α_2-Noradrenergic receptors activation enhances excitability and synaptic integration in rat prefrontal cortex pyramidal neurons via inhibition of HCN currents

David B. Carr, Glenn D. Andrews, William B. Glen and A. Lavin

Department of Neurosciences, Medical University of South Carolina, Charleston, SC 29425, USA

Stimulation of α_2-noradrenergic (NA) receptors within the PFC improves working memory performance. This improvement is accompanied by a selective increase in the activity of PFC neurons during delay periods, although the cellular mechanisms responsible for this enhanced response are largely unknown. Here we used current and voltage clamp recordings to characterize the response of layer V–VI PFC pyramidal neurons to α_2-NA receptor stimulation. α_2-NA receptor activation produced a small hyperpolarization of the resting membrane potential, which was accompanied by an increase in input resistance and evoked firing. Voltage clamp analysis demonstrated that α_2-NA receptor stimulation inhibited a caesium and ZD7288-sensitive hyperpolarization-activated (HCN) inward current. Suppression of HCN current by α_2-NA stimulation was not dependent on adenylate cyclase but instead required activation of a PLC–PKC linked signalling pathway. Similar to direct blockade of HCN channels, α_2-NA receptor stimulation produced a significant enhancement in temporal summation during trains of distally evoked EPSPs. These dual effects of α_2-NA receptor stimulation – membrane hyperpolarization and enhanced temporal integration – together produce an increase in the overall gain of the response of PFC pyramidal neurons to excitatory synaptic input. The net effect is the suppression of isolated excitatory inputs while enhancing the response to a coherent burst of synaptic activity.

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Corresponding author A. Lavin: Department of Neurosciences, Medical University of South Carolina, 173 Ashley Avenue, Suite 403 BSB, Charleston, SC 29425, USA. Email: lavina@musc.edu

Pyramidal cells within the prefrontal cortex (PFC) are thought to be key processing elements in neuronal networks responsible for complex executive functions such as working memory. Through their recurrent synaptic connections, these networks are thought to hold information online in order to guide future behaviour (Fuster, 1997; Durstewitz *et al.* 2000). This action requires the suppression of other irrelevant stimuli that may interfere with the active maintenance of this memory trace. The ability to maintain such sustained attention is critically dependent on the proper functioning of noradrenergic (NA) afferents to the PFC (Aston-Jones & Cohen, 2005). Based on recordings from behaving primates, it has been postulated that NA acts to enhance the signal to noise ratio of PFC neuronal firing during working memory tasks by either increasing task-related activity (Li *et al.* 1999; Wang *et al.* 2007), or decreasing background activity (Sawaguchi *et al.* 1990). However, the mechanisms responsible for these effects are poorly understood.

Administration of the psychostimulant methylphenidate produces an enhancement in working memory performance in human subjects (Elliott *et al.* 1997; Mehta *et al.* 2000). We have recently shown that application of methylphenidate produces a substantial increase in the excitability of PFC pyramidal neurons recorded *in vitro* (Andrews & Lavin, 2006). This effect was produced by increased activation of α_2-noradrenergic (α_2-NA) receptors due to blockade of NA reuptake by methylphenidate. In the present paper, we sought to determine the ionic mechanisms responsible for the facilitating effects of α_2-NA receptor stimulation on PFC pyramidal neurons. Utilizing a combination of voltage and current clamp studies in acute PFC slices, the experiments described here demonstrate that the effects of α_2-NA receptor activation are mediated by the inhibition of hyperpolarization/cyclic nucleotide gated (HCN) channels through a PLC–PKC linked signalling cascade. Inhibition of these channels by α_2-NA receptors produces a hyperpolarization of the resting membrane potential, but a significant enhancement in the temporal integration of distally evoked EPSPs. The net effect is the suppression of isolated excitatory inputs while enhancing the response to a coherent burst of synaptic activity. Thus, inhibition of HCN channels may be an important cellular

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mechanism mediating the enhanced signal to noise ratio produced by NA in the PFC.

Methods

Slice preparation and aCSF solutions

All experimental protocols were approved by the institutional animal care and use committee of the Medical University of South Carolina. Male Sprague–Dawley rats (P16–25) were deeply anaesthetized with chloral hydrate (400 mg kg^{-1} I.P.) and rapidly decapitated. The brain was quickly removed and submerged in a 0°C sucrose solution containing (mM): sucrose, 200; KCl, 1.9; Na_2HPO_4, 1.2; $NaHCO_3$, 33; $MgCl_2$, 6; $CaCl_2$, 0.5; dextrose, 10; ascorbic acid, 0.4. Coronal slices (300–350 μm) including the infralimbic and prelimbic cortices (Paxinos & Watson, 1998) were cut using an oscillating tissue slicer (Leica, VT1000) and transferred to a holding chamber for a minimum of 1 h at room temperature (22–24°C) prior to recording. The holding buffer contained (mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 25; $MgCl_2$, 4; $CaCl_2$, 1; sucrose, 15; glucose, 10; ascorbic acid, 0.4; ~310 mosmol l^{-1}. Slices were transferred to a submersion-type recording chamber and perfused with aCSF containing (mM): NaCl 125; KCl 2.5; $NaHCO_3$, 25; $MgCl_2$, 1.3; $CaCl_2$, 2; glucose 10; ascorbic acid, 0.4; ~300 mosmol l^{-1} at a rate of 1–2 ml min^{-1}. All aCSF solutions were constantly aerated with a mixture of 95% O_2–5% CO_2 to maintain pH ~7.4.

