

Nicotinic $\alpha 4\beta 2$ Cholinergic Receptor Influences on Dorsolateral Prefrontal Cortical Neuronal Firing during a Working Memory Task

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The primate dorsolateral prefrontal cortex (dlPFC) subserves top-down regulation of attention and working memory abilities. Depletion studies show that the neuromodulator acetylcholine (ACh) is essential to dlPFC working memory functions, but the receptor and cellular bases for cholinergic actions are just beginning to be understood. The current study found that nicotinic receptors comprised of $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\beta 2$ -nAChR) enhance the task-related firing of delay and fixation cells in the dlPFC of monkeys performing a working memory task. Iontophoresis of $\alpha 4\beta 2$ -nAChR agonists increased the neuronal firing and enhanced the spatial tuning of delay cells, neurons that represent visual space in the absence of sensory stimulation. These enhancing effects were reversed by coapplication of a $\alpha 4\beta 2$ -nAChR antagonist, consistent with actions at $\alpha 4\beta 2$ -nAChR. Delay cell firing was reduced when distractors were presented during the delay epoch, whereas stimulation of $\alpha 4\beta 2$ -nAChR protected delay cells from these deleterious effects. Iontophoresis of $\alpha 4\beta 2$ -nAChR agonists also enhanced the firing of fixation cells, neurons that increase firing when the monkey initiates a trial, and maintain firing until the trial is completed. These neurons are thought to contribute to sustained attention and top-down motor control and have never before been the subject of pharmacological inquiry. These findings begin to build a picture of the cellular actions underlying the beneficial effects of ACh on attention and working memory. The data may also help to explain why genetic insults to $\alpha 4$ subunits are associated with working memory and attentional deficits and why $\alpha 4\beta 2$ -nAChR agonists may have therapeutic potential.

Key words: *in vivo* single-cell recording; iontophoresis; nicotinic alpha4beta2 receptor; nonhuman primate; prefrontal cortex; working memory

Significance Statement

The acetylcholine (ACh) arousal system in the brain is needed for robust attention and working memory functions, but the receptor and cellular bases for its beneficial effects are poorly understood in the newly evolved primate brain. The current study found that ACh stimulation of nicotinic receptors comprised of $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\beta 2$ -nAChR) enhanced the firing of neurons in the primate prefrontal cortex that subserve top-down attentional control and working memory. $\alpha 4\beta 2$ -nAChR stimulation also protected neuronal responding from the detrimental effects of distracters presented during the delay epoch, when information is held in working memory. These results illuminate how ACh strengthens higher cognition and help to explain why genetic insults to the $\alpha 4$ subunit weaken cognitive and attentional abilities.

Introduction

Acetylcholine (ACh) has been known to have important influences on working memory and attentional abilities for decades

(Bartus and Johnson, 1976; Granon et al., 1995; Sarter et al., 1997; Levin et al., 2006; Mansvelder et al., 2009), but the specific receptor and molecular mechanisms underlying its various roles in cognitive function have only begun to be understood. ACh acts at a variety of metabotropic muscarinic and ionotropic nicotinic receptor subtypes, the latter comprised of five α ($\alpha 2$ – $\alpha 10$) and/or

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β ($\beta 2$ – $\beta 4$) subunits. The $\alpha 4\beta 2$ nicotinic receptor ($\alpha 4\beta 2$ -nAChR) is of interest given its high affinity for nicotine and its relationship to cigarette smoking (Picciotto and Kenny, 2013; McClure-Begley et al., 2016), its widespread distribution in brain (Han et al., 2000), and its links to cognitive processing. For example, genetic alterations in the $\alpha 4$ subunit in humans have been associated with altered cognitive and attentional abilities (Reinvang et al., 2009; Espeseth et al., 2010; Mobascher et al., 2016) and possibly to greater incidence of attention deficit hyperactivity disorder (ADHD) (Kent et al., 2001; Winterer et al., 2007; Wallis et al., 2009). Similarly, genetic knockdown or blockade of nicotinic $\alpha 4$ and/or $\beta 2$ nicotinic subunits impairs, whereas stimulation improves, attentional processing in rodent models (Guillem et al., 2011; Bloem et al., 2014; Wood et al., 2016) and stimulation of the cholinergic basal forebrain can enhance visual processing in the visual cortex through muscarinic receptors (Goard and Dan, 2009). Research in monkeys has also shown prominent muscarinic effects on attentional enhancement of visual processing in V1 (Herrero et al., 2008), but only limited influences of nicotinic receptor stimulation (Disney et al., 2007). However, systemic administration of the $\alpha 4\beta 2$ -nAChR agonist ABT-418 to monkeys improved working memory performance under distracting conditions (Prendergast et al., 1998), suggesting that these receptors may have beneficial effects in primate cortex in more anterior cortical regions.

The dorsolateral prefrontal cortex (dlPFC) plays a fundamental role in working memory and attentional processing. Lesions to the dlPFC in monkeys impair top-down attentional control (Rossi et al., 2007) and produce profound deficits in working memory, particularly when distractors are presented during the delay period (Grueninger and Pribram, 1969; Bartus and Levere, 1977). More recent physiology studies have provided a cellular basis for this phenomenon, finding that dlPFC neurons are uniquely capable of maintaining firing to a relevant stimulus and suppressing responses to distractors (Miller et al., 1996; Suzuki and Gottlieb, 2013). dlPFC neurons also fire in relationship to top-down attentional control, whereas bottom-up attention emanates from more posterior cortices (Buschman and Miller, 2007). The cognitive functioning of the dlPFC is remarkably dependent on modulatory influences by ACh because ACh depletion from dlPFC produces working memory deficits comparable to dlPFC ablation (Croxson et al., 2011). This can also be seen at the cellular level, where cholinergic stimulation of nicotinic $\alpha 7$ receptors ($\alpha 7$ -nAChRs) has a critical, permissive effect on dlPFC NMDA receptor (NMDAR) actions needed for neuronal firing during a working memory task (Yang et al., 2013). However, there have been no studies examining how $\alpha 4\beta 2$ -nAChRs influence the firing patterns of dlPFC neurons.

The current study examined the roles of $\alpha 4\beta 2$ -nAChR stimulation on dlPFC neuronal firing in monkeys performing an oculomotor delayed response (ODR) task, a test of visuospatial working memory (Fig. 1A), while recording single units from the dlPFC (Fig. 1D). In addition, we tested the role of $\alpha 4\beta 2$ -nAChR under distracting conditions, when irrelevant stimuli were presented during the delay period (Fig. 1B). The dlPFC contains a variety of working memory task-related neurons (Funahashi et al., 1991), including fixation cells that sustain firing across each trial, neurons that fire in relationship to the sensory cue (cue cells), or to the saccadic response (response cells), as well as delay cells, which maintain firing across the delay epoch in a spatially tuned manner with greater firing for their “preferred direction” (Fig. 1E). Delay cells are thought to carry the neuronal representations needed to maintain goals in working memory and to protect these goals from the interference of distraction (Goldman-Rakic, 1995) (Goldman-Rakic, 1995); therefore,

these cells were the focus of the current study. We also examined drug effects on fixation cells, given their potential role in sustained attention, as well as task-related cue and response cells for comparison. We found that $\alpha 4\beta 2$ -nAChR stimulation enhanced the firing of delay cells and fixation cells and protected delay cell firing from the detrimental effects of distraction.

Materials and Methods

Subjects. Three adult male rhesus monkeys (*Macaca mulatta*) were used for the current study and were cared for under the guidelines of the National Institutes of Health and the Yale University Institutional Animal Care and Use Committee.

