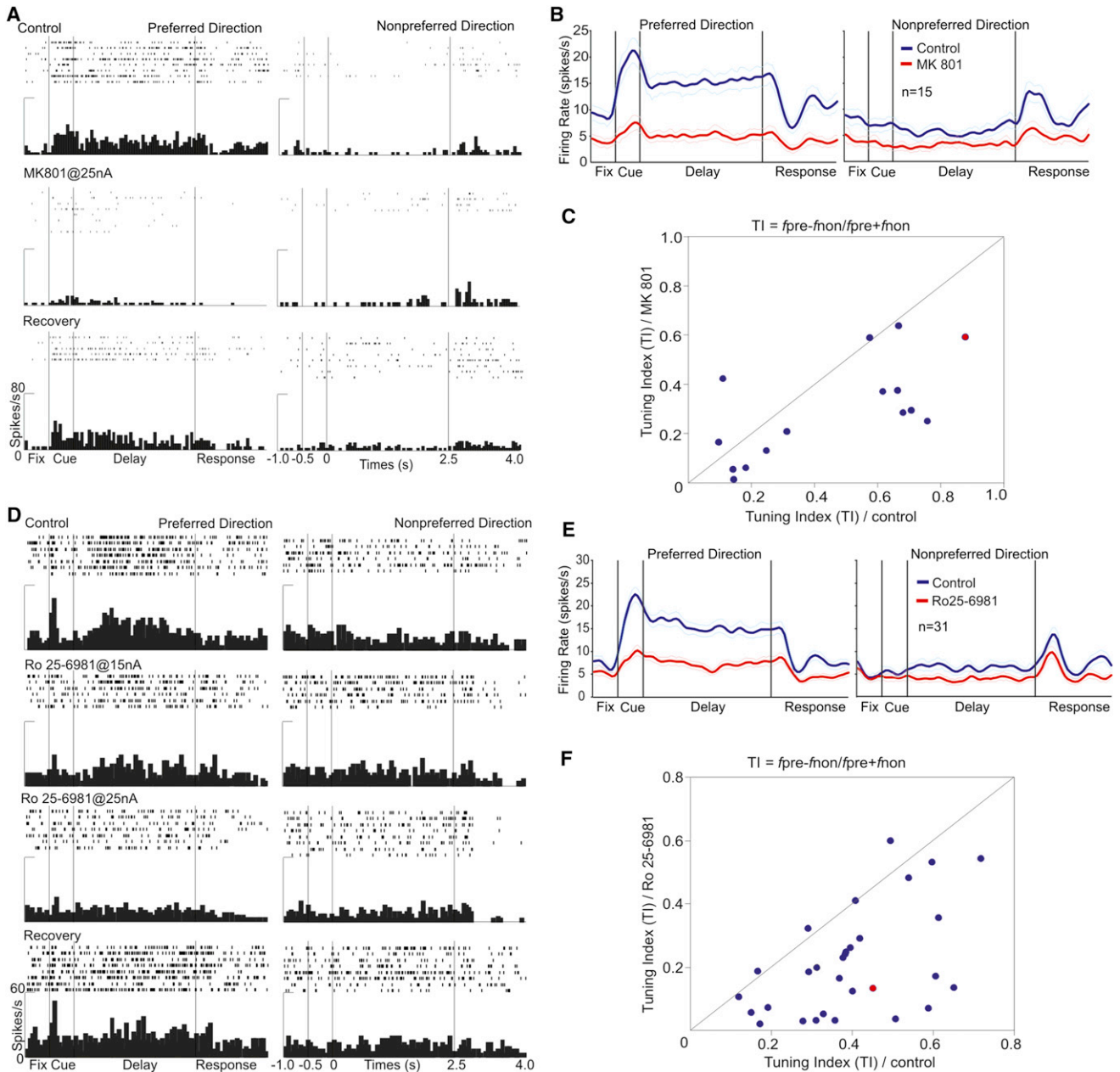
#### Iontophoresis of AMPA Receptor Antagonists

The influence of AMPARs on task-related firing was examined by iontophoresis of the selective AMPA blockers NBQX or CNQX disodium salt.

#### Effects of AMPA Receptor Blockade on Delay Cells

AMPA antagonists had mixed effects on Delay cell firing (Figures 5A and 5B). Iontophoresis of AMPAR antagonists significantly reduced the task-related firing of 10 out of 16 Delay cells (an example in Figure 5A, 1-ANOVA-R,  $p < 0.05$ ), while it increased the task-related firing of 3 of the 16 Delay cells. Overall, there was a significant decrease in task-related neuronal firing (Figure 5B, Tdep for the average,  $p < 0.005$ ) and a significant reduction in the spatial tuning index (Figure 5C,  $p < 0.05$ ; Wilcoxon,  $p = 0.013$ ). The proportion of neurons with reduced tuning did not significantly differ between AMPAR and NMDAR blockade ( $p = 0.13$  with chi-square). However, the magnitude of the reduction produced by AMPAR blockade was not as large as that seen with NMDA blockade (Figure 6A, right; Tdep,  $p = 0.001$ ), and NMDA blockade reduced firing in a greater proportion of neurons (Figure 6A, left; Wilcoxon,  $p = 0.046$ ).

Eight delay cells were sufficiently stable to test the effects of both NMDA and AMPAR blockade within the same neuron. A single neuron example is shown in Figure 6B, where task-related firing was markedly suppressed by the iontophoresis of the



**Figure 3. The Effects of Intra-PFC Iontophoresis of the NMDA Antagonists MK801 or Ro25-6981 on the Task-Related Firing of Delay Cells in the Primate dIPFC**

(A) An example of an individual dIPFC Delay cell under control conditions and after iontophoresis of MK801 (25 nA). The rasters and histograms show firing patterns for the neuron's preferred direction and the nonpreferred direction opposite to the preferred direction. Iontophoresis of MK801 markedly reduced task-related firing; firing returned toward control levels when delivery of MK801 was stopped (recovery;  $p < 0.05$ ).

