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Dopamine Receptors Differentially Enhance Rule Coding in Primate Prefrontal Cortex Neurons

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SUMMARY

Flexibly applying abstract rules is a hallmark feature of executive functioning represented by prefrontal cortex (PFC) neurons. Prefrontal networks are regulated by the neuromodulator dopamine, but how dopamine modulates high-level executive functions remains elusive. In monkeys performing a rule-based decision task, we report that both dopamine D1 and D2 receptors facilitated rule coding of PFC neurons, albeit by distinct physiological mechanisms. Dopamine D1 receptor stimulation suppressed neuronal firing while increasing responses to the preferred rule, thereby enhancing neuronal rule coding. D2 receptor stimulation, instead, excited neuronal firing while suppressing responses to the nonpreferred rule, thus also enhancing neuronal rule coding. These findings highlight complementary modulatory contributions of dopamine receptors to the neuronal circuitry mediating executive functioning and goal-directed behavior.

INTRODUCTION

Flexibly applying abstract rules is a hallmark feature of executive functioning represented by the activity of prefrontal cortex (PFC) neurons (Wallis et al., 2001; Miller and Cohen, 2001). The PFC receives particularly strong projections from dopamine neurons in the midbrain (Björklund and Dunnett, 2007) that regulate frontal lobe functions (Robbins and Arnsten, 2009). Prefrontal dopamine is essential for spatial working memory (Brozoski et al., 1979; Sawaguchi and Goldman-Rakic, 1991) and the learning of associations and rules (Crofts et al., 2001; Puig and Miller, 2012; Puig and Miller, 2014).

On a cellular level, dopamine influences PFC neurons via the D1 (D1R) and the D2 receptor (D2R) families (Lidow et al., 1998; de Almeida and Mengod, 2010). Prefrontal D1Rs modulate spatial working memory performance (Sawaguchi and Goldman-Rakic, 1991; Müller et al., 1998). In rhesus monkeys engaged in a spatial working memory task, PFC neurons active in the delay period of the task showed improved tuning to preferred remembered locations when stimulated with D1R agonists (Vijayraghavan et al., 2007) and showed impaired tuning when D1Rs were blocked (Sawaguchi, 2001). Interestingly,

blocking D1Rs has also been reported to improve spatial tuning (Williams and Goldman-Rakic, 1995), complicating the current understanding of D1Rs in spatial coding. While an impact of D1Rs on modulating spatial working memory processes in the PFC is established (Arnsten, 2011), the precise role of D1Rs in modulating cognitive signals remains elusive.

D2Rs, on the other hand, do not modulate spatial persistent mnemonic-related activity in the PFC (Sawaguchi and Goldman-Rakic, 1994; Wang et al., 2004). Instead, D2Rs selectively modulate neuronal activities associated with memory-guided saccades in oculomotor delayed-response tasks (Wang et al., 2004). In addition, rodent studies suggest that D2Rs are involved in flexible behavior. Blockade of D2Rs impairs the ability of rats to switch between different response strategies (Floresco and Magyar, 2006). In humans, D2R stimulation increases blood-oxygen-level-dependent activity in the PFC when flexibly switching between rules (Stelzel et al., 2013). Both prefrontal D1Rs and D2Rs are critical for learning new association rules. Blocking D1Rs or D2Rs impairs neural selectivity to learned saccade directions (Puig and Miller, 2012; Puig and Miller, 2014). This suggests a cooperative role for D1Rs and D2Rs in modulating cognitive flexibility (Puig and Miller, 2014).

We hypothesized that both D1Rs and D2Rs play a crucial role in regulating rule-guided decision-making, a hallmark feature of executive control and central to flexible behavior. Executive control is required for processing numbers and quantity information according to abstract principles, or rules, of how to structure, process, and evaluate numerical information. PFC neurons represent these semantic aspects of numerical quantities (Nieder et al., 2002; Nieder, 2012, 2013; Viswanathan and Nieder, 2013; Jacob and Nieder, 2014) and quantitative rules (Bongard and Nieder, 2010; Vallentin et al., 2012; Eiselt and Nieder, 2013). Here, we therefore studied the activity of individual PFC neurons in rhesus monkeys required to flexibly switch between "greater than"/"less than" rules. By selectively activating or blocking D1Rs or D2Rs in the PFC, we report that dopamine modulates the neuronal coding of abstract rules through both receptor families by distinct physiological mechanisms.

RESULTS

To determine if and how the dopaminergic system modulates abstract rule coding in the PFC, we trained two macaque monkeys to apply numerical rules to numerosities and to flexibly switch between the rules based on cues shown during each trial
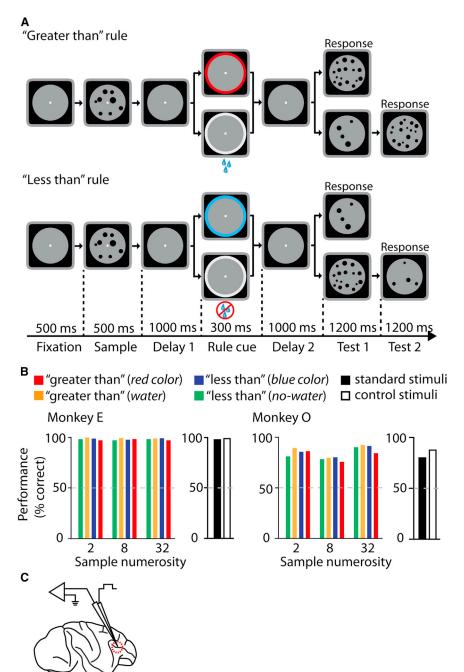
A

"Greater than" rule

"Less than" rule

| 500 ms | 500 ms | 1000 ms | 300 ms | 1000 ms | 1200 ms | 1200 ms |
| Fixation | Sample | Delay 1 | Rule cue | Delay 2 | Test 1 | Test 2 |

B

■ "greater than" (*red color*) ■ "less than" (*blue color*) ■ standard stimuli
■ "greater than" (*water*) ■ "less than" (*no-water*) □ control stimuli

C

ple-item dot displays. Average correct performances were 98% for monkey E and 85% for monkey O (Figure 1B).

We recorded 384 randomly selected single neurons from the lateral PFC of two macaque monkeys (246 from monkey E, 138 from monkey O) (Figure 1C) performing the rule-switching task. To directly assess the impact of dopamine receptor targeting agents, control conditions without drug application alternated with drug conditions in each recording session. In each session, we tested one of three different substances that selectively targeted the D1R or the D2R: the D1R agonist SKF81297, the D1R antagonist SCH23390, and the D2R agonist quinpirole. Physiological NaCl solution was used as control.