ODR task. The monkeys were seated in primate chairs with their heads fixed and faced a 27 inch computer monitor 30 inches in front of them. The monkeys’ eye positions were monitored with the ISCAN eye movement monitoring system. The monkeys were trained in the visuospatial ODR task, which required the subject to make a memory-guided saccade to a remembered visuospatial target. Patients with schizophrenia have been shown to be impaired on a human version of this task (Keedy et al., 2006). The ODR task was generated by the PICTO system (custom-designed Windows-based data acquisition software). The task is illustrated in Figure 1A. A central small white circle was illuminated on the computer monitor, which served as the fixation target. To initiate a trial, the animal fixated this central target and maintained fixation for 0.5 s (fixation period), whereupon a cue (the same sized white circle) was illuminated for a period of 0.5 s (cue period) at 1 of 8 peripheral targets located at an eccentricity of 13° with respect to the fixation spot. After the cue was extinguished, a 2.5 s delay period followed. The subject was required to maintain central fixation throughout both the cue presentation and the delay period. At the end of the delay, the fixation spot was extinguished, which instructed the monkey to make a memory-guided saccade to the location where the cue had been shown before the delay period. A trial was considered successful if the subject’s response was completed within 0.5 s of the offset of the fixation spot and was within 2° around the correct cue location. The subject was rewarded with fruit juice immediately after every successful response. The position of the stimulus was randomized over trials such that it had to be remembered on a trial-by-trial basis. The intertrial intervals (ITIs) were at least 3 s. The subject performed 1000–1500 trials per session.

ODR task with distractors. During distractor trials, a red distractor (0.5 s duration) was presented during the delay period, as shown in Figure 1B. Because the cue was a white circle, the red color of the distractor indicated that the stimulus was irrelevant. The distractor occurred randomly at 1 of 3 locations opposite to the cue 1 s after the delay period was initiated. Distractors were only tested during recordings from delay cells.

Recording locus. Before recording, the animals underwent an MRI scan to obtain exact anatomical coordinates of brain structures, which guided placement of the chronic recording chambers. MRI-compatible materials were used for the implant so that another MRI could be performed after implantation to confirm the position of the recording chambers. The recording wells were placed over the caudal principal sulcus as illustrated in Figure 1D.

In vivo single-unit recordings and iontophoresis. This study used iontophoresis to apply the $\alpha 4\beta 2$ -nAChR agonists ABT-418 and RJR2403 and/or the $\alpha 4\beta 2$ -nAChR antagonist DH β E near dlPFC neurons. ABT-418 was from Sigma-Aldrich. DH β E and RJR2403 were from Tocris Bioscience. Drug solutions (5–10 mM, pH 3–4) were made using sterilized water. Iontophoretic electrodes were constructed with a 20 μ m-pitch carbon fiber (ELSI) inserted in the central barrel of a seven-barrel nonfilamented capillary glass (Friedrich and Dimmock). The assembly was pulled using a multipipette electrode puller (PMP-107L; MicroData Instrument) and the tip was beveled to obtain the finished electrode (Fig. 1C). Finished electrodes had impedances of 0.3–1.5 M Ω (at 1 kHz) and tip sizes of 30–40 μ m. The outer barrels of the electrode were then filled with three drug solutions (two consecutive barrels each) and the solutions were pushed to the tip of the electrode using compressed air. A Neurophore BH2 iontophoretic system (Medical Systems) was used to control the delivery of the drugs. The drug was ejected at currents that

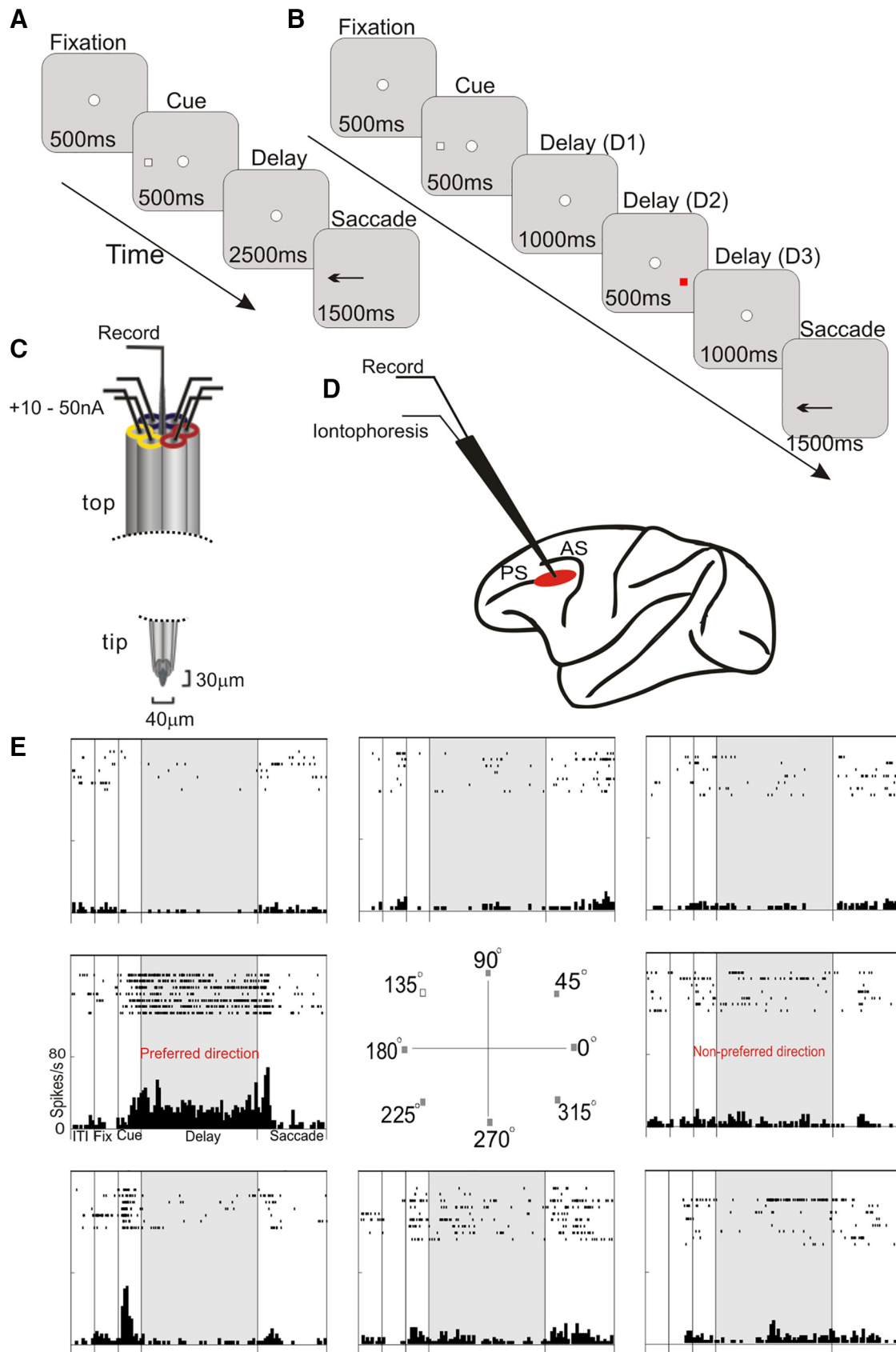


Figure 1. Paradigm used for iontophoretic recordings in monkeys performing a spatial working memory task. **A**, ODR spatial working memory task. **B**, Distractor version of the ODR task. **C**, Schematic of the iontophoresis/recording electrode with six micropipettes surrounding the carbon fiber electrode, where different compounds can be delivered by currents through different pipettes; for example, drug 1 (in two consecutive yellow pipettes) versus drug 2 (in two red pipettes). **D**, Region of the caudal principal sulcal (PS) PFC, where recordings are performed. AS, Arcuate sulcus. **E**, Typical dIPFC delay cell with spatially tuned, delay-related firing. Rasters and histograms are arranged to indicate the location of the corresponding cue; the preferred direction for this neuron was 180°. Please note that, in subsequent figures, only the preferred direction and one nonpreferred direction will be shown for neurons with delay-related firing to conserve space.

varied from 10 to 25 nA. Retaining currents of -5 to -10 nA were used in a cycled manner (1 s on, 1 s off) when not applying drugs. Drug ejection did not create noise in the recording and there was no systematic change in either spike amplitude or time course at any ejection current.

The electrode was mounted on a MO-95 micromanipulator (Narishige) in a 25-gauge stainless steel guide tube. The dura was then punctured using the guide tube to facilitate access of the electrode to cortex. Extracellular voltage was amplified using an AC/DC differential preamplifier (Model 3000; A-M Systems) and band-pass filtered (180 Hz–6 kHz, 20 dB gain, 4-pole Butterworth; Kron-Hite). Signals were digitized (15 kHz, micro 1401; CED) and acquired using Spike2 software (CED). Neural activity was analyzed using waveform sorting by a template-matching algorithm. Poststimulus time histograms (PSTHs) and rastergrams were constructed online to determine the relationship of unit activity to the task. Unit activity was measured in spikes per second. Four different kinds of ODR task-related cells were classified: fixation cells, cue cells, delay cells, and response cells. If the rastergrams showed that a neuron displayed task-related activity, then recording continued and pharmacological testing was performed. A total of 101 task-related cells were recorded and tested with $\alpha 4\beta 2$ -nAChR agents (72 from Monkey C, 24 from Monkey A, and 5 from Monkey P). The percentages of task-related neurons were as follows: fixation cells 22 (22%), delay cells 43 (43%), cue cells 6 (6%), and response cells 30 (30%).