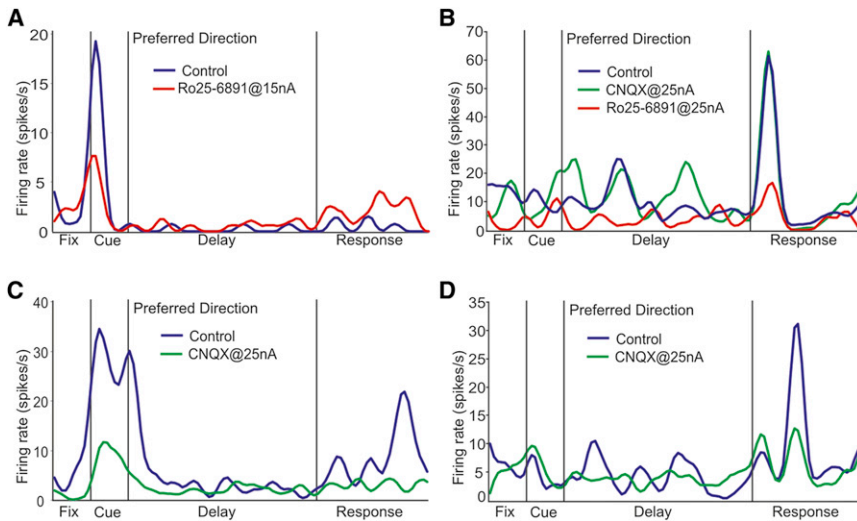
(B) Average response showing the mean  $\pm$  SEM firing patterns of 15 dIPFC Delay cells for their preferred versus nonpreferred directions under control conditions (blue) and after iontophoresis of MK801 (red). MK801 markedly decreased task-related firing, especially for the neurons' preferred direction.

(C) The spatial tuning index (TI) comparing each neuron's firing for its preferred versus nonpreferred directions to examine the neuron's spatial tuning. Iontophoresis of MK801 significantly weakened spatial tuning by reducing TI.

(D) An example of an individual dIPFC Delay cell under control conditions and after iontophoresis of Ro25-6981 (15–25 nA). Iontophoresis of Ro25-6981 markedly reduced task-related firing in a dose-dependent manner; firing returned toward control levels when delivery of Ro25-6981 was stopped (recovery;  $p < 0.05$ ).

(E) Average response showing the mean  $\pm$  SEM firing patterns of 31 dIPFC Delay cells for their preferred versus nonpreferred directions under control conditions (blue) and after iontophoresis of Ro25-6981 (red). Ro25-6981 markedly decreased task-related firing, especially for the neurons' preferred direction.

(F) Iontophoresis of Ro25-6981 significantly weakened spatial tuning by reducing TI.



**Figure 4. The Effects of NMDA versus AMPAR Blockade on the Task-Related Firing of Cue and Response Cells in the Primate dlPFC**

(A) Example of a Cue cell under control conditions (blue) and after iontophoresis of the NMDA NR2B antagonist, Ro25-6891 (15 nA; red). NMDA blockade significantly reduced task-related firing of the Cue cell.

(B) Example of a perisaccadic Response cell under control conditions (blue) and after iontophoresis of the AMPA antagonist, CNQX (25 nA; green), and Ro25-6891 (25 nA; red). Perisaccadic-related firing of the Response cell was reduced by NMDA but not AMPAR blockade.

(C) Example of a Cue cell under control conditions (blue) and after iontophoresis of CNQX (25 nA; green). AMPA blockade significantly reduced task-related firing of the Cue cell.

(D) Example of a postsaccadic Response cell under control conditions (blue) and after iontophoresis of CNQX (25 nA; green). In contrast to the Response cell shown in (B), the postsaccadic-related firing of this Response cell was reduced by AMPAR blockade.

NMDA NR2B blocker Ro25-6891 (25 nA; red). After cessation of drug delivery, the neuron recovered its normal level and pattern of task-related firing (light blue). Subsequent application of the AMPAR blocker CNQX (40 nA) produced only a modest reduction in delay-related firing, which developed over the delay period (green). This pattern was also evident in the average of the eight neurons (Figure 6C). A more detailed analysis of the delay period (Figure 6D) showed that AMPAR blockade had little effect early in the delay period ( $p > 0.2$ ) but had significant reductions later (i.e., starting at 1.0 s;  $p < 0.05$ ). In contrast, NMDA blockade significantly reduced delay-related firing throughout the entire delay period compared to both control conditions (all  $p < 0.01$ ) and AMPAR blockade (all  $p < 0.05$ ). These results suggest that AMPARs may provide background depolarization needed to maintain firing but do not mediate the moment-by-moment synaptic activity mediating the persistent firing of Delay cell networks.

#### Effects of AMPA Receptor Blockade on Cue and Response Cells

CNQX or NBQX markedly reduced the firing of Cue cells (an example in Figure 4C, 1-ANOVA-R,  $p < 0.05$  for 4 out of 4 cells). In contrast, AMPA antagonists had a mixed effect on Response cells, decreasing some but not others (Figures 4B and 4D). Eight Response cells were tested with CNQX or NBQX; these compounds decreased response-related firing in the five Response cells with postsaccadic firing (an example in Figure 4D, 1-ANOVA-R,  $p < 0.05$ ) but had no effect on the three Response cells with perisaccadic firing (an example in Figure 4B, 1-ANOVA-R,  $p > 0.05$ ). These data suggest that AMPARs may mediate the feedback from motor cortices to postsaccadic Response neurons.