Rule-selective neurons were identified based on a significant main effect of the behavioral rule on the discharge rate in the delay 2 period using a four-way ANOVA (with main factors iontophoresis condition [control/drug], sample numerosity ["2"/"8"/"32"], behavioral rule ["greater than"/"less than"], and rule-cue modality [red/blue versus water/no-water]; $p < 0.05$). To ensure that neuronal responses varied with the rule rather than

(Bongard and Nieder, 2010; Eiselt and Nieder, 2013) (see Figure 1A for protocol and details). Rule-related activity was investigated in the delay 2 period, after the behavioral rule was indicated via the rule cues, but before the monkeys could prepare a motor plan. Simultaneous neuronal recordings and micro-iontophoretic drug application started after the monkeys had learned to proficiently apply the "greater than/"less than" rules, irrespective of the absolute values of the three sample numerosities ("2," "8," or "32"), the two rule-cue modalities (red/blue versus water/no-water), and the visual appearance of the multi-

Drug	Total Neurons	Rule-Selective (Greater/Less)
SKF81297 (D1R agonist)	123	20 (12/8)
SCH23390 (D1R antagonist)	112	18 (8/10)
Quinpirole (D2R agonist)	79	16 (7/9)
NaCl	70	10 (7/3)
Sum	384	64

with the rule cue, we excluded neurons that showed a significant interaction of the main factors rule and rule-cue modality. A total of 17% (64/384) of all tested neurons encoded abstract numerical rules (Table 1) and entered subsequent analyses. A similar number of neurons preferred the "greater than" rule (34 neurons with higher discharge for the "greater than" rule) and the "less than" rule (30 neurons exhibiting higher response rates for the "less than" rule).

D1Rs and D2Rs Modulated Single Neurons Encoding Abstract Numerical Rules

The coding properties of rule-selective neurons were modulated by drugs targeting either D1Rs or D2Rs. Figure 2A shows a "less than"-rule-selective neuron that differentiated more between "greater than" and "less than" rules (irrespective of rule-cue modalities) after stimulation with D1R agonist SKF81297 (Figure 2A). In contrast, blocking the D1R with SCH23390 strongly reduced rule selectivity of a different neuron that preferred the "greater than" rule in control conditions (Figure 2C). When targeting the D2Rs, rule selectivity was also affected. Stimulation of the D2R with quinpirole increased selectivity in a "less than"-rule-selective neuron (Figure 2E).

To analyze population responses, the responses of neurons classified as "greater than"- or "less than"-rule-selective neurons were normalized and averaged. Stimulating the D1R with SKF81297 increased the differentiation between the preferred rule (red trace) and the nonpreferred rule (blue trace) in the population of rule-selective neurons tested with SKF81297 (Figure 2B) by increasing the mean difference in normalized discharge rates ($\Delta R = +0.37 \pm 0.12$ [SEM], $p = 0.01$, $n = 20$, Wilcoxon test). Conversely, blocking the D1R with SCH23390 significantly reduced the rule selectivity of rule-selective neurons recorded with SCH23390 (Figure 2D; $\Delta R = -0.17 \pm 0.05$, $p = 0.01$, $n = 18$, Wilcoxon test). Stimulating the D2R with quinpirole also increased the differentiation between the preferred rule and the nonpreferred rule in the population of all rule-selective neurons recorded with quinpirole (Figure 2F; $\Delta R = +0.29 \pm 0.078$, $p = 0.001$, $n = 16$, Wilcoxon test). After terminating iontophoretic drug application, neuronal rule selectivity returned to the same levels as prior to the first drug application, i.e., the drug effects washed out (see Figures S1A, S1C, S1E, and S1G; see Supplemental Information available online). Iontophoretic application of NaCl did not change rule-selective responses (Figures S2A and S2B, $p = 0.1$, $n = 10$, Wilcoxon test), confirming drug-specific effects.

D1R and D2R Stimulation Enhanced Abstract Rule Coding of PFC Neurons

We characterized the quality of rule coding for each rule-selective neuron (identified by the ANOVA) during control and drug conditions by determining the area under the receiver operator characteristic (AUROC) (see Experimental Procedures) using the discharge rates in the same analysis window as for the ANOVA. Stimulating the D1R increased the coding strength (AUROCs) in 75% (15/20) of all rule-selective neurons tested with SKF81297 (Figure 3A; mean ΔAUROC $= +0.080 \pm 0.023$ [SEM], $p = 0.004$, $n = 20$, Wilcoxon test). In contrast, blocking the D1R with SCH23390 decreased the AUROCs in 83% (15/18) of the rule-selective neurons, thus impairing rule coding (Figure 3C; ΔAUROC $= -0.044 \pm 0.016$, $p = 0.01$, $n = 18$, Wilcoxon test). Stimulation of the D2R with quinpirole also increased the AUROCs in almost all rule-selective neurons (88%, or 14/16) (Figure 3E, ΔAUROC $= +0.050 \pm 0.012$, $p = 0.002$, $n = 16$, Wilcoxon test). After terminating iontophoretic drug application, AUROCs returned to the same levels as prior to the first drug application phase, i.e., the drug effects washed out (Figures S1B, S1D, S1F, S1H; Supplemental Information). Iontophoretic application of NaCl did not change AUROCs and thus left rule coding unaffected (Figure S2C; $p = 0.8$, $n = 10$, Wilcoxon test). In summary, both D1R and D2R activation facilitated rule coding in the PFC.

We used a sliding ROC analysis to assess the time course of rule coding after rule-cue presentation and throughout the entire delay 2 period (Figures 3B, 3D, and 3F). In general, coding quality increased during the delay 2 period. D1R stimulation with SKF81297 caused a more prominent increase of AUROCs compared to control conditions, particularly in the second half of the delay 2 period (Figure 3B, left panel). The average latency of rule coding, defined as the time to the first significant rule coding from delay 2 onset (see Experimental Procedures), did not change after D1R stimulation (Figure 3B; right panel, mean Δlatency $= 90$ ms ± 103 ms [SEM], $p = 0.6$, Wilcoxon test testing Δlatency against zero). Blocking D1Rs with SCH23390 impaired AUROCs during the delay period (Figure 3D, left panel) but left the average latency unchanged (Figure 3D; right panel, Δlatency $= -13$ ms ± 75 ms, $p = 0.8$, Wilcoxon test). Stimulating D2Rs with quinpirole resulted in elevated AUROCs in particular in the second half of the delay phase (Figure 3F, left panel), while not changing average latency (Figure 3F, right panel, Δlatency $= -93$ ms ± 50 ms, $p = 0.3$, Wilcoxon test). Thus, the temporal profile during the delay 2 period was not modulated by dopamine receptor stimulation.