Neuronal activities were first collected from the cell under a control condition in which at least eight trials at each of eight cue locations was obtained. Upon establishing the stability of the cells' activity, this control condition was followed by iontophoretic application of drug(s). Dose-dependent effects of the drug were tested in two or more consecutive conditions, which was followed by a recovery condition or a reversal condition. Drugs were applied continuously at a relevant current throughout a given condition. Each condition had ~ 8 (range 6–12) trials at each location to allow for statistical analyses of drug effects. In the distractor experiments, neuronal firing was first recorded under control classic ODR condition (no distraction). If the cell showed spatially tuned delay-related activity from the online PSTHs and rastergrams, then the control condition was followed by ODR with distractor condition and then by iontophoretic application of the $\alpha 4\beta 2$ -nAChR agonist condition as the monkey performed the distractor version of the task.

Data analysis. For purposes of data analysis, each trial in the ODR task was divided into four epochs—initial fixation, cue, delay, and response (saccade). The initial fixation epoch lasted for 0.5 s. The cue epoch lasted for 0.5 s and corresponds to the stimulus presentation phase of the task. The delay lasted for 2.5 s and reflects the mnemonic component of the task. The response phase started immediately after the delay epoch and lasted ~ 1.5 s. Data analysis was performed in MATLAB and SPSS. Spike density functions were constructed in 50 ms windows. Two-way ANOVA was used to examine the spatially tuned task-related activity with regard to different periods of the task (fixation, cue, delay, and response vs ITI) and different cue locations. This study mainly focused on delay cells that represent working memory. Many delay cells fire during the cue and/or response epochs as well as the delay epoch; given their variable responding to the cue and response epochs, data analyses focused on the delay epoch. One-way ANOVA or two-tailed paired t test were used to assess the effects of drug application on task-related activity. In the interest of brevity, figures often show the neurons' preferred direction compared with just one nonpreferred direction, the "antipreferred" direction directly opposed to the neurons' preferred direction. For delay cells, spatial tuning was assessed by comparing firing levels for the neuron's preferred direction vs its nonpreferred directions. Quantification of spatial tuning was performed by calculating a measure of d' using the following formula:

$$d' = (\text{mean}_{\text{pref}} - \text{mean}_{\text{nonpref}}) / \sqrt{(sd_{\text{pref}}^2 + sd_{\text{nonpref}}^2) / 2}$$

Results

The results of iontophoresing $\alpha 4\beta 2$ -nAChR compounds on task-related neurons in the primate dlPFC are summarized below. Please note that the iontophoresis procedure itself has no effect on neuronal firing on its own; that is, iontophoresis of vehicle

(saline, pH 4) with either a positive or negative current has no effect on neuronal firing (Vijayraghavan et al., 2007). In addition, iontophoresis releases very small amounts of drug, which likely influence a mini-column of nearby neurons, but are inadequate to influence behavioral performance. Behavioral performance was maintained at optimal levels unless otherwise noted, that is, during conditions of distraction.

Stimulation of $\alpha 4\beta 2$ -nAChR enhances the delay-related firing and spatial tuning of dlPFC delay cells

We first tested the effect of $\alpha 4\beta 2$ -nAChR agonists on the delay-related (0–2.5 s) firing and spatial tuning of dlPFC delay cells in monkeys performing the classic ODR task (Fig. 1A). We found that iontophoresis of $\alpha 4\beta 2$ -nAChR agonists significantly enhanced the delay-related firing and spatial tuning of delay cells. As seen in a single-cell example in Figure 2A, iontophoretic application of the $\alpha 4\beta 2$ -nAChR agonist ABT-418 produced a dose-related enhancement in delay-related firing for the neuron's preferred direction, but not for its nonpreferred direction. Similarly, in another single-cell example shown in Figure 2B, iontophoretic application of another $\alpha 4\beta 2$ -nAChR agonist, RJR2403, enhanced delay-related firing and firing returned to control levels when the drug was no longer applied (Fig. 2B, recovery condition, light blue line). The enhancing effects of $\alpha 4\beta 2$ -nAChR agonists were consistent in all 25 delay cells recorded. Figure 2C shows the averaged effect of $\alpha 4\beta 2$ -nAChR agonist on delay cell firing at the population level, where $\alpha 4\beta 2$ -nAChR agonist at doses of 10–25 nA significantly enhanced delay cell firing during the delay period for the neurons' preferred directions. Given the similarities between the two agonists, these averaged data include neurons administered either ABT-418 or RJR2403. Statistical analysis showed that $\alpha 4\beta 2$ -nAChR agonist application significantly increased neuronal firing during the delay epoch for the neurons' preferred direction ($t_{\text{dep}(24)} = 10.49$, $p < 0.0001$, two-tailed paired t test; Fig. 2D), but not for the nonpreferred direction ($t_{\text{dep}(24)} = 1.948$, $p = 0.063$, two-tailed paired t test; Fig. 2D). Because this enhancement in delay-related firing by $\alpha 4\beta 2$ -nAChR agonists is direction selective, we investigated whether $\alpha 4\beta 2$ -nAChR agonists also enhance the spatial tuning of delay cells as assessed by d' (see formula in Materials and Methods). This measure captures how well a delay cell can represent a spatial position over the delay epoch in the absence of sensory stimulation, and thus is particularly important to the strength of working memory. Figure 2E shows a scatter plot of d' values comparing each neuron's firing for its preferred versus nonpreferred directions to illustrate a neuron's spatial tuning under control versus $\alpha 4\beta 2$ -nAChR agonist conditions. A greater d' value indicates greater directional selectivity; that is, greater spatial tuning. A total of 20–25 delay cells showed higher d' in the $\alpha 4\beta 2$ -nAChR agonist condition compared with the control condition. Overall, iontophoresis of $\alpha 4\beta 2$ -nAChR agonists significantly enhanced the spatial tuning of delay cell firing during the delay epoch by increasing d' ($t_{\text{dep}(24)} = 3.712$, $p = 0.001$, two-tailed paired t test; Fig. 2F).

In addition to enhancing cell firing during the delay epoch, $\alpha 4\beta 2$ -nAChR agonists produced a small but significant increase in firing during the initial fixation period (-1 to -0.5 s). This small increase in firing was evident for both the preferred and nonpreferred directions (preferred: $t_{\text{dep}(24)} = 2.25$, $p = 0.033$; nonpreferred: $t_{\text{dep}(24)} = 2.38$, $p = 0.02$, two-tailed paired t test; Fig. 2C,D). Because the cue had yet to occur during this epoch, it is not surprising that drug-induced increases in delay cell firing during the initial fixation period were not direction selective.