#### Systemic Administration of the NMDA Antagonist Ketamine

The effects of systemic ketamine administration (0.5–1.5 mg/kg, i.m.) were examined to see whether there would be signs of

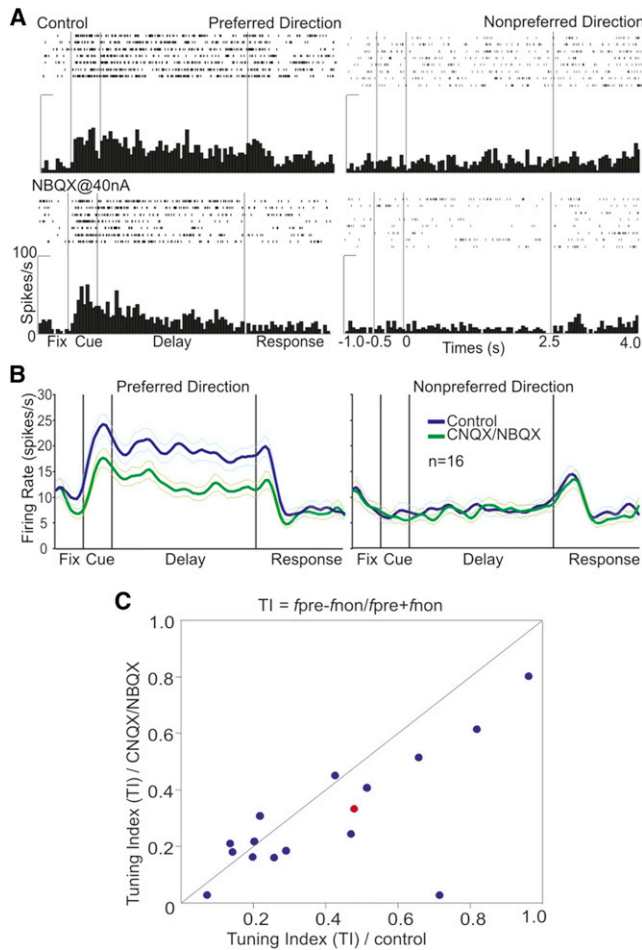
reduced persistent firing and increased spontaneous firing as has been seen in rodents (Jackson et al., 2004). Subanesthetic doses were chosen that impair spatial working memory in monkeys (Roberts et al., 2010). As chronic NMDA antagonist administration can have serious consequences (Linn et al., 1999), ketamine treatments were limited in number and spaced at intervals of  $>1$  week. Ketamine produced a dose-related reduction in the accuracy of ODR performance (Figure 7A, Wilcoxon,  $p = 0.01$ ,  $n = 7$  experiments). At higher doses (1.0–1.5 mg/kg), the monkeys initially exhibited nystagmus that interfered with performance of the ODR task. In these cases, normal eye movement control returned about 30 min postinjection, and cognitive testing resumed with accurate eye movements but impaired cognitive performance (percent correct: control:  $87\% \pm 4\%$  versus ketamine  $56\% \pm 9\%$ ;  $n = 5$ ). Lower doses (0.5 mg/kg) usually did not produce nystagmus but induced modest cognitive impairment (percent correct: control: 70% versus ketamine 66%;  $n = 2$ ). Recording sessions with ketamine examined Delay cell and Response cell firing; no Cue cells were found during these recording sessions.

#### Delay Cell Firing

Systemic ketamine had no effect on the spontaneous firing of Delay cells (Figure 7B) but significantly reduced the task-related firing of Delay cells (Figures 7C and 7D, Wilcoxon,  $p = 0.014$ ). The effects of systemic ketamine were more subtle than those observed with direct iontophoretic application of NMDA antagonists, consistent with the use of low, subanesthetic doses.

#### Response Cells

In contrast to Delay cells, systemic ketamine significantly increased the firing of postsaccadic Response cells. Ketamine increased both their spontaneous firing rate (Figure 7B, Wilcoxon,  $p = 0.025$ ) and their task-related firing (Figures 7E and 7F, Wilcoxon,  $p = 0.028$ ). Increases in Response cell firing were not seen with iontophoresis of NMDA antagonists.



**Figure 5. The Effects of AMPAR Blockade on the Task-Related Firing of Delay Cells in the Primate dlPFC**

(A) An example of an individual dlPFC Delay cell under control conditions and after iontophoresis of NBQX (40 nA). Iontophoresis of NBQX reduced task-related firing as the delay period progressed.

(B) Average response showing the mean  $\pm$  SEM firing patterns of 16 dlPFC Delay cells under control conditions (blue) and after iontophoresis of CNQX or NBQX (green), with the drug effects being most prominent late in the delay period.

(C) Iontophoresis of CNQX/NBQX weakened spatial tuning by reducing TI.

## DISCUSSION

The persistent firing of dlPFC neurons in monkeys performing a spatial working memory task is considered the neurophysiological basis for the mental representation of visual space (Goldman-Rakic, 1995). These elementary representational operations are the building blocks of more complex, dlPFC executive functions, including top-down regulation of attention, high-order decision making, and cognitive control (e.g., Buschman and Miller, 2007; Kim et al., 2008; Wallis et al., 2001). Working memory is generated by the momentary activation of a precise pattern of cortical networks, including recurrent excitation of pyramidal cell microcircuits in deep layer III (Goldman-Rakic, 1995), the neurons that expand most in primate evolution (Elston, 2003). Working