D1Rs and D2Rs Differentially Modulated Preferred and Nonpreferred Rule-Related Activity

To investigate whether the dopaminergic system differentially modulates neuronal responses to the preferred and the nonpreferred rule, we calculated a drug modulation index (MI). The MI indicated if discharges to the preferred and/or nonpreferred rule were modulated by the drug, in comparison to the baseline (see Experimental Procedures). Stimulating the D1R with SKF81297 specifically increased neuronal responses to the preferred rule (mean MI $= +0.35 \pm 0.13$ [SEM], $p = 0.01$, Wilcoxon
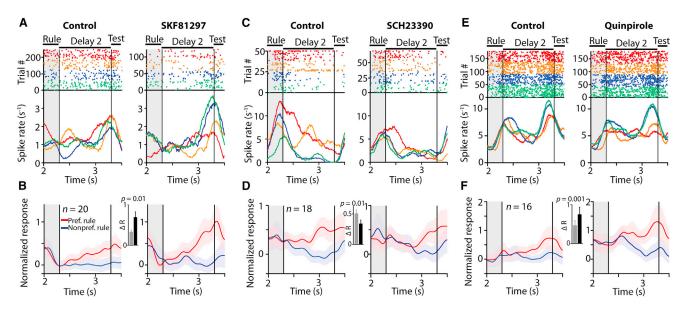
Figure 2. Modulation of Rule-Selective Neurons by Dopamine Receptors

(A) Dot raster and PSTH of a single neuron recorded during control conditions (left panel) and after application of SKF81297 (right panel) from the time of rule-cue presentation (gray shaded area). After D1R stimulation, the neuron responded more strongly to the "less than" rule (blue and green trace) as compared to the "greater than" rule (red and orange trace).

(C) Same conventions as in (A), showing a single neuron modulated by SCH23390. Blocking the D1R reduced rule-related neuronal responses.

(E) Same conventions as in (A), showing a single neuron that was modulated by quinpirole. Stimulating the D2R enhanced rule-related neuronal responses.

(B, D, and F) Averaged normalized responses of all rule-selective neurons recorded with the three drugs for the preferred rule (red trace) and the nonpreferred rule (blue trace) during control conditions (left panels) and drug conditions (right panels). Insets show differences in normalized responses ΔR between the preferred and the nonpreferred rule for control conditions (gray bars) and drug conditions (black bars). Error bars represent SEMs, n denotes sample size, p values of Wilcoxon tests.

test against zero MI), but not to the nonpreferred rule (MI = +0.015 ± 0.090, p = 0.9) (Figure 4A; p = 0.01 Wilcoxon test between MIs for the preferred and nonpreferred rules). Consequently, blocking the D1R with SCH23390 reduced neuronal responses to the preferred rule (MI = −0.23 ± 0.083, p = 0.02), while leaving neuronal responses to the nonpreferred rule unaffected (MI = −0.057 ± 0.071, p = 0.9) (Figure 4B; p = 0.01). In contrast, stimulating the D2R with quinpirole reduced neuronal responses to the nonpreferred rule (MI = −0.13 ± 0.071, p = 0.02), but not to the preferred rule (MI = +0.015 ± 0.060, p = 0.3) (Figure 4C; p = 0.03), thus enlarging the differentiation between the preferred and nonpreferred rule as witnessed in previous analysis (Figure 2F).
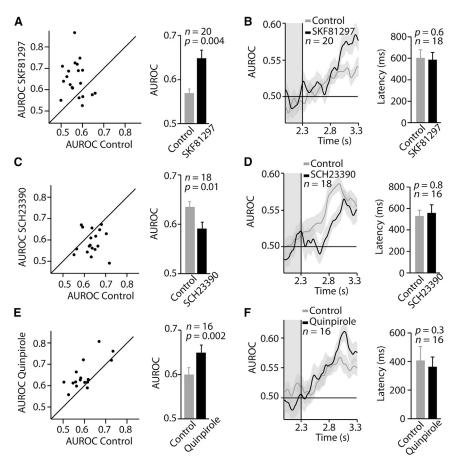
Differences in the modulation indices could be caused by changes of the discharge rates or by changes in the variability of neuronal discharges. We therefore computed the Fano factor as a measure of the trial-by-trial variability of neuronal discharges (Nawrot et al., 2008). None of the tested drugs changed the Fano factors in the baseline period (Figures S3A–S3D; Supplemental Information) or the delay 2 period for either preferred or nonpreferred rules (Figures S3E–S3H; Supplemental Information). This confirms that changes in rule-related firing rates (relative to the overall firing rates) rather than changes in discharge variability drive the changes in modulation indices.

Taken together, stimulation of both D1Rs and D2Rs improved rule selectivity, but in distinct ways: D1Rs specifically modulated the neuronal responses to the preferred rule (but not to the nonpreferred rule); D2Rs, on the other hand, modulated neuronal response to the nonpreferred rule (but not to the preferred rule).

D1R Stimulation Enhanced Numerosity Coding Strength

Because the monkeys were required to apply rules to numerosities, we also analyzed whether prefrontal dopamine receptors might modulate the encoding of numerical values. We quantified the coding strength of the numerical value in numerosity-selective neurons during the sample period by comparing responses to preferred and nonpreferred sample numerosities. D1R stimulation increased AUROCs of numerosity-selective neurons tested with SKF81297, significantly enhancing sample numerosity coding (Figure 5A; mean ΔAUROC = +0.04 ± 0.02 [SEM], n = 28, p = 0.02, Wilcoxon test). Blocking D1Rs with SCH23390 did not significantly modulate AUROCs (Figure 5C; ΔAUROC = +0.001 ± 0.02, n = 13, p = 0.7, Wilcoxon test). D2R stimulation with quinpirole did not systematically change AUROCs (Figure 5E; ΔAUROC = +0.02 ± 0.03, n = 22, p = 0.6, Wilcoxon test). Thus, D1R stimulation modulated sample numerosity coding, while D2R stimulation did not.

To study these effects in more detail, we separately analyzed drug impact on the responses to the preferred and nonpreferred numerosity. Application of SKF81297 did not modulate neuronal responses to nonpreferred (mean MI = −0.8 ± 0.4 [SEM], p = 0.2, Wilcoxon test against zero MI) or preferred

Figure 3. Modulation of Neuronal Rule Coding by Dopamine Receptors

(A) Distribution of AUROCs in control conditions and after application of SKF81297 (left panel, each dot corresponds to one neuron). SKF81297 increased AUROCs compared to control conditions in almost all rule-selective neurons. The mean AUROC was increased (right panel) by SKF81297 (black bar) compared to control conditions (gray bar).