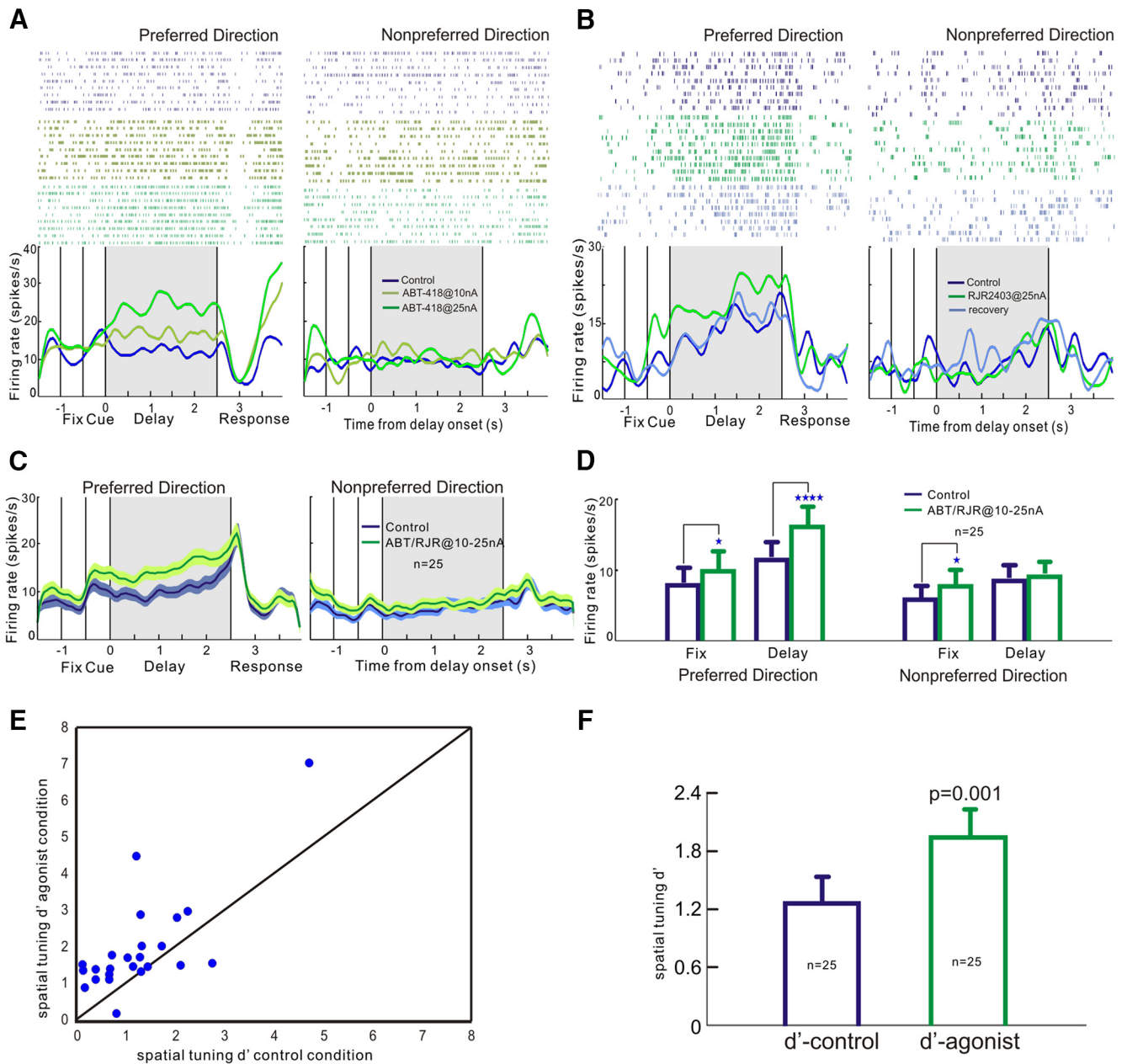


Figure 2. Effects of $\alpha 4\beta 2$ -nAChR agonists on the task-related firing of dlPFC delay cells. **A**, Single-neuron example of the dose-dependent effects of the $\alpha 4\beta 2$ -nAChR agonist ABT-418 on delay-related activity. The top part of the figure shows spike raster plots for the neuron's preferred versus nonpreferred direction. The bottom part of the figure shows the spike density function of this neuron. Data are shown for three different conditions: control conditions (blue), ABT-418 at 10 nA (Martian green) and ABT-418 at 25 nA (green). ABT-418 produced a dose-dependent increase in delay-related firing selectively for the neuron's preferred direction. **B**, Single-neuron example of the enhancing effects of another $\alpha 4\beta 2$ -nAChR agonist, RJR2403. Iontophoresis of RJR2403 at 25 nA significantly enhanced delay-related firing for the neuron's preferred direction, but not for the nonpreferred direction (green). Activity was reduced to control levels after drug application was terminated during the recovery condition (light blue). **C**, Population spike density functions for the average of 25 delay cells showing firing for their preferred versus their nonpreferred directions under control conditions (blue) and after iontophoresis of an $\alpha 4\beta 2$ -nAChR agonist (green). **D**, Mean \pm SEM firing rate of these same 25 delay cells during the initial fixation versus delay epochs of the task. Statistical analysis shows that $\alpha 4\beta 2$ agonist significantly enhance the delay firing for the preferred direction, but not for the nonpreferred direction, whereas the $\alpha 4\beta 2$ -nAChR agonist slightly but significantly enhanced the fixation period firing for both the preferred and the nonpreferred direction. **E**, Scatter plot of d' comparing each neuron's firing of delay period for its preferred versus nonpreferred directions to examine the neuron's spatial tuning. Greater d' value indicates greater directional selectivity. **F**, Iontophoresis of $\alpha 4\beta 2$ -nAChR agonists significantly enhanced the spatial tuning of delay cell by increasing d' . Error bars indicate SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

We next tested whether the enhancing effects of ABT-418 occurred at $\alpha 4\beta 2$ -nAChR by determining whether coapplication of the $\alpha 4\beta 2$ -nAChR antagonist Dh β E would block the enhancing effects of ABT-418. We found that co-iontophoresis of Dh β E reversed the enhancing effects of ABT-418 on delay-related firing of delay cells. As shown in Figure 3, **A** and **B**, ABT-418 at 25 nA significantly increased delay-related firing for the neurons' preferred direction and these enhancing effects of ABT-418 were

reversed by co-iontophoresis of Dh β E (overall: $\chi^2/df = 9.3/2$, $p = 0.009$, Friedman test; pairwise comparisons: control vs ABT418: $S = -10.5$, $p = 0.03$; control vs ABT418+Dh β E: $S = -1.5$, $p = 0.84$; ABT418 vs ABT418+Dh β E: $S = -10.5$, $p = 0.03$, Wilcoxon signed-rank test; Fig. 3*B*). These results are consistent with ABT-418 acting at $\alpha 4\beta 2$ -nAChR.

We also found that the converse was true: co-iontophoresis of RJR2403 could reverse the reduction in delay-related firing in-