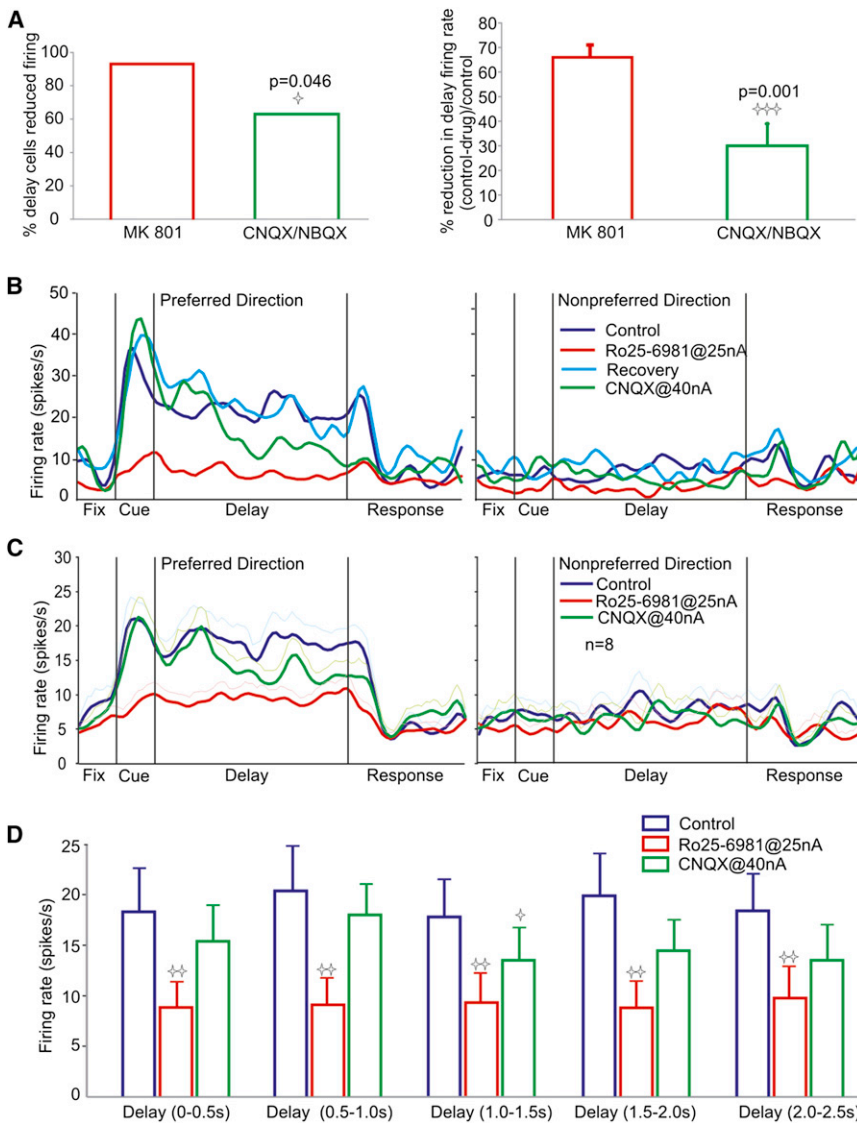
memory is fundamentally different from long-term memory consolidation, in which events are stored through architectural changes in “classic” synapses (Arnsten et al., 2012). In classic, neuroplastic synapses, the insertion of AMPAR into the membrane modulates the strength of synaptic reactivity (Lüscher and Malenka, 2012), and NMDA NR2B receptors often play an extrasynaptic role (Dumas, 2005). Computational models predicted that the persistent firing underlying working memory and mental representation would require qualitatively different glutamate actions than those needed for classic plasticity: the kinetics of AMPAR are too rapid to sustain firing and lead to network collapse, while the slower kinetics of NR2B are optimal for prolonged network firing (Compte et al., 2000; Wang, 1999). Consistent with these predictions, the current study found that the highly evolved, recurrent excitatory layer III dlPFC synapses underlying working memory contain NMDA NR2B subunits exclusively within the postsynaptic density and that persistent firing during mental representation requires NMDA NR2B stimulation.

### The Critical Role of NMDAR for the Task-Related Firing of dlPFC Delay Cells

The present study showed that blockade of NMDARs in the dlPFC rapidly reduced the task-related neuronal firing in monkeys performing a spatial working memory task, irrespective of whether the antagonist was applied locally or by systemic injection. Delay cell firing was reduced for all task epochs, consistent with a sustained loss of recurrent excitation after NMDAR blockade. These results were predicted by the computational model, in which reduced NMDAR conductance decreased firing for all task epochs, with even a 30% reduction in NMDAR conductance leading to a complete loss of persistent firing in affected neurons. Thus, even a modest reduction in NMDAR stimulation in dlPFC (e.g., due to drug or genetic insult) would dramatically reduce persistent activity and impair mental representation. Indeed, this study—as well as others—has found significant working memory impairment with local PFC or systemic administration of NMDAR antagonists in rodents, monkeys, and humans (e.g., Honey et al., 2004; Krystal et al., 2005; Moghaddam and Adams, 1998; Roberts et al., 2010). This sensitivity to NMDAR actions helps to explain why dlPFC Delay neurons comprise the “weakest link” in the circuits underlying cognitive behavior. The results further suggest that any cognitive operation relying on dlPFC recurrent firing would be compromised by insults to NMDAR transmission.

The immediate effects of NMDA blockade differed from the slow “run down” of cell firing across the delay period after AMPAR blockade, which suggests that AMPARs provide an underlying depolarization that permits NMDA actions in Delay cells. However, AMPAR are known to have prominent excitatory effects on GABAergic interneurons in mouse PFC (Rotaru et al., 2011), and thus a reduction in lateral inhibition may also have contributed to the relatively subtle changes in Delay cell firing after AMPAR blockade. Depolarizing influences on NMDAR are also provided by cholinergic stimulation of nicotinic  $\alpha 7$  receptors in the primate dlPFC (Y.Y., L.E.J., A.F.T.A., and M.W., unpublished data).