(B) Sliding ROC analysis showing the temporal evolution of rule coding from rule-cue onset during the delay 2 period (left panel). Gray shaded box corresponds to rule-cue presentation. The latency of rule coding was unchanged (right panel).

(C) Same conventions as in (A), showing that SCH23390 reduced AUROCs.

(D) Same conventions as in (B) for SCH23390.

(E) Same conventions as in (A), showing that quinpirole increased AUROCs.

(F) Same conventions as in (B) for quinpirole. Error bars represent SEMs, n denotes sample size, p values of Wilcoxon tests.

of drug-influenced baseline activity that differed significantly (Figure 6F; p = 10^{-7}, Kruskal-Wallis test). Dopamine receptor manipulation did not change neuronal trial-by-trial variability measured by the Fano factor (Nawrot et al., 2008) in the baseline period (Figures S3A–S3D; Supplemental Information). In sum, stimulating D1Rs inhibited neurons, while blocking D1Rs excited neurons. Strong excitation was observed after stimulating D2Rs.

(MI = −0.8 ± 0.4, p = 0.2) sample numerosities alone (Figure 5B; p = 0.4, Wilcoxon test between MIs for nonpreferred and preferred sample numerosities). Application of SCH23390 increased neuronal responses to nonpreferred sample numerosities (MI = +1.6 ± 0.8, p = 0.02), but also tended to increase responses to preferred sample numerosites (MI = +1.3 ± 0.6, p = 0.07), thus resulting in no coding differences (Figure 5D; p = 0.7). Application of quinpirole did not modulate neuronal responses to nonpreferred (MI = +0.3 ± 0.3, p = 0.2) or preferred (MI = +0.7 ± 0.7, p = 0.3) sample numerosities (Figure 5F; p = 0.5). In sum, sample coding was not modulated by specific changes of neuronal responses to preferred or nonpreferred sample numerosities.

D1Rs and D2Rs Modulated Baseline Activity in Opposite Directions

D1Rs and D2Rs modulated baseline discharge rates (during the fixation period) of the population of all neurons. SKF81297 slightly decreased baseline discharge rates (Figure 6A; ΔFR = −0.27 Hz, p = 0.04, n = 123, Wilcoxon test), and SCH23390 mildly increased baseline activity (Figure 6B; ΔFR = +0.75 Hz, p = 0.05, n = 112, Wilcoxon test), whereas quinpirole enhanced baseline rates (Figure 6C; ΔFR = +1.1 Hz, p = 10^{-5}, n = 79, Wilcoxon test). No baseline modulation was found after applying NaCl solution as a control (Figure 6D; ΔFR = +0.060 Hz, p = 0.5, n = 70, Wilcoxon test). Figure 6E displays the average time courses

Dopaminergic Modulation of Behavior

Next, we asked if modulation of prefrontal dopamine receptors influenced the monkeys' behavior. Since monkeys did not show any switch costs (Figure S4; Supplemental Information) consistent with findings reported in task-switching paradigms (Stoet and Snyder, 2009), we focused our behavioral analysis on changes in performance and reaction times. Iontophoretic drug application is highly focal (Herz et al., 1969), and most primate studies that iontophoretically applied drugs to the cortex did not report any behavioral changes (Williams and Goldman-Rakic, 1995; Sawaguchi, 2001; Wang et al., 2004, 2013; Vijayraghavan et al., 2007). However, small modulations of reaction times were reported in some studies (Herrero et al., 2008, 2013). Due to extensive training, behavioral performance was at ceiling levels (see Figure 1) and did not change after drug application (Figure 7A; p > 0.1 for all drugs, Wilcoxon test over recording sessions). However, drug application slightly modulated behavioral reaction times (Figure 7B). Stimulating D1Rs with SKF81297 increased reactions times (ΔRT = +3.2 ms, p = 0.004, Mann-Whitney U test). Accordingly, blocking D1Rs with SCH23390 decreased reaction times (ΔRT = −2.8 ms, p = 0.03, Mann-Whitney U test). Stimulating D2Rs with quinpirole increased reaction times (ΔRT = +1.8 ms,
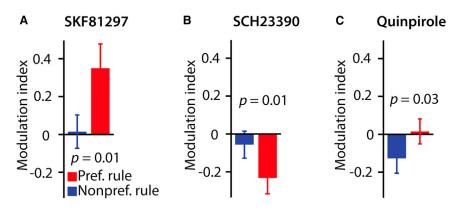
Figure 4. Differential Modulation of Preferred and Nonpreferred Rule-Related Activity by D1Rs and D2Rs

(A) SKF81297 enhanced the modulation indices for the preferred rule (red bar), but not the nonpreferred rule (blue bar).

(B and C) Same conventions as in (A), showing that SCH23390 reduced modulation indices for the preferred rule, whereas quinpirole reduced modulation indices for the nonpreferred rule. Error bars represent SEMs, n denotes sample size, p values of Wilcoxon tests.

$p = 0.04$, Mann-Whitney U test). As a control, application of NaCl did not produce changes in reaction times (ΔRT = -0.1 ms, $p = 0.3$, Mann-Whitney U test). Thus, manipulation of both prefrontal D1Rs and D2Rs produced changes in the monkeys' behavior.

DISCUSSION

Our findings highlight that dopaminergic input to the PFC is essential for mediating executive functions. We show that D1Rs and D2Rs assume complementary roles in enhancing neuronal representations of rule-guided decision-making at the microcircuit level. D1R stimulation suppresses neuronal baseline firing while enhancing the neurons' responses to the preferred rule. D2R stimulation, on the other hand, excites neuronal baseline firing while suppressing responses to the nonpreferred rule. Thus, two distinct physiological mechanisms that are dissociable at the dopamine receptor level modulate rule coding in the PFC.