Current clamp recordings

Deep layer pyramidal neurons (layers V–VI) were targeted for recording using an upright microscope equipped with Nomarski differential interference contrast optics, a 40× water immersion objective, and an infrared video imaging camera. Whole-cell current-clamp recordings of targeted cells were made using glass microelectrodes (3–7 MΩ) filled with internal solution containing (mM): potassium gluconate or $KMeSO_4$, 125; KCl, 20; Hepes, 10; EGTA, 1; $MgCl_2$, 2; ATP, 4.0; GTP, 0.3; pH 7.2–7.4; 298 mosmol l^{-1}. After gigaohm seal formation and patch rupture, neurons were given at least 5 min to stabilize before data were collected. In experiments in which drugs were included in the internal solution, recordings began > 10 min after patch rupture to allow perfusion of the drug into the recorded cell. The signal was amplified using an AxoPatch 200B or MultiClamp 700B (Axon Instruments) and stored for off-line analysis using a custom-made, LabView based program. Recordings were performed at room temperature unless stated otherwise.

Voltage clamp recordings

For voltage clamp recordings, glass micropipettes were filled with an internal solution containing (mM): $KMeSO_4$, 130; KCl, 5; MgATP, 2; Na_2GTP, 0.5; Hepes, 5; $CaCl_2$, 0.5; EGTA, 5; pH 7.3, 270 mosmol l^{-1}. The pipette resistance, as measured in the bath, was typically 4 ± 0.5 MΩ. Following patch rupture, series resistance was compensated 50–70% and continually monitored throughout the experiment. Cells were discarded if series resistance increased by > 15%. Voltage clamp recordings were obtained using a Multiclamp 700B amplifier, a Digidata 1322 A/D converter and pCLAMP software (Axon Instruments). An inline heater was used to maintain the temperature of the aCSF in the bath at 33 ± 1°C.

Drugs

All reagents were obtained from Sigma (St Louis, MO) with the exception of $KMeSO_4$ (ICN Biochemicals), ZD7288 (Tocris), SQ22536, MDL-12 330 A, 2′,5′-dideoxy-adenosine, U73122, calphostin C, and chelrythrine chloride (Calbiochem). All adrenergics, channel blockers, and signal transduction reagents were prepared as concentrated stock solutions and either added immediately to the recording aCSF or internal solution at working concentrations or aliquotted and frozen at −20°C until use.

Catecolamine depletion

For the experiments with catecholamine depletions, animals were injected with reserpine (5 mg kg^{-1}, I.P.) 24 h before slice procedure. Previous reports in the literature indicate that this procedure reduces basal levels of catecholamines by 60–96% (Kannari *et al.* 2000; Hatip-Al-Khatib *et al.* 2001; Yoshitake *et al.* 2004).

Statistics

Data are presented as means ± standard error unless stated otherwise. Statistical comparisons between groups were performed using either Student's paired t test or a one-way repeated measures ANOVA with Fisher's *post hoc* test. The significance level was $P < 0.05$ unless stated otherwise.

Results

α_2-NA receptor stimulation increases the excitability of PFC pyramidal neurons

Bath application of the α_2-NA agonist clonidine produced three consistent effects on deep layer mPFC pyramidal neurons recorded in current clamp: (1) a small (3–5 mV) hyperpolarization in resting membrane potential, (2) a significant increase in input resistance, and (3) an increase in the number of evoked spikes per depolarizing pulse (Fig. 1*A*). To quantify these changes in neuronal