In contrast to Delay cells, the firing of Cue cells was rapidly reduced by either AMPAR or NMDAR blockade. Response cells



**Figure 6. A Comparison of AMPA versus NMDAR Blockade on the Task-Related Firing of Delay Cells in the Primate dlPFC**

(A) Left: the percentage of neurons showing significant reduction in firing rate after iontophoresis of the NMDA antagonist, MK801 compared to the AMPA antagonists CNQX or NBQX. Right: the maximal degree of reduction in delay-related firing induced by the NMDA antagonist MK801 compared to the AMPA antagonists CNQX or NBQX. The reduction in firing rate was measured by the following ratio: (control-drug)/control.

(B) An example of an individual Delay cell treated with NMDA versus AMPA antagonists. Under control conditions, the neuron showed prominent, spatially tuned, delay-related firing (dark blue). Subsequent iontophoresis of the NMDA NR2B antagonist Ro25-6981 (25 nA; red) led to a large reduction in task-related firing. The iontophoretic current was then turned off and the neuron recovered normal rates of firing (light blue). After recovery, the AMPA antagonist CNQX (40 nA; green) was iontophoresed onto the neuron. CNQX had little effect on firing early in the delay epoch but reduced firing in the later portion of the delay epoch.

(C) Average response showing the mean  $\pm$  SEM firing patterns of the eight dlPFC Delay cells under control conditions (dark blue), during iontophoresis of Ro25-6981 (25 nA; red), and during iontophoresis CNQX (40 nA; green). Ro25-6981 produced a marked reduction in task-related firing, and CNQX had more subtle effects, reducing firing only in the later aspects of the delay epoch.

(D) A comparison of mean  $\pm$  SEM firing rates in the five successive 0.5 s epochs of the 2.5 s delay period under control, MK801, and CNQX conditions. \* $p < 0.05$ ; \*\* $p < 0.01$ .

also reduced firing to NMDAR blockade but only postsaccadic Response cells responded to AMPAR blockade. Overall, these data suggest that neurons engaged in recurrent excitatory circuits are especially reliant on NMDAR rather than AMPAR stimulation, while neurons receiving “sensory-motor” information from sensory or motor circuits are influenced by both types of receptors.

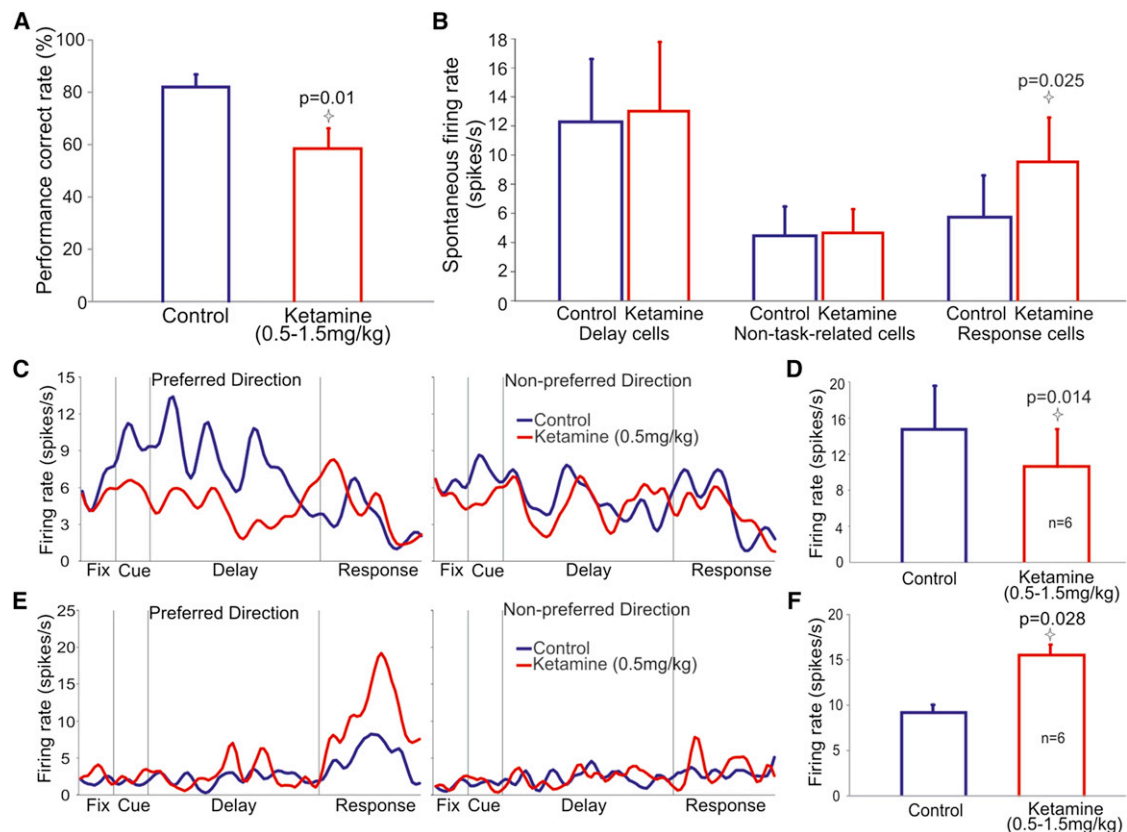
The current data are the first physiological recordings during NMDA blockade in animals engaged in a high-order cognitive task, when NMDAR are most important for network firing. The important role of NMDARs in PFC network firing in monkeys is in partial agreement with data from rodents, where systemic administration of NMDA blockers reduced medial PFC neuronal burst firing in vivo (Jackson et al., 2004), and local application reduced EPSCs in vitro (Rotaru et al., 2011; Wang et al., 2008). A recent in vitro study of mouse PFC identified the NMDA-responsive neurons as pyramidal cells (Rotaru et al., 2011). It should be noted that most neurons in rodent medial PFC are

probably Response-like cells, or hybrid progenitors of Delay-like and Response-like cells (Arnsten et al., 2012), and thus direct comparisons to dlPFC Delay cells in primates must be done with caution. However, a prominent role of NR2B subunits has been seen in in vitro recordings from rodent medial PFC, which showed greater NR2B conductance in medial PFC than in V1 cortex (Wang et al., 2008). These findings are consistent with a recent study showing that overexpression of forebrain NR2B improves working memory performance in mice (Cui et al., 2011). Thus, some aspects of NMDAR signaling in working memory circuits can be observed across species.