Modulation of Rule-Related Activity via D1Rs

D1Rs have been demonstrated to modulate the responsiveness of PFC neurons via a variety of cellular mechanisms (Seamans and Yang, 2004). We find that D1R activation suppresses neuronal baseline activity of PFC neurons. Mechanistically, this can be explained either by D1R stimulation reducing the efficacy of excitatory neurotransmission in PFC slices (Gao et al., 2001), amplifying inhibitory currents (Trantham-Davidson et al., 2004), or weakening non-NMDA-glutamatergic responses (Seamans et al., 2001). A predominantly inhibitory effect on PFC neurons has also been reported in studies iontophoretically applying D1R agonists in the PFC of monkeys engaged in a spatial working memory task (Vijayraghavan et al., 2007). This inhibition enhanced the neurons' spatial selectivity in the memory period of the task, "sculpting" their spatial memory fields (Arnsten, 2011). In agreement with this finding, blockade of D1Rs has been shown to impair spatial memory fields (Sawaguchi, 2001) (but see also Williams and Goldman-Rakic, 1995, for opposite findings). At the same time, D1R stimulation increases excitability of PFC neurons in vitro by potentiating NMDA-evoked responses (Seamans et al., 2001; Tseng and O'Donnell, 2004). Together, these findings lead to the proposal that D1R stimulation enhances

NMDA-dependent persistent activity in prefrontal networks and reduces baseline activity by controlling recurrent glutamatergic connections (Seamans and Yang, 2004; Durstewitz and Seamans, 2008; Wang et al., 2013). Our results are in agreement with this model because we find D1R stimulation to increase the neurons' sustained responses to the preferred rule while generally suppressing baseline activity. In contrast, previous studies reported that prefrontal D1Rs primarily modulate neural responses to remembered nonpreferred spatial directions (Vijayraghavan et al., 2007) or neural responses to nonpreferred associations (Puig and Miller, 2012). These findings might reflect differences in spatial and cognitive coding in the PFC. Blocking D1Rs decreased the neurons' sustained responses to the preferred rule while generally enhancing baseline activity. Thus, physiological activation of D1Rs is necessary to maintain rule coding in the PFC.

While prefrontal D1Rs modulate working memory in monkeys (Sawaguchi and Goldman-Rakic, 1991, 1994) and humans (Müller et al., 1998; McNab et al., 2009), emerging evidence also suggests a broader role of D1Rs in prefrontal functions. Blocking prefrontal D1Rs in monkeys impairs learning of new association rules and reduces corresponding neural selectivity to learned saccade directions (Puig and Miller, 2012). In rodent studies, blocking D1Rs impairs flexibly switching between different response strategies (Ragozzino, 2002; Floresco and Magyar, 2006). Similarly, D1R availability in human PFC is positively correlated with flexibly shifting between rules in a Wisconsin card sorting test (Takahashi et al., 2008; Takahashi et al., 2012). By strengthening rule signals in the PFC, our results provide a possible cellular basis for a role of D1Rs in flexible decision-making. Thus, our findings further argue for a role of D1Rs beyond working memory (Floresco and Magyar, 2006), including cognitive control processing such as rule-based decision-making.

Modulation of Rule-Related Activity via D2Rs

Our data demonstrate a D2R-mediated excitation of PFC cells. Consistently, D2R-mediated excitation was reported by in vitro studies showing that D2Rs increase excitability of PFC cells by decreasing postsynaptic inhibitory currents (Trantham-Davidson et al., 2004) as well as with in vivo studies (Wang and Goldman-Rakic, 2004). In behaving monkeys, iontophoretic D2R stimulation in PFC predominantly excited neurons when
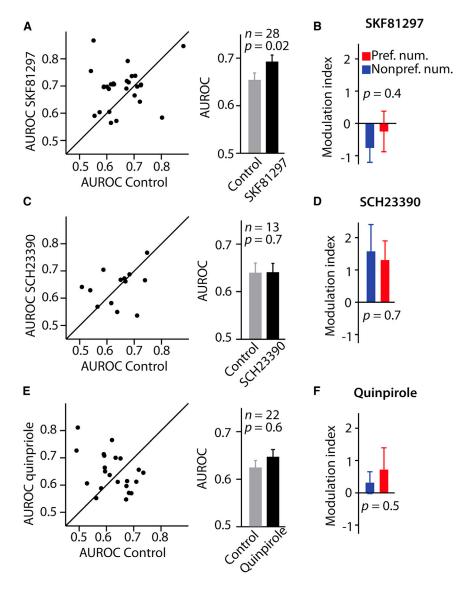
Figure 5. Modulation of Numerosity Coding Strength by Dopamine Receptors

(A) Distribution of AUROCs in control conditions and after application of SKF81297 (left panel, each dot corresponds to one neuron) during the sample period. The mean AUROC was increased (right panel) by SKF81297 (black bar) compared to control conditions (gray bar).

(B) Modulation index for nonpreferred (blue bar) and preferred (red bar) responses during the sample period induced by SKF81297.

(C) Same conventions as in (A) for SCH23390.

(D) Same conventions as in (B) for SCH23390.

(E) Same conventions as in (A) for quinpirole, showing no modulation of sample preference.

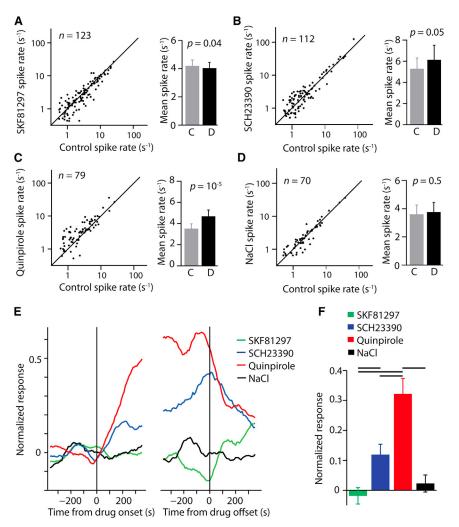(F) Same conventions as in (B) for quinpirole. Error bars represent SEMs, n denotes sample size, p values of Wilcoxon tests.

blocking prefrontal D2Rs in monkeys impairs learning of new association rules and reduces neural selectivity for the learned saccade direction particularly for the nonpreferred direction (Puig and Miller, 2014). Furthermore, blocking D2Rs increased preservation errors, thus impairing behavioral flexibility (Puig and Miller, 2014). In addition, rodent studies suggest that D2Rs modulate behavioral flexibility and decision-making (Floresco and Magyar, 2006). After blocking D2Rs in the PFC, rats were impaired in switching between different response strategies (Floresco et al., 2006), and blocking D2Rs impaired set-shifting in humans (Mehta et al., 1999). Stimulating D2Rs increased BOLD signals in frontal cortex during rule switching in humans (Stelzel et al., 2013) and improved performance of monkeys in a delayed response

monkeys made a saccade toward a remembered location (Wang et al., 2004). Sustained activity during the spatial memory period of the task, however, was not affected (Wang et al., 2004). The authors thus concluded that D2R manipulation has little or no effect on the persistent mnemonic-related activity (Wang et al., 2004). Consistent with these physiological results, D2R manipulation does not produce changes in spatial working memory performance in monkeys (Sawaguchi and Goldman-Rakic, 1994) or humans (Müller et al., 1998).