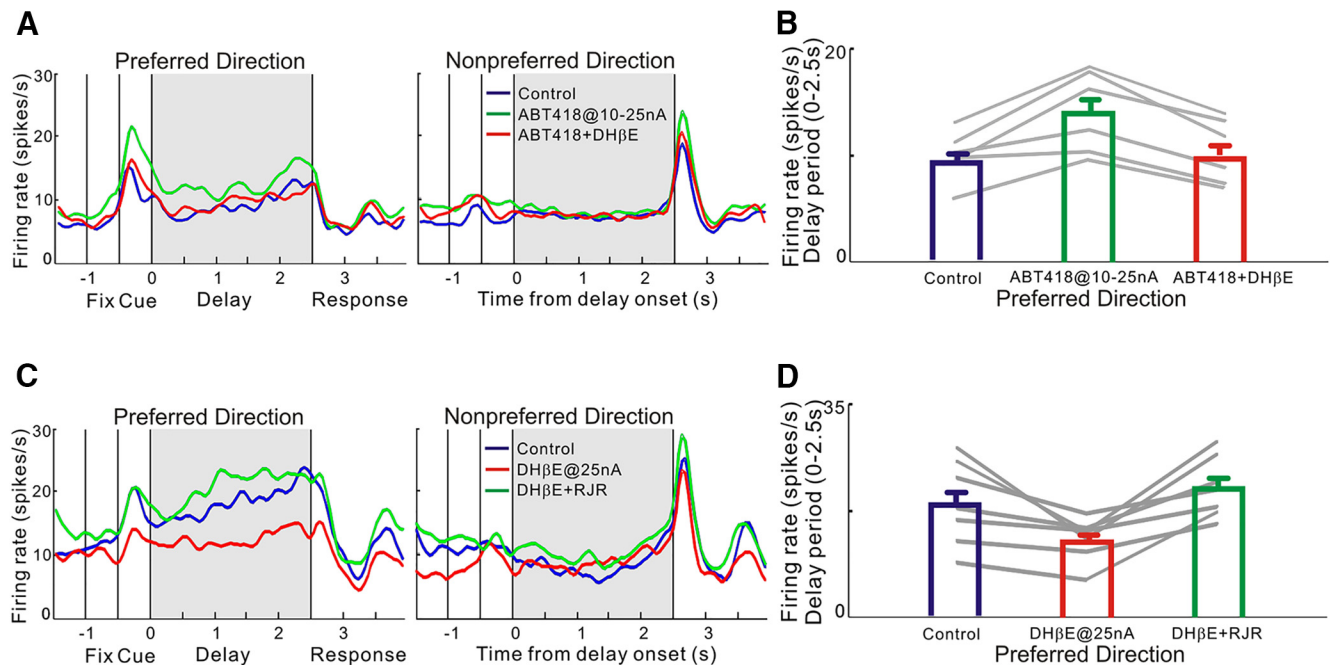


Figure 3. $\alpha 4\beta 2$ -nAChR agonist versus antagonist reverse each other's actions. **A, B,** An $\alpha 4\beta 2$ -nAChR antagonist reversed the enhancing actions of an $\alpha 4\beta 2$ -nAChR agonist. **A,** The $\alpha 4\beta 2$ -nAChR agonist ABT-418 significantly increased delay-related firing (green) and the enhancement was reversed by co-iontophoresis of the $\alpha 4\beta 2$ -nAChR antagonist DH β E at 15 nA (red), consistent with ABT-418 actions on $\alpha 4\beta 2$ -nAChR. **B,** Mean \pm SEM firing rate (delay period of preferred direction) of six delay cells and a scatter plot of delay-related firing of each neuron showing that DH β E + ABT-418 reversed the enhancement of ABT-418. **C, D,** $\alpha 4\beta 2$ -nAChR agonist reversed the reduction in firing induced by an $\alpha 4\beta 2$ -nAChR antagonist. **C,** $\alpha 4\beta 2$ -nAChR antagonist DH β E significantly decreased delay-related firing (red) and the reduction was reversed by co-iontophoresis of the $\alpha 4\beta 2$ -nAChR agonist ABT-418. **D,** Mean \pm SEM firing rate (delay period of preferred direction) of seven delay cells and a scatter plot of delay-related firing of each neuron showing that ABT-418 + DH β E reversed the reduction of DH β E.

duced by the $\alpha 4\beta 2$ -nAChR antagonist, DH β E. As shown in Figure 3, **C** and **D**, DH β E at 25 nA significantly reduced delay-related firing for the neurons' preferred direction and these reductions were then reversed by co-iontophoresis of RJR2403 (overall: $\chi^2/df = 11.1/2$, $p = 0.004$, Friedman test; pairwise comparisons: control vs DH β E: $S = 14$, $p = 0.016$; control vs DH β E + RJR2403: $S = -11$, $p = 0.078$; DH β E + RJR2403 vs DH β E: $S = 14$, $p = 0.016$, Wilcoxon signed-rank test; Fig. 3D). Therefore, the $\alpha 4\beta 2$ -nAChR agonist versus antagonist had opposing, competitive effects on delay cell firing.

Presentation of distractors impairs working memory performance and interrupts dlPFC delay cell firing

To further test the effects of $\alpha 4\beta 2$ -nAChR agonists under attention-demanding conditions, we modified the ODR task by adding a distractor on each trial during the delay period. The modified version of the ODR task is shown in Figure 1B. Assessment of behavioral performance showed that the presentation of a distractor during the delay epoch significantly impaired the accuracy of spatial working memory performance. As shown in Figure 4A, the average percentage correct for 18 test sessions with classic ODR trials was 90%, whereas the average percentage correct for 18 test sessions with distractor trials was only 75% ($t_{dep(33)} = 6.624$, $p < 0.001$, $n = 18$ sessions, two-tailed t test).

At the neuronal level, the presentation of a distractor also altered delay cell firing during the delay period. As shown in Figure 4B, a single-cell example, and Figure 4C, a population analysis, under control conditions without distraction (blue), the neuron showed strong persistent firing throughout the delay epoch for its preferred direction and little firing for its nonpreferred direction. The presentation of a distractor (red) during the delay period from 1.0–1.5 s (indicated by pink shading) reduced the

delay-related firing for the neuron's preferred direction, specifically reducing the firing in the later delay period (1.0–2.5 s, during and after the distractor presentation; $t_{dep(17)} = 3.564$, $p = 0.002$, two-tailed paired t test). In contrast, the presentation of a distractor produced a transient activation during the 1.0–1.5 s epoch of the delay period for the neuron's nonpreferred direction ($t_{dep(17)} = 3.077$, $p = 0.006$, two-tailed paired t test). Therefore, the presentation of the distractor reduced the spatial tuning of delay cells, especially for the latter portion of the delay epoch 1.0–2.5 s. A total of 16 of 18 delay cells showed reduced d' in the ODR-distractor condition compared with the control ODR condition (Fig. 4D) and the reduction of spatial tuning was highly significant ($t_{dep(17)} = 4.258$, $p = 0.0005$, two-tailed paired t test; Fig. 4E) due to both decreasing the firing for the neurons' preferred direction and increasing the firing for the nonpreferred direction. Therefore, the presentation of a distractor during the delay epoch weakened delay representations of visual space.

Stimulation of $\alpha 4\beta 2$ -nAChR restores delay-related firing during distracting conditions in dlPFC delay cells

The effects of $\alpha 4\beta 2$ -nAChR stimulation on delay cell firing were next investigated during distracting conditions to determine whether they could rescue neuronal firing patterns. As seen in a single-cell example in Figure 5A, iontophoresis of the $\alpha 4\beta 2$ -nAChR agonist RJR2403 (green) restored the loss of firing for the neuron's preferred direction caused by the presentation of a distractor. This rescue of firing for a neuron's preferred direction could be seen for the population of delay cells ($n = 10$; Fig. 5B) and was statistically significant compared with distraction in the absence of the $\alpha 4\beta 2$ -nAChR agonist (distractor vs distractor + RJR2403 condition: $t_{dep(9)} = 3.212$, $p = 0.01$, two-tailed paired t test). However, the distraction-induced firing for the neurons' nonpre-