### Contrasts between Mechanisms Mediating Working Memory versus Long-Term Plasticity

NMDAR and AMPAR mechanisms have been a major focus of classic neuroplasticity research, e.g., in synapses in the primary sensory cortices and in CA1 neurons of the hippocampus (e.g., Cho et al., 2009; Lüscher and Malenka, 2012). NMDA NR2B receptors are important for synaptic plasticity during development but move to extrasynaptic locations in mature circuits





**Figure 7. The Effects of Systemic Ketamine Administration on the Working Memory Performance and the Physiological Responses of Delay Cells and Response Cells in the Primate dlPFC**

(A) The systemic administration of ketamine significantly impaired the accuracy of spatial working memory performance on the ODR task. Data represent mean  $\pm$  SEM collapsed across all doses (0.5–1.5 mg/kg). See text for breakdown in performance between lower and higher doses.  
 (B) The effects of systemic ketamine administration on the spontaneous firing rate of Delay cells ( $n = 6$ ), Response cells ( $n = 6$ ), and nontask-related cells ( $n = 4$ ) when the monkeys were resting and not performing the task. Ketamine had no significant effect on the spontaneous firing of Delay cells or nontask cells but significantly increased the spontaneous firing of Response cells.  
 (C) An example of the effects of ketamine on the task-related firing of an individual Delay cell in the dlPFC. This neuron showed pronounced task-related firing for its preferred direction under control conditions (blue) but reduced task-related firing after injection of ketamine (red).  
 (D) Systemic administration of ketamine significantly reduced the task-related firing of the six Delay cells found in the monkey dlPFC. Results represent mean  $\pm$  SEM firing rate during the delay epoch.  
 (E) An example of the effects of ketamine on the task-related firing of an individual Response cell in the dlPFC. This neuron showed increased postsaccadic firing under control conditions (blue), which was markedly increased after injection of ketamine (red).  
 (F) Systemic administration of ketamine significantly increased the task-related firing of six Response cells in the monkey dlPFC. All of these Response cells showed postsaccadic firing patterns. Results represent mean  $\pm$  SEM firing rate during the response epoch.

when plasticity is governed by more rapid NR2A NMDAR (Dumas, 2005). In contrast, the current study found that NR2B are expressed exclusively in the postsynaptic density in the adult dlPFC, with no extrasynaptic localization, consistent with their prominent role in persistent network firing. The reliance of highly evolved, dlPFC networks on NMDA NR2B mechanisms may render them especially vulnerable to degeneration, as calcium entry through NR2B is particularly excitotoxic (Liu et al., 2007).

Plasticity in classic synapses is regulated by the numbers of AMPAR inserted into the postsynaptic density, where they have permissive effects on NMDAR opening and can rapidly alter synapse strength (Lüscher and Malenka, 2012). In contrast, the current study found that AMPAR blockade had mixed effects on working memory neuronal firing in primate dlPFC. Although

AMPA blockade arrested firing in dlPFC sensory-motor neurons (i.e., the Cue and postsaccadic Response cells), it had less effect on Delay cell firing, primarily decreasing firing at the end of the delay period, consistent with a slow “run down” in neuronal depolarization. These permissive AMPAR actions are probably combined with excitatory neuromodulation to engage NMDAR and coordinate dlPFC network activity with arousal state (Arnsten et al., 2012).

**Local versus Systemic NMDA Receptor Blockade**

An important finding of the current study was that dlPFC neurons were differentially influenced by systemic ketamine administration, whereby ketamine decreased the firing of Delay cells but increased the firing of a subset of Response cells.

The reduction of mnemonic firing in Delay cells was most prominent when the monkeys were engaged in the working memory task, indicating that the role of NMDARs is best observed under conditions of cognitive engagement. This surprising heterogeneity indicates that current models of NMDA actions in PFC need to be refined, particularly as they relate to cognitive changes in schizophrenia (Homayoun and Moghaddam, 2007). A prevalent model of NMDA actions in PFC has focused on predominate NMDA actions on interneurons, whereby NMDAR blockade decreases GABAergic inhibition leading to a disinhibition of pyramidal cell firing (Homayoun and Moghaddam, 2007; Murray et al., 2012). On the other hand, NMDA receptors in pyramidal cells have long been proposed to play a critical role in reberatory synaptic excitation underlying the maintenance of persistent activity (Wang, 1999), and this theoretical prediction received support from a recent study of the adult mouse PFC by Rotaru et al. (2011) showing that NMDA actions are actually more prevalent on pyramidal cells than interneurons. These results suggest that the action of ketamine is more complex than previously thought, and the functional consequences of altered NMDA signaling in the PFC needs to be analyzed by taking into account a combination of effects on the NMDA receptors in both pyramidal cells and interneurons. Indeed, a recent work showed that, in the same prefrontal local circuit, a relatively small reduction of NMDA receptor-dependent excitation in these two cell types can lead to either disinhibition or the abolishment of persistent activity (Murray et al., 2012). Further experimental and computational work will be needed to provide clarity on this important issue.