We show here, however, that a different type of sustained activity, namely rule-selective responses during a delay period, is indeed influenced by D2Rs. D2R stimulation enhances rule coding by suppressing responses to the nonpreferred rule while leaving responses to the preferred rule unchanged. This relative suppression of responses to the nonpreferred rule might be mediated by specific inhibitory D2R actions in prefrontal neurons reported by several in vitro studies (Tseng and O'Donnell, 2004). Our findings are in agreement with a recent study showing that

task (Arnsten et al., 1995). Thus, our finding that D2R activation enhances rule coding in the PFC provides a cellular basis for D2R modulation of cognitive functions. Our results highlight that D2Rs—while not being involved in spatial mnemonic processing—do play an important role during flexible decision-making.

Consistent with the electrophysiological findings, both D1R and D2R stimulation caused changes in the monkeys' behavior in the same direction. The monkeys needed slightly longer to respond after D1R and D2R stimulation, whereas blocking D1Rs mildly decreased reaction times. The magnitude of the effect was comparable to previous studies reporting changes in reaction times after iontophoretic drug application (Herrero et al., 2008, 2013). Prolonged reaction times during D1R and D2R stimulation might reflect the increased stability in rule coding in the PFC. In addition to cognitive variables, prefrontal dopamine receptors also modulate motor-related signals (Wang et al., 2004). While we did not investigate motor-related

Figure 6. Drug Effects on Neuronal Baseline Activity

(A) Comparison of individual neurons' baseline spike rates during SKF81297 application and control conditions (left panel) and mean baseline spike rates during control and SKF81297 conditions (right panel). SKF81297 induced a small reduction in baseline spike rates. C, control conditions; D, drug conditions.

(B) Same conventions as in (A), showing that SCH23390 induced a small increase in baseline spike rates.

(C) Same conventions as in (A), showing that quinpirole increased baseline spike rates.

(D) Same conventions as in (A), showing that NaCl did not change absolute spike rates.

(E) Average time courses (wash-in and wash-out effects) of normalized baseline activity for all neurons aligned to onset (left) and offset (right) of drug application.

(F) Mean normalized neuronal response in the drug phase. SCH23390 (blue bar) and quinpirole (red bar) increased baseline activity, whereas SKF81297 (green bar) and NaCl (black bar) did not. Black horizontal bars indicate pairwise significant differences ($p < 0.05$, Kruskal-Wallis test with post hoc Tukey's comparisons). Error bars represent SEM, n denotes sample size, p values of Wilcoxon tests.

tiple prefrontal functions and at different time scales (Schultz, 2007). In contrast, D2Rs did not modulate numerosity coding strength, just as it did not modulate spatial working memory processes (Wang et al., 2004), although D2Rs modulate neural signatures of associative learning (Puig and Miller, 2014). Therefore, prefrontal D2Rs might assume a more specific role in cognitive processing.

influences, we speculate that our pharmacological interventions also affected motor selection signals. The precise mechanisms by which manipulation of prefrontal dopamine receptors affects behavior surely require further investigation.

D1R Modulation of Coding Strength to Sample Numerosity

Midbrain dopamine neurons fire phasic bursts in response to behaviorally relevant sensory events (Schultz, 1998; Matsumoto and Hikosaka, 2009; de Lafuente and Romo, 2012). In the PFC, dopamine enhances visual signals (Jacob et al., 2013), possibly gating neuronal representations of relevant stimuli (D'Ardenne et al., 2012). Consistently, we found that D1R stimulation enhanced neuronal representation of sample numerosity that is needed to solve numerical tasks (Nieder, 2012, 2013; Jacob and Nieder, 2014). Thus, D1Rs might mediate dopamine's function of supporting the detection of relevant sensory events (Redgrave et al., 2008; de Lafuente and Romo, 2011). Together with studies demonstrating D1R modulation of spatial working memory processes (Williams and Goldman-Rakic, 1995; Sawaguchi, 2001; Vijayraghavan et al., 2007) and associative learning (Puig and Miller, 2012), prefrontal D1Rs are also involved in mul-

Complementary Roles of D1Rs and D2Rs in Behavioral Flexibility

We find that D1Rs and D2Rs modulated spontaneous firing in opposite directions, with D1Rs and D2Rs strengthening rule coding in complementary ways. This is consistent with the idea that the ratio between D1R and D2R activation determines excitability in prefrontal networks (Seamans and Yang, 2004). In recent monkey experiments, both prefrontal D1Rs and D2Rs influenced saccadic target selection (Noudoost and Moore, 2011a) possibly underlying attentional processes (Noudoost and Moore, 2011b; Clark and Noudoost, 2014), while only D1Rs seemed to control cortical visual signals. Interestingly, dopamine depletions impair not only spatial working memory (Brozoski et al., 1979) but also the learning of rules in monkeys (Crofts et al., 2001). Both prefrontal D1R and D2R activation contribute to learning new associative rules, suggesting a cooperative role in cognitive flexibility of both receptor families (Puig and Miller, 2012, 2014). This is in agreement with the finding that midbrain dopamine neurons signal the cognitive component
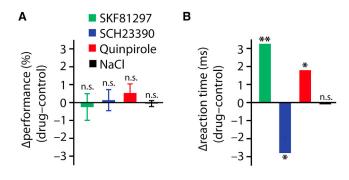
A

B

Figure 7. Drug Effects on the Monkeys' Behavior
(A) Difference in performance (% correct trials) between control and drug conditions. Error bars represent SEMs over recording sessions. n.s., not significant (Wilcoxon test, p > 0.05).
(B) Difference in mean normalized reaction times between control and drug conditions pooled over all recording sessions. n.s., not significant (p > 0.05), *p < 0.05, **p < 0.01 (Mann-Whitney U test).

of a task (Stefani and Moghaddam, 2006; Matsumoto and Takada, 2013) and are correlated with the monkeys' decisions (de Lafuente and Romo, 2011). Complementary roles of D1Rs and D2Rs for behavioral flexibility are thus present in both primates and rodents (Floresco and Magyar, 2006; Takahashi et al., 2012; Floresco, 2013; Puig and Miller, 2014). Our data extend these findings and show that dopamine influences executive functions in the PFC through both D1Rs and D2Rs, enhancing rule-based decision-making. These findings might contribute to interpreting drug effects in psychiatric disorders with disturbed prefrontal dopamine signaling (Arnsten, 2011; Winterer and Weinberger, 2004).

EXPERIMENTAL PROCEDURES

Animals and Surgical Procedures
Two male rhesus monkeys (*Macaca mulatta*) were implanted with a titanium head post and one recording chamber centered over the principal sulcus of the lateral PFC, anterior to the frontal eye fields (right hemispheres in both monkeys). Surgery was conducted using aseptic techniques under general anesthesia. Structural magnetic resonance imaging was performed before implantation to locate anatomical landmarks. All experimental procedures were in accordance with the guidelines for animal experimentation approved by the authority, the Regierungspräsidium Tübingen, Germany.