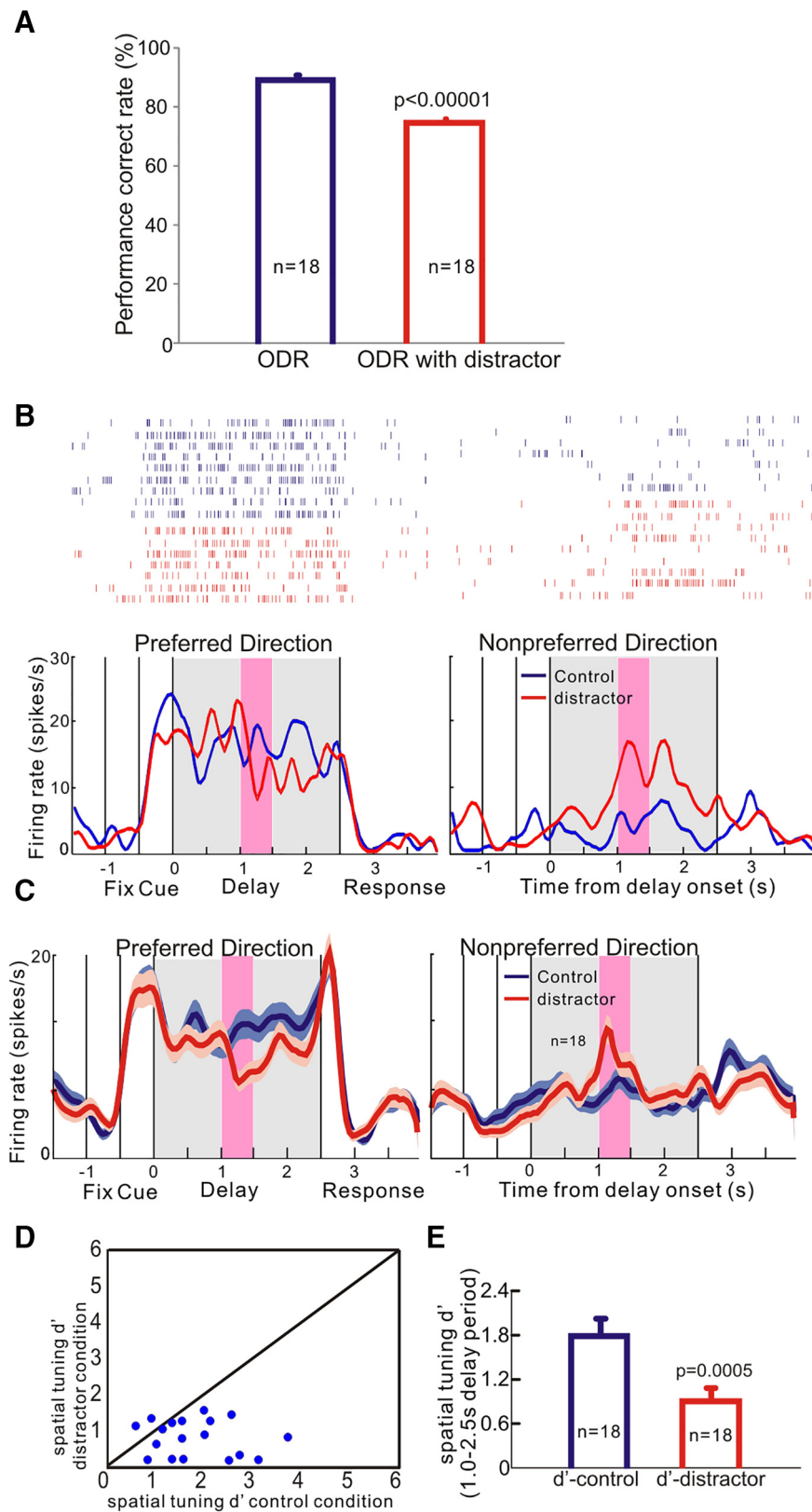


Figure 4. Effects of presenting distractors during the delay epoch on working memory performance and dIPFC delay cell firing. **A**, Presentation of distractors significantly impaired the accuracy of spatial working memory performance on the ODR task. Data are shown as mean \pm SEM. ODR correct rate: $90 \pm 2\%$; ODR with distractor correct rate: $75 \pm 2\%$. **B**, Single-neuron example of the effects of distractor presentation on delay cell firing. Under control conditions (blue), there were no distractors and the neurons showed strong, spatially tuned persistent firing throughout the delay epoch. The presentation of a distractor during the delay period (pink shading) reduced the delay-related firing for the preferred direction and produced the distractor-related activation for the nonpreferred direction (red). **C**, Consistent effects of distractors on the firing of 18 delay cells. **D**, Scatter plot of d' comparing

preferred direction was unchanged by drug treatment (distractor vs distractor+RJR2403 condition: $t_{\text{dep}(9)} = 1.846$, $p = 0.098$, two-tailed paired t test).

A more detailed temporal analysis of neural activity during the delay period can be seen in Figure 5C. Compared with the classic ODR task without distractors, the additions of a distractor significantly reduced neuronal firing during the presentation of the distractor (1–1.5 s period, control vs distractor condition: $t_{\text{dep}(9)} = 2.404$, $p = 0.03$, two-tailed paired t test), as well as the epochs after distractor presentation (1.5–2 s period, control vs distractor condition: $t_{\text{dep}(9)} = 2.627$, $p = 0.027$, two-tailed paired t test; 2–2.5 s periods, $t_{\text{dep}(9)} = 2.439$, $p = 0.037$, two-tailed paired t test). Subsequently, iontophoresis of the $\alpha 4\beta 2$ -nAChR agonist RJR2403 restored neuronal firing levels during the distractor (1–1.5 s period, distractor vs distractor+RJR2403 condition: $t_{\text{dep}(9)} = 2.757$, $p = 0.02$, two-tailed paired t test) and in the time period immediately after distraction (1.5–2 s period, distractor vs distractor+RJR2403 condition: $t_{\text{dep}(9)} = 2.991$, $p = 0.02$, two-tailed paired t test).

In contrast to the pattern of firing for the neurons' preferred direction, neuronal firing for a neuron's nonpreferred direction significantly increased only during the presentation of the distractor (1–1.5 s period, control vs distractor condition: $t_{\text{dep}(9)} = 3.919$, $p = 0.003$, two-tailed paired t test), but returned to normal, low levels of firing in the subsequent epochs (1.5–2 period, control vs distractor condition: $t_{\text{dep}(9)} = 1.472$, $p = 0.17$, two-tailed paired t test; 2–2.5 s period, control vs distractor condition: $t_{\text{dep}(9)} = 1.957$, $p = 0.08$, two-tailed paired t test). Iontophoresis of an $\alpha 4\beta 2$ -nAChR agonist had no effect on neuronal firing for the nonpreferred direction (1–1.5 s period, distractor vs distractor+RJR2403 condition control vs distractor condition: $t_{\text{dep}(9)} = 0.3663$, $p = 0.722$, two-tailed paired t test). Overall, stimulation of $\alpha 4\beta 2$ -nAChR rescues the loss of delay-related firing caused by distraction, but only by enhancing firing for the neurons' preferred direction.

The next set of experiments examined the effect of $\alpha 4\beta 2$ -nAChR agonists on

←

each neuron's firing (1.0–2.5 s of delay period) for its preferred versus nonpreferred directions. **E**, Presentation of the distractor significantly decreased the spatial tuning of delay cell by decreasing the firing for the preferred direction and increasing the firing for the nonpreferred direction. Error bars indicate SEM.