The current data emphasize the unique pharmacology of the postsaccadic Response cells, which increased their firing with systemic ketamine and were sensitive to AMPAR blockade, similar to neurons recorded from rodent PFC (Homayoun and Moghaddam, 2007; Jackson et al., 2004). Response cells are thought to be large, layer V pyramidal cells and are very prevalent in both the primate (Funahashi et al., 1991) and especially the rodent (Caetano et al., 2012) PFC. Thus, drug effects on these neurons may predominate in many neuronal recordings and in fMRI BOLD signals. For example, systemic ketamine has been shown to disinhibit dIPFC neuronal firing in monkeys performing an associative task, irrespective of memory conditions, consistent with Response-like cells (Skoblenick and Everling, 2012). Systemic administration of NMDA antagonists to human subjects can increase the BOLD response and increase signs of glutamate release (Honey et al., 2004; Rowland et al., 2005), which may involve increases in Response cell firing. The marked disinhibition of Response cells after systemic NMDAR blockade may obscure the simultaneous decrease in the firing of cognitive Delay cell circuits. This may distort views of NMDAR “inhibitory” actions and confuse our understanding of NMDAR contributions to cognitive disorders (Fitzgerald, 2012).

What causes the increase in Response cell firing with systemic ketamine? As increased firing only occurred with systemic drug administration, but not local NMDAR blockade, increased firing probably arose from drug actions outside the PFC or beyond the column of PFC neurons influenced by iontophoretic application. One possibility is that systemic NMDAR blockade activates dopamine mechanisms that increase Response cell firing. Layer

V pyramidal cells have unique patterns of dopamine receptor expression, with high levels of D2R mRNA (Lidow et al., 1998). Response cells are uniquely activated by D2R stimulation (Wang et al., 2004), and systemic NMDA blockade increases dopamine release in rat PFC (Jentsch et al., 1997; Verma and Moghaddam, 1996). Thus, increased D2 receptor stimulation may contribute to increased Response cell firing after systemic ketamine. Response cells may also increase firing due to reduced inhibition from GABAergic neurons (Homayoun and Moghaddam, 2007), e.g., those interneurons that are normally driven by NMDA-dependent Delay cell networks (Funahashi et al., 1991). They may also be driven by ketamine actions in thalamus (Dawson et al., 2011) that disrupt feedback to this subset of neurons.

Interestingly, the disinhibited Response cells in the ketamine experiments all showed postsaccadic neuronal firing, i.e., they fired *during or after* the monkey had made its response, likely due to feedback from the motor system via the thalamus (Funahashi et al., 1991; Sommer and Wurtz, 2008). Alterations in the firing of this class of Response cells may produce cognitive changes in healthy human subjects given ketamine, interfering with the accuracy of responses (Murray et al., 2012) and possibly contributing to the delusional thinking induced by NMDAR antagonists (Corlett et al., 2006). These are intriguing areas for future research. However, as described below, patients with schizophrenia show *reduced* BOLD signals during the Delay and Response epochs in a spatial working memory task (Driesen et al., 2008), indicating that ketamine’s suppressive effects on Delay cells, rather than its disinhibition of Response cells, are more relevant to working memory deficits in schizophrenia.

### Relevance to Mental Illness

NMDAR signaling is of particular relevance to mental illness, as NMDA blockers such as ketamine are used as a model of schizophrenia (Krystal et al., 2003; Malhotra et al., 1997) but are currently being developed for the treatment of severe, medication-resistant depression (Skolnick et al., 2009). The current physiological data may help elucidate these seemingly inconsistent actions.

Schizophrenia has been linked to genetic insults that weaken NMDAR signaling (Banerjee et al., 2010; Javitt, 2010), and post mortem studies show evidence of altered NR2B NMDAR expression and trafficking (Kristiansen et al., 2010a, 2010b), including links between allelic alterations in NR2B and impaired reasoning abilities in patients with schizophrenia (Weickert et al., 2012). Neuropathological studies of schizophrenia have shown extensive changes to dIPFC layer III, including loss of neuropil and spines (Glantz and Lewis, 2000; Selemon et al., 1995) and reductions in glutamate terminals onto GABAergic interneurons (Bitanirhwe et al., 2009). Deep layer III of dIPFC is the sublayer that contains the most extensive recurrent circuits thought to underlie Delay cell firing (Kritzer and Goldman-Rakic, 1995) and the NR2B synapses documented in the current study. Imaging studies also point to the importance of dIPFC for fundamental deficits in schizophrenia. Patients with schizophrenia show impaired working memory abilities and reduced dIPFC BOLD response, which correlate with measures of thought

disorder (Perlstein et al., 2001). Indeed, patients performing a spatial working memory task similar to the ODR task used in monkeys show reduced dIPFC BOLD response during the delay and early response epochs (Driesen et al., 2008), consistent with the reduced firing of Delay and perisaccadic Response cells after NMDAR blockade in the current study.