Task
Monkeys learned to flexibly perform numerical "greater than" versus "less than" comparisons. They initiated a trial by grasping a lever and maintaining central fixation on a screen. After a pure fixation period (500 ms), a sample stimulus (500 ms) cued the animals for the reference numerosity (i.e., number of dots) they had to remember for a brief time interval. The first memory interval (delay 1, 1,000 ms) was followed by a rule cue (300 ms) that instructed the monkeys to select either a larger number of dots ("greater than" rule) or a smaller number of dots ("less than" rule) than the sample numerosity in the subsequent test phase. The test phase was preceded by a second delay (delay 2, 1,000 ms) requiring the monkeys to assess the rule at hand for the subsequent choice. In the following test 1 phase, the monkeys had to release the lever in a "greater than" trial, if the number of items in the test display was larger than the number of items in the sample display (match trial), or to keep holding the lever for another 1,200 ms until the appearance of a second test display (test 2), if the number of items in the test display was smaller than the number of items in the sample display (nonmatch trial). In a "less than" trial,

these conditions were reversed. Monkeys got a liquid reward for a correct choice. Thus, only test 1 required a decision; test 2 was used so that a behavioral response was required in each trial, ensuring that the monkeys were paying attention during all trials. Because both sample and test numerosities varied randomly, the monkeys could only solve the task by assessing the numerosity of the test display relative to the three possible numerosities of the sample display together with the appropriate rule in any single trial. To test a range of numerosities, both monkeys were presented with numerosities 2 (smaller test numerosity = 1, larger test numerosity = 4), 8 (4:16), and 32 (16:64). For any sample numerosity, test numerosities were either larger or smaller with equal probability (p = 0.5). Because the monkeys' numerosity discrimination performance obeys the Weber-Fechner law (Nieder and Miller, 2003), numerosities larger than a sample numerosity need to be numerically more distant than numerosities smaller than the sample numerosity to reach equal discriminability. Based on this design, any test numerosity (except the smallest and largest used) served as test numerosities for different sample numerosities, thus precluding the animals from learning systematic relations between numerosities.

To prevent the animals from exploiting low-level visual cues (e.g., dot density, total dot area), a standard numerosity protocol (with dot sizes and positions pseudorandomized) and a control numerosity protocol (with equal total area and average density of all dots within a trial) were each presented in 50% of the trials in a pseudorandomized fashion. To dissociate the rule-related cellular responses from responses to the sensory features of the rule cue, each rule was signified by two different rule cues in two different sensory modalities: a red circle ("greater than" rule, red color) or a white circle with a drop of water ("greater than" rule, water) signified the rule "greater than." The "less than" rule was cued by a blue circle ("less than" rule, blue color) or a white circle with no water ("less than" rule, no water). We showed in previous studies that monkeys generalize the numerical principles "greater than" and "less than" to numerosities they had never seen before (Bongard and Nieder, 2010; Eiselt and Nieder, 2013). Before each session, the displays were generated anew using MATLAB (Mathworks). Trials were randomized and balanced across all relevant features ("greater than" and "less than" rules, rule-cue modalities, sample numerosities, standard and control stimuli, match and nonmatch trials). Monkeys had to keep their gaze within 1.75° of the fixation point from the fixation interval up to the onset of the first test stimulus (monitored with an infrared eye-tracking system; ISCAN, Burlington, MA).

Electrophysiology and Iontophoresis
Extracellular single-unit recording and iontophoretic drug application were performed as described previously (Jacob et al., 2013). In each recording session, up to three custom-made tungsten-in-glass electrodes flanked by two pipettes each were inserted transdurally using a modified electrical microdrive (NAN Instruments). Single neurons were recorded at random; no attempt was made to preselect the neurons to any task-related activity or based on drug effects. Signal acquisition, amplification, filtering, and digitalization were accomplished with the MAP system (Plexon). Waveform separation was performed offline (Offline Sorter; Plexon). Drugs were applied iontophoretically (MVCS iontophoresis system; npi electronic) using custom-made tungsten-in-glass electrodes flanked by two pipettes each (Jacob et al., 2013; Thiele et al., 2006). Electrode impedance and pipette resistance were measured after each recording session. Electrode impedances were 0.8–3 MΩ (measured at 500 Hz; Omega Tip Z; World Precision Instruments). Pipette resistances depended on the pipette opening diameter, drug, and solvent used. Typical resistances were 15–50 MΩ (full range, 12–160 MΩ). As in previous experiments (Jacob et al., 2013), we used retention currents of −7 nA to hold the drugs in the pipette during control conditions. The ejection current for SKF81297 (10 mM in double-distilled water [pH 4.0] with HCl; Sigma-Aldrich) was +15 nA, the ejection current for SCH23390 (10 mM in double-distilled water [pH 4.0] with HCl; Sigma-Aldrich) was +25 nA, and the ejection current for quinpirole (10 mM in double-distilled water [pH 4.0] with HCl; Sigma-Aldrich) was +40 nA. In control experiments with 0.9% physiological NaCl (pH 4.0) with HCl, the ejection current was +25 nA. We did not investigate dosage effects and chose ejection currents to match the values reported to be maximally effective, i.e., in the peak range

of the "inverted-U function" (Wang et al., 2004; Vijayraghavan et al., 2007). One pipette per electrode was filled with drug solution (either SKF81297, SCH23390, quinpirole, or NaCl), and the other always contained 0.9% NaCl. In each recording session, control conditions using the retention current alternated with drug conditions using the ejection current. Drugs were applied continuously for 12–15 min (drug conditions), depending on the number of trials completed correctly by the animal. Each control or drug application block consisted of 72 correct trials to yield sufficient trials for analysis. The first block (12–15 min) was always the control condition. Given that iontophoretic drug application is fast and can quickly modulate neuronal firing properties (Jacob et al., 2013), we did not exclude data at the current switching points.