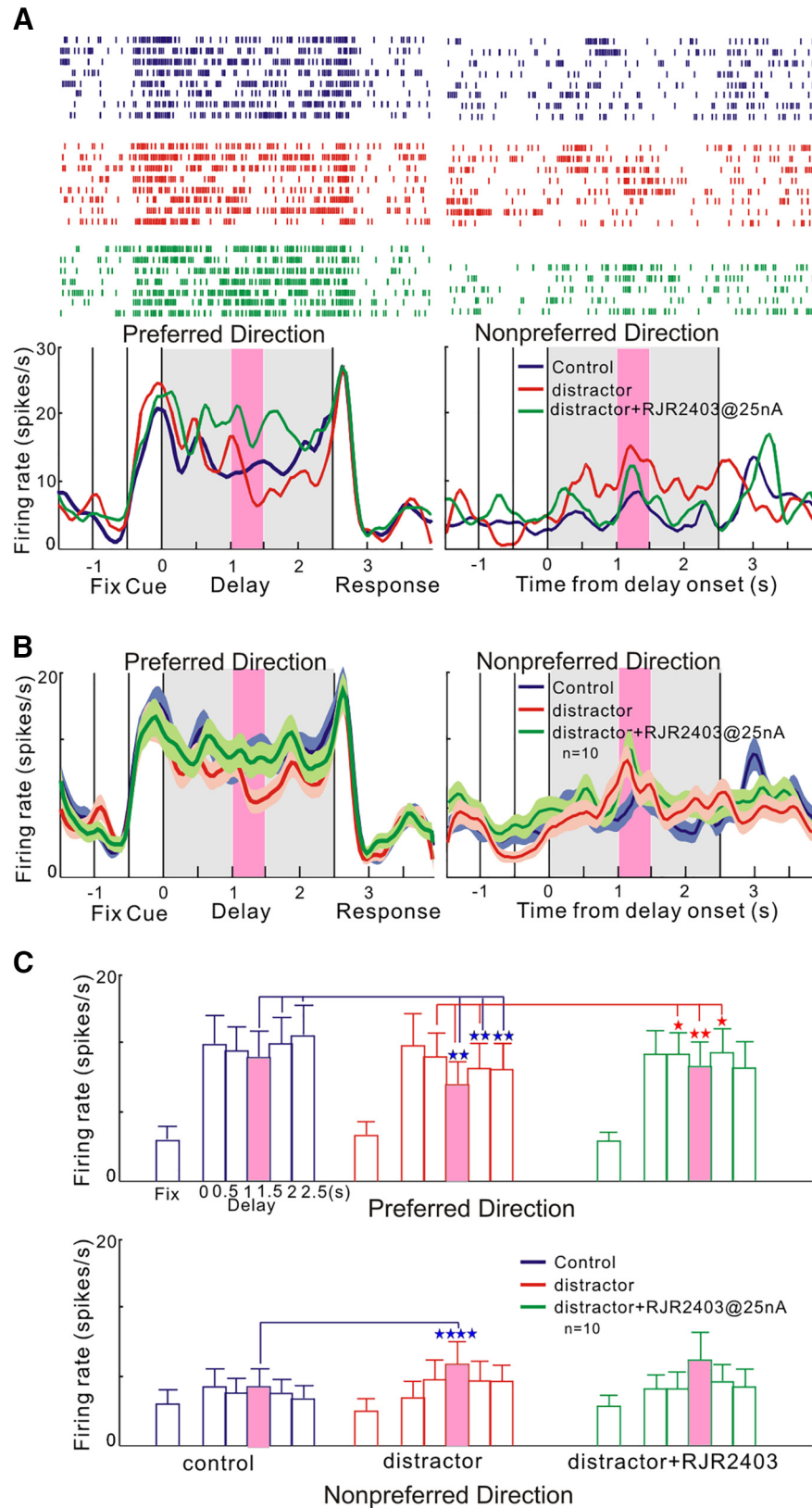


Figure 5. Effects of $\alpha 4\beta 2$ -nAChR agonist on the reduced delay-related firing by distractor presentation. **A**, Example of an individual neuron. The presentation of a distractor during the delay period (pink shading) reduced the delay-related firing for the preferred direction (red), and the following iontophoretic application of the $\alpha 4\beta 2$ -nAChR agonist RJR2403 significantly restored delay-related activity for the preferred direction (green). **B**, $\alpha 4\beta 2$ -nAChR agonist (green) rescued the reduction of delay-related firing by the distractor presentation (red) at the population level ($n = 10$). **C**, Detailed analysis of the delay period showing that the distractor reduced the firing significantly during the presentation of the distractor

other types of task-related dlPFC neurons: fixation cells, cue cells, and response cells.

Stimulation of $\alpha 4\beta 2$ -nAChR enhances the firing of dlPFC fixation cells

In this experiment, monkeys had to fixate on a central spot of light to initiate a trial and had to maintain fixation throughout the extent of the trial until the fixation point was turned off and the monkey could make a saccadic response to the remembered location for juice reward. Fixation cells in dlPFC initiate firing when the monkey begins fixation regardless of the cue's subsequent spatial position and maintain firing until the fixation point is exterminated (Fig. 6A,B). These cells may be related to the sustained attention and top-down motor control needed to perform the task. Similar to the effect on delay cells, we found that stimulation of the $\alpha 4\beta 2$ -nAChR by ABT-418 or RJR2403 markedly enhanced fixation-related firing. A single neuron example in Figure 6A shows that the $\alpha 4\beta 2$ -nAChR agonist RJR2403 produced a dose-related increase in fixation-related firing. Neuronal firing levels returned toward control levels after removal of drug; for example, ABT-418 in Figure 6B. These enhancing effects were seen in all fixation cells tested ($n = 22$), with ABT-418 or RJR2403 significantly increasing task-related firing regardless of the spatial position of the cue ($t_{dep(21)} = 6.01, p < 0.0001$, two-tailed paired t test; Fig. 6C).

Stimulation of $\alpha 4\beta 2$ -nAChR has no significant change on the firing of cue cells and response cells

In contrast to fixation and delay cells, we found that $\alpha 4\beta 2$ -nAChR agonists had only subtle effects on the firing of cue or response cells. Given the robust firing of these neurons under control conditions, this may have been due to ceiling effects. The effects of $\alpha 4\beta 2$ -nAChR agonists on the neuronal firing of cue cells are shown in Figure 6D (this cue cell neuron also exhibits response-related firing) and in Figure 6E (averaged response of all cue cells). Application of agonist had no significant change on neuronal firing to the

(1–1.5 s period) and after the presentation (1.5–2 s and 2–2.5 s period) and $\alpha 4\beta 2$ -nAChR agonist restored the firing significantly during the presentation of the distractor (1–1.5 s period) and after the presentation (1.5–2 s) for the preferred direction. In contrast, the distractor increased the firing significantly during the presentation of the distractor (1–1.5 s period) for the nonpreferred direction. Error bars indicate SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

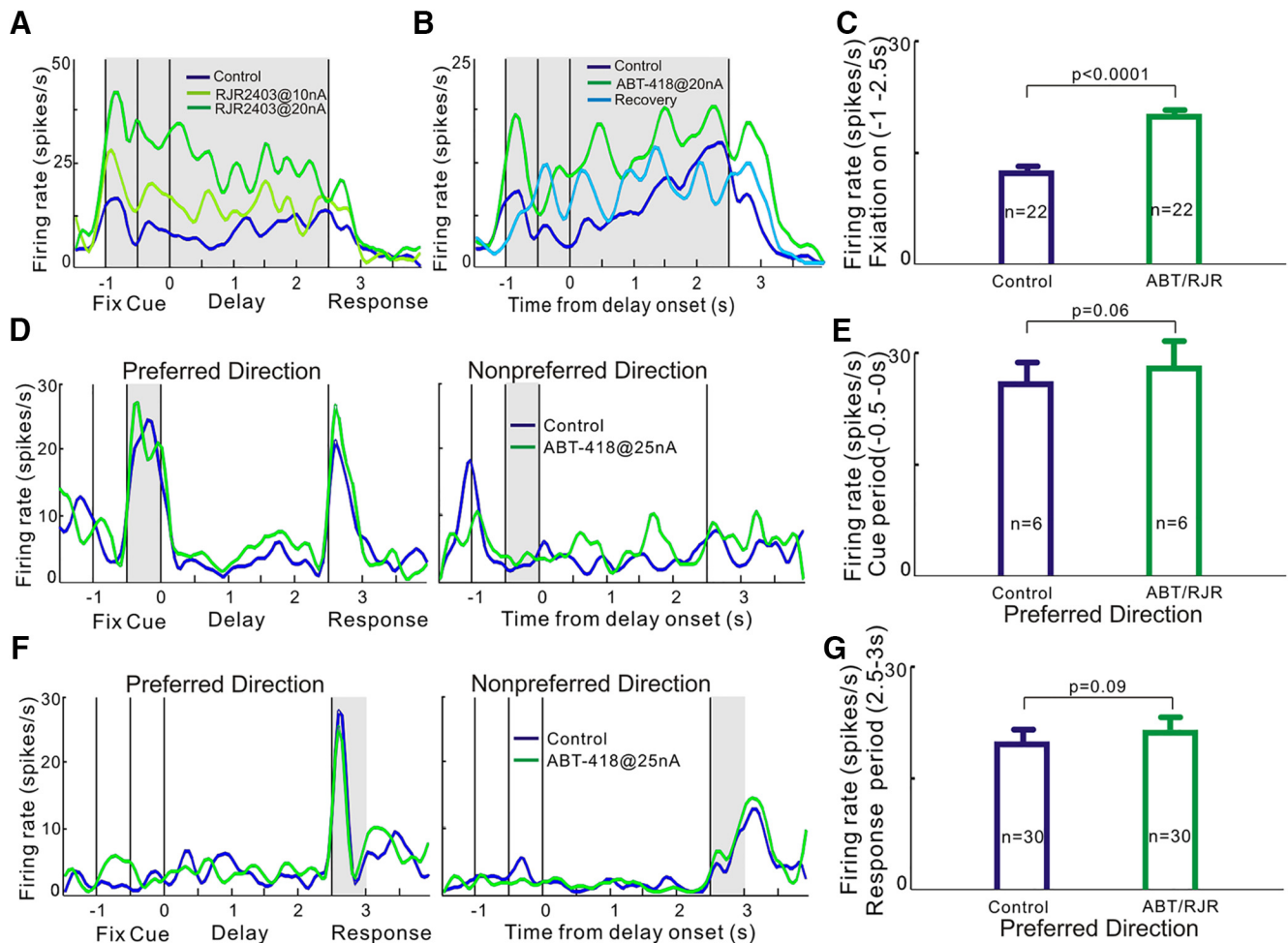


Figure 6. Effects of the $\alpha 4\beta 2$ -nAChR agonists on other task-related neuronal firing. **A–C**, Effects of the $\alpha 4\beta 2$ -nAChR agonists on fixation-related neuronal firing. **A**, Single-neuron example showing that iontophoretic application of RJR2403 significantly enhanced fixation-related activity in the dose-dependent manner. **B**, Single-neuron example showing that the enhancing effects of ABT-418 could be recovered to control levels during the recovery condition (light blue). **C**, Mean \pm SEM firing rate of total 22 fixation cells during fixation-on. $\alpha 4\beta 2$ agonist significantly enhance the fixation-related firing. **D, E**, Effects of the $\alpha 4\beta 2$ -nAChR agonists on cue-related neuronal firing. Both a single-neuron example and population data show that $\alpha 4\beta 2$ agonists have no significant change on the cue-related firing for the preferred direction. **F, G**, Effects of the $\alpha 4\beta 2$ -nAChR agonists on response-related neuronal firing. Both a single-neuron example and population data show that $\alpha 4\beta 2$ agonists have no significant change on the response-related firing for the preferred direction. Error bars indicate SEM.

cue for the neurons' preferred direction ($t_{\text{dep}(5)} = 2.407, p = 0.06$, two-tailed paired t test; Fig. 6E). Similarly, $\alpha 4\beta 2$ agonists had no significant effect on response-related firing ($t_{\text{dep}(29)} = 1.757, p = 0.09$, two-tailed paired t test; Fig. 6F,G). Therefore, $\alpha 4\beta 2$ -nAChR stimulation enhanced the firing of delay and fixation cells in the dlPFC, but had little effect on cue and response cells.