The current findings also help illuminate apparent discrepancies between data showing reduced NMDAR actions in schizophrenia and hyperglutamate theories of the disease. Recent findings indicate that impaired cognitive abilities in patients with schizophrenia are associated with reduced NMDAR glutamate signaling (Bustillo et al., 2011), rather than the hyperglutamate signaling that has been the focus of recent theories (reviewed in Kantrowitz and Javitt, 2012). Hyperglutamate theories have arisen from studies of NMDAR actions in rodent PFC, where systemic NMDA antagonists increase neuronal firing and glutamate release (Jackson et al., 2004). The current data show that systemic administration of NMDA antagonists increases the firing of Response cells, and as Response cells are prevalent in rodent PFC, these actions probably account for the increased neuronal firing and hyperglutamergergia observed in rodents. However, rodents do not appear to have the highly evolved Delay cells that exhibit *reduced* firing with systemic or local NMDAR blockade. Thus, the loss of firing in the circuits mediating higher cognition in primates would not be evident in rodent models. The reduction in dIPFC activity with systemic ketamine can also be observed in healthy humans performing a spatial working memory task: ketamine impaired working memory performance, reduced the dIPFC BOLD response during the Delay epoch, and reduced dIPFC functional connectivity (Anticevic et al., 2012; N. Driesen and J. Krystal, personal communication). Thus, in primates, NMDAR blockade leads to impaired working memory and reduced cognitive brain activity. These data suggest that treatments for schizophrenia should try to strengthen the activity of dIPFC NMDA recurrent circuits to restore cognitive abilities. The data also explain why treatments that reduce NMDAR actions, based on the hyperglutamate theory of schizophrenia, have failed or even worsened symptoms (Goff et al., 2007; Lieberman et al., 2009).

In contrast to schizophrenia in which ketamine worsens symptoms (Malhotra et al., 1997), acute ketamine treatment rapidly ameliorates symptoms in patients with treatment-resistant depression (Zarate et al., 2006). Rodent models suggest that these beneficial actions of ketamine may occur via increased AMPA-mTOR signaling, leading to increased spines in medial PFC (Li et al., 2010). Based on both clinical and basic findings, NR2B antagonists are being developed for the treatment-resistant depression (Skolnick et al., 2009). The current data caution that these agents may markedly worsen the higher cognitive functions of the dIPFC and thus would not be appropriate for long-term treatment or for treatment of schizophrenia. Interestingly, the positive response to ketamine in severely depressed patients has been related to their anterior cingulate response to fearful faces pretreatment (Salvadore et al., 2009). Neurons in the anterior cingulate of monkeys have been shown to represent negative emotions such as symbolic punishment (Seo and Lee, 2009), as well as the loss of expected reward (Rushworth and Behrens, 2008). Thus, it is also possible that ketamine treat-

ment may be helpful by reducing the firing of NMDAR-dependent, recurrent excitatory circuits in the anterior cingulate and/or in other ventromedial PFC circuits (e.g., Brodmann's area 25; Mayberg et al., 2005) that represent negative emotions and instigate mental suffering. Interrupting the activity of these circuits could underlie the immediate beneficial effects of ketamine in some patients.

### Relevance to Aging and Alzheimer's Disease

Reductions in NMDA signaling may also contribute to age-related cognitive disorders. NMDA NR2B expression declines in dIPFC with advancing age (Bai et al., 2004), although it is not yet known whether this simply reflects age-related loss of dendritic spines. Internalization of NMDA NR2B receptors may underlie early cognitive decline in AD. Recent studies of the etiology of cognitive deficits in AD have focused on the toxic effects of soluble A $\beta$  oligomers on synaptic transmission, prior to end stage plaque formation. Importantly, A $\beta$  induces the internalization of NMDA NR2B receptors and a reduction in NMDAR currents (Snyder et al., 2005) via STEP signaling, and STEP actions are increased in the PFC of Alzheimer's disease patients (Kurup et al., 2010). The current study shows that reduced NMDA NR2B receptor signaling in the PFC would probably lead to a reduction in persistent network firing and thus impaired cognition. However, excitotoxicity arising from cell death probably occurs later in the course of the illness. This might explain why memantine would be effective in late, but not early, stage AD (van Dyck, 2004).

### Conclusion

Traditionally, the function of the NMDAR has been almost exclusively emphasized in terms of its critical role in long-term synaptic plasticity. However, computational work suggests that NMDAR-dependent recurrent excitation may also be important for "cognitive-type" online computations, such as working memory, cognitive control (Lo et al., 2009), and decision making (Wang, 2002). The present work provides direct evidence in support of this idea, offering a new perspective for understanding the cellular and circuit mechanisms of higher cognition. The predominant role of NMDAR in dIPFC pyramidal cell circuits should also inform glutamate theories of schizophrenia and explain why insults to these NMDAR synapses can lead to working memory deficits and thought disorder (Arnsten et al., 2012).

### EXPERIMENTAL PROCEDURES

All procedures were approved by the IACUC's of Yale University and Mount Sinai School of Medicine.

#### Immunoelectron Microscopy

The antibodies used in this study were selective for NMDA NR2B and are described in detail in the [Supplemental Experimental Procedures](#). Details of the immuno-EM methods can also be found in Janssen et al. (2005).

#### Computational Modeling

Please see details described in Brunel and Wang (2001), Compte et al. (2000), and Wang (1999).

#### Single-Neuron Recording in Monkeys Performing the ODR Task

Studies were performed on two adult male rhesus monkeys trained on the spatial ODR task (Figure 1). Iontophoretic electrodes, neuronal recording,

and drug delivery were as described in Wang et al. (2004, 2007) and also are provided in the Supplemental Experimental Procedures. Drugs MK801 and Ro 25-6981 (Tocris) were dissolved at 0.01 M in triple-distilled water (pH 3.5–4.0), and the AMPA antagonists CNQX disodium salt and NBQX disodium salt (Tocris) were dissolved at 0.01 M in triple-distilled water (pH 8.0–8.5). Two-way ANOVA was used to examine the spatial-tuned task-related activity with regard to (1) different periods of the task (cue, delay, and response versus fixation) and (2) different cue locations. One-way ANOVAs were employed to assess the effect of the drug application on cells displaying task-related activity; paired comparisons of drug versus control for the average response were assessed with a dependent t test. The spatial tuning was examined by calculating the tuning index (TI, 0 = no tuning; 1 = strongest tuning): TI = firing rate at (preferred direction – nonpreferred direction)/firing rate at (preferred direction + nonpreferred direction).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2012.12.032>.

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