Data Analyses
Rule-Selective Neurons
All well-isolated recorded single units with a baseline spike rate above 0.5 Hz (determined in the 500 ms fixation period preceding sample presentation) entered the analyses. Neurons were not included based on drug effects. We calculated a four-way ANOVA for each neuron to determine if a neuron's response was correlated with the numerical rules. We used spike rates in a 600 ms window beginning 500 ms after offset of the rule-cue, i.e., in the second half of the delay 2 period. We chose this window because previous studies found the most prominent rule coding during this period (Bongard and Nieder, 2010; Eiselt and Nieder, 2013). The main factors were iontophoresis condition (control conditions/drug conditions), sample numerosity ("2"/"8"/"32"), rule to apply ("greater than"/"less than") and the rule-cue modality (red/blue versus water/no-water). We identified rule-selective neurons by a significant main factor of the rule that the monkeys had to apply ($p < 0.05$). To ensure that neuronal responses varied with the abstract numerical rules rather than with the rule cues, we excluded neurons with a significant interaction of the main factors rule and rule-cue modality ($p < 0.05$). Since the monkeys' behavior did not show any differences for standard and control stimuli (Figure 1), and because we have shown previously that neuronal responses in the PFC do not differentiate between standard and control stimuli (Bongard and Nieder, 2010; Eiselt and Nieder, 2013), we pooled over standard and control stimuli trials. A similar number of neurons preferred the "greater than" (34 neurons) and the "less than" rule (30 neurons), and neurons in the PFC encode both numerical rules about equally well (Bongard and Nieder, 2010; Eiselt and Nieder, 2013). In general, nine trials was the minimum number of trials in one of the four rule conditions for a neuron to enter the analyses. The maximum number was 70 trials per rule condition, with an average of 25 trials per one of the four rule conditions (i.e., the average neuron was recorded for 200 trials: four rule conditions for control and drug conditions, respectively).

Single-Cell and Population Responses
For plotting single-cell spike density histograms, the average firing rate in trials with one of the four different rule-cues (correct trials only) was smoothed with a Gaussian kernel (bin width of 200 ms, steps of 1 ms). For the population responses, trials with rule cues signifying the same numerical rule were pooled. A neuron's preferred rule was defined as the numerical rule yielding the higher average spike rate in the analysis window used for the ANOVA. The nonpreferred rule was defined as the numerical rule resulting in lower average spike rate. Neuronal activity was normalized by subtracting the mean baseline firing rate in the control condition and dividing by the standard deviation of the baseline firing rates in the control condition. For population histograms, normalized activity was averaged and smoothed with a Gaussian kernel (bin width of 200 ms, step of 1 ms). To quantify a neuron's selectivity to its preferred rule, we calculated the difference ΔR between the normalized response to the preferred and the nonpreferred rule in the same analysis window used for the ANOVA.

Receiver Operating Characteristic Analysis
Rule-coding quality was quantified using receiver operating characteristic (ROC) analysis derived from Signal Detection Theory (Green and Swets, 1966). The AUROC is a nonparametric measure of the discriminability of two distributions. It denotes the probability with which an ideal observer can tell apart a meaningful signal from a noisy background. Values of 0.5 indicate no separation, and values of 1 signal perfect discriminability. The AUROC takes into account both the difference between distribution means as well as their

widths and is therefore a suitable indicator of signal quality. We used AUROCs to quantify the quality of numerical rule coding. We calculated the AUROC for each neuron using the spike rate distributions of the preferred and the nonpreferred rule in the same analysis window used for the ANOVA. Sliding ROC analysis was performed from rule-cue onset until the end of the delay 2 period with overlapping 100 ms windows stepped in 10 ms increments. For each window, we calculated the AUROC comparing spike rates for the preferred and nonpreferred rule. We performed a permutation test for each window, estimating the null distribution of AUROCs by randomly relabeling trials to the preferred or nonpreferred group with 999 repetitions. Latency of rule coding was defined as the time of the first of three consecutive significant windows in the permutation test ($p < 0.05$, two-sided) beginning from the onset of the delay 2 period. Four neurons were excluded from the analysis, because no latency could be computed for both control and drug conditions.

Drug Modulation Index
To quantify if a drug specifically modulated the discharge of a neuron to the preferred or the nonpreferred rule, we calculated a drug MI for each drug and neuron separately for the preferred and the nonpreferred rule. The MI was computed by first subtracting the mean baseline spike rate (500 ms fixation period preceding sample presentation) from each trial separately for control and drug conditions and dividing by the standard deviation of baseline spike rates to account for general shifts in baseline spike rates induced by the drugs (see Figure 6). Next, we calculated the MI for the preferred rule defined as the difference between the mean response to the preferred rule in the drug condition and the mean response to the preferred rule in the control condition for each neuron and drug. The MI for the nonpreferred rule was calculated in the same way. Thus, the MI reflects the amount by which the drug modulates the preferred or the nonpreferred rule, respectively, in comparison to the neuron's baseline activity.

Analysis of Sample Numerosity Modulation
We calculated a two-way ANOVA with main factors sample numerosity (sample numerosities "2," "8," "32") and iontophoresis condition (control or drug condition) in the sample phase, a 500 ms window beginning 100 ms after sample onset (Bongard and Nieder, 2010) and selected sample-selective neurons with a significant main effect of sample numerosity ($p < 0.05$). The preferred sample numerosity was defined as the numerosity yielding the highest spike rate, the nonpreferred sample item was defined as the numerosity yielding the lowest spike rate in the sample phase. AUROCs were calculated using the distribution of spike rates for preferred and nonpreferred numerosities in the same analysis window. Modulation indices were calculated in the same analysis window and calculated as described for rule-selective neurons.

Modulation of Neuronal Baseline Activity
Baseline spike rates (500 ms fixation period preceding sample presentation) were normalized for each neuron by subtracting the mean baseline spike rate in control conditions and dividing by the standard deviation of baseline spike rates in control conditions. Thus, the mean normalized activity in control conditions is by definition zero. The amplitude of drug modulation is then given by the mean normalized activity in drug conditions. We assessed the time course of baseline modulation throughout one block (12–15 min) of drug administration by aligning normalized baseline activity to the time point when the iontophoretic drug application was switched on and off, respectively. We used bins of 10 s (about the time of two trials) to average the population activity and smoothed the population time course with a Gaussian kernel (width of 60 s).

Behavioral Modulation by Drug Application
Behavioral performance was calculated for each recording session for control and drug conditions and compared using a paired Wilcoxon test (n = 63 for SKF81297, n = 50 for SCH23390, n = 39 for quinpirole, and n = 27 for NaCl). Behavioral reaction times were normalized for each recording session by subtracting the mean reaction time for the respective recording session from each reaction time (Herrero et al., 2013). Normalized reaction times were pooled over recording sessions for control and drug conditions and compared with a Mann-Whitney U test (n = 4,886, n = 4,778 for control and SKF81297 conditions; n = 5,234, n = 4,998 for control and SCH23390 conditions; n = 2,995, n = 3,830 for control and quinpirole conditions; n = 2,912, n = 2,914 for control and NaCl conditions). Only correct match trials were used.

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