Discussion

This is the first study of $\alpha 4\beta 2$ -nAChR influences on dlPFC neuronal physiology in primates, including the very first study of modulatory influences on dlPFC fixation cells. The data showed that iontophoretic stimulation of $\alpha 4\beta 2$ -nAChR in monkey dlPFC enhanced the task-related firing of both fixation and delay cells, with delay cells showing improved spatial tuning and stronger resistance to distractors after $\alpha 4\beta 2$ -nAChR agonist application. These data are consistent with a larger literature indicating a role of $\alpha 4\beta 2$ -nAChR in working memory and attentional control. In addition, the study also revealed an unexpected dissociation in neuronal response, where $\alpha 4\beta 2$ -nAChR agonists rescued delay cell firing during distraction for the neurons' preferred direction, but not for the nonpreferred direction. These data suggest unknown dlPFC circuit mechanisms when the initial cue and

the distractors are in similar locations. Despite this unexpected response, the overall d' was improved by $\alpha 4\beta 2$ -nAChR agonist stimulation during distracting conditions, indicating a beneficial role in top-down attention.

Importance of $\alpha 4\beta 2$ -nAChR mechanisms to cognitive circuits

Ach plays a widespread role in coordinating arousal with cognitive state. For example, Ach in the rodent medial PFC has been shown to regulate Ach release in posterior cortex (Nelson et al., 2005), consistent with a cholinergic orchestration of attentional and executive circuits (Hasselmo and Sarter, 2011). Ach actions at $\alpha 4\beta 2$ -nAChR likely contribute to this arrangement because mice lacking $\beta 2$ -nAChR subunits have impaired attention (Guillem et al., 2011). Conversely, mice administered the $\alpha 4\beta 2$ -nAChR agonist ABT-418 showed improvement in a test of sustained attention (Young et al., 2013). Similarly, monkeys administered ABT-418 showed improvement in a working memory task, particularly under conditions of distraction (Prendergast et al., 1998).

Genetic variations in the $\alpha 4$ subunit in human subjects have been related to a variety of differences in cognitive operations

thought to be mediated by the PFC. For example, performance of the Stroop interference and Trails B tasks are related to $\alpha 4$ genotype and these tasks both depend on PFC function and require suppression of distraction and inhibition of a prepotent response (Reinvang et al., 2009). The $\alpha 4$ genotype interacts with the dopamine D2 receptor genotype in mediating nicotine's effects on performance of an attention task with distracters (Ahrens et al., 2015). Similarly, the genotype for the noradrenergic synthetic enzyme dopamine β hydroxylase interacts with the $\alpha 4$ genotype in relationship to working memory abilities with varying attentional load (Greenwood et al., 2009). The $\alpha 4$ genotype has also been related to activity of the anterior cingulate and posterior parietal cortex during an oddball task (Winterer et al., 2007) and to alterations in posterior attentional circuits in tasks that require the reorienting of attention (Giessing et al., 2012; Greenwood et al., 2012). Because $\alpha 4\beta 2$ -nAChR expression is widespread in the brain, these relationships likely involve receptors in multiple brain circuits. However, the current data suggest that genetic differences in $\alpha 4\beta 2$ -nAChR within dlPFC may contribute to variations in cognitive and attentional abilities in human subjects.

Speculations on cellular mechanisms within dlPFC

Single-unit recordings in awake, behaving monkeys do not allow identification of the neuronal cell type or laminar location of the neuron under study, but previous studies by Goldman-Rakic and colleagues strongly suggest that delay cells likely reside in deep layer III of dlPFC. Delay cell firing arises from the recurrent excitation of pyramidal cell microcircuits that excite each other via NMDAR on spines to maintain persistent firing across the delay period in the absence of sensory stimulation (Goldman-Rakic, 1995; Wang et al., 2013; Riley and Constantinidis, 2015). In contrast, the spatial tuning of delay cells arises from lateral inhibition from fast-spiking GABAergic interneurons (Goldman-Rakic, 1995; Rao et al., 2000). Although superficial layer V shows some evidence of extended horizontal projections, these are not as extensive as layer III (Kritzer and Goldman-Rakic, 1995). In contrast, the laminar identity of fixation cells is unknown. There is also no knowledge of the ultrastructural localization of $\alpha 4\beta 2$ -nAChR in the primate dlPFC, which hampers hypotheses regarding cellular mechanisms. Studies from rodent medial PFC suggest that $\beta 2$ -subunit-containing receptors reside on both non-fast-spiking interneurons and layer VI pyramidal cells (Bloem et al., 2014), as well as on presynaptic thalamocortical terminals, where they increase glutamate release (Lambe et al., 2003). Given the great evolutionary expansion of layer III dlPFC in primates (Elston, 2003; Defelipe, 2011), there are likely to be species differences in some of these mechanisms. However, $\alpha 4\beta 2$ -nAChR influences on catecholamine release in cortex may be shared across species. In rat PFC, stimulation of $\beta 2$ -containing nAChR can increase release of norepinephrine (NE) (Kennett et al., 2012) or dopamine (Livingstone et al., 2009). If the same is true in primate dlPFC, then some of the excitatory effects of $\alpha 4\beta 2$ -nAChR stimulation could arise through these mechanisms. For example, NE can increase the firing of dlPFC delay cells through stimulation of postsynaptic $\alpha 2A$ -adrenoceptors on layer III dendritic spines, which inhibit cAMP-PKA- K^+ channel signaling (Wang et al., 2007; Wang et al., 2011). Low levels of dopamine release could also increase delay cell firing through D1R phosphorylation of NMDAR, although this mechanism in primates is currently only speculative (Arnsten et al., 2015). Therefore, catecholamine actions, as well as increases in glutamate release and direct postsynaptic action, may all contribute to $\alpha 4\beta 2$ -nAChR's enhancing effects on delay cell firing. Mechanisms underlying the

enhancing effects on fixation cells are unknown because there has been so little previous research on these neurons.

Clinical relevance

Understanding $\alpha 4\beta 2$ -nAChR actions may also be relevant to the etiology and treatment of ADHD (Wilens and Decker, 2007). There is extensive evidence that ADHD involves dysfunction and slowed and/or impaired maturation of the PFC (Rubia et al., 1999; Shaw et al., 2009; Arnsten and Rubia, 2012; Shaw et al., 2013). There are also potential links between the incidence of ADHD symptoms and alterations in the $\alpha 4$ nAChR genotype, although data have been mixed (Kent et al., 2001; Lee et al., 2008). $\alpha 4\beta 2$ -nAChR agonists have been considered as potential therapies for ADHD patients (Wilens and Decker, 2007), although there are concerns about possible addictive potential (Henningfield and Zeller, 2009; Picciotto and Kenny, 2013). An initial pilot study of ABT-418 in adults with ADHD showed subtle improvement with an inverted U dose–response (Wilens et al., 1999). Although further studies of a partial $\alpha 4\beta 2$ -nAChR agonist, ABT-089, had no effects in children (Wilens et al., 2011; Childress and Sallee, 2014) or adults (Bain et al., 2012) with ADHD, preliminary results with a more selective $\alpha 4\beta 2$ -nAChR agonist, ABT-894, are promising (Bain et al., 2013). The current data, showing that $\alpha 4\beta 2$ -nAChR stimulation enhances dlPFC neuronal activity needed to sustain goals in the presence of distraction, suggest that stronger PFC function may contribute to potential therapeutic actions.

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