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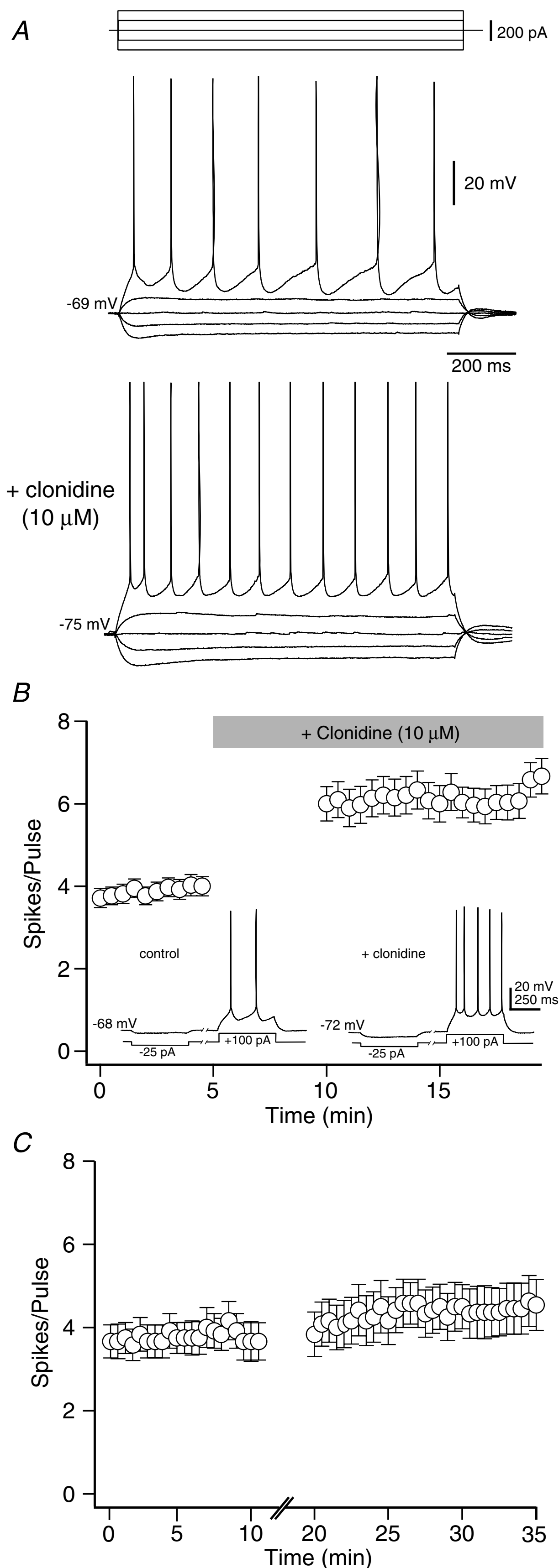

excitability, 1 s depolarizing current pulses were delivered every 30 s, with the current amplitude adjusted for each cell to elicit an average of three to four action potentials per pulse. Once a stable baseline was established (> 5 min) clonidine (10 μM) was bath applied for 5 min without stimulation. During this period, clonidine induced a small but significant hyperpolarization of the resting membrane potential (control: -68.3 ± 0.56 mV; clonidine: -71.8 ± 0.68 mV, $P < 0.001$, $n = 18$). However, when stimulation was resumed, a significant increase in the average number of spikes per depolarizing pulse was observed (baseline: 4.0 ± 0.7 spikes/pulse; clonidine: 8.8 ± 1.9 spikes/pulse, $P < 0.05$, $n = 18$; Fig. 1*B*). This increase in excitability was accompanied by an increase in input resistance, as measured by the peak amplitude of the hyperpolarization produced by a small (25–50 pA, 250 ms) negative current pulse (baseline: 245 ± 34 MΩ; clonidine: 286 ± 43 MΩ, $P < 0.05$; $n = 7$). The effects of clonidine were not due to the hyperpolarization of the resting membrane potential, as they persisted even when V_m was held constant during the experiment using DC injection (excitability – baseline: 3.7 ± 0.2 spikes/pulse; clonidine: 6.2 ± 0.4 spikes/pulse, $P < 0.001$, $n = 39$; input resistance – baseline: 243 ± 31 MΩ; clonidine: 321 ± 38 MΩ, $P < 0.05$, $n = 15$). These effects were also not due to repeated stimulation, intracellular perfusion, or any other time dependent process as no significant changes in V_m, R_{in}, or evoked firing were observed during repeated stimulation in the absence of clonidine (Fig. 1*C*). Similar results were also obtained if recordings were performed at 32 ± 1°C rather than room temperature (membrane potential – baseline: -68.9 ± 0.62 mV; clonidine: -73.2 ± 0.53 mV, $P < 0.05$, $n = 6$; input resistance – baseline: 138 ± 22; clonidine: 169 ± 31, $P < 0.05$, $n = 6$; excitability – baseline: 3.2 ± 0.5 spikes/pulse; clonidine: 7.1 ± 0.8 spikes/pulse, $P < 0.05$, $n = 6$).

Figure 1. The α_2-NA receptor agonist clonidine increases excitability of deep layer PFC pyramidal neurons *in vitro*
A, sweeps from a representative neuron showing the response to hyperpolarizing and depolarizing current injection before and after clonidine (10 μM). Clonidine produced a small hyperpolarization but an increase in the number of spikes evoked by depolarizing current pulses. The increase in cell excitability was accompanied by an increase in input resistance. *B*, timeline showing the change in excitability of 39 PFC pyramidal neurons following clonidine application (grey bar). Each point represents the mean ± S.E.M. For each cell, the amplitude of a 1 s depolarizing current pulse was adjusted to evoke 3–4 spikes per pulse and the membrane potential was held constant with DC injection (see text). Inset, sweeps from a representative neuron showing the response to bath applied clonidine when membrane potential was allowed to float. *C*, timeline showing no change in the average number of spikes per depolarizing current pulse during prolonged recording without drug application ($n = 12$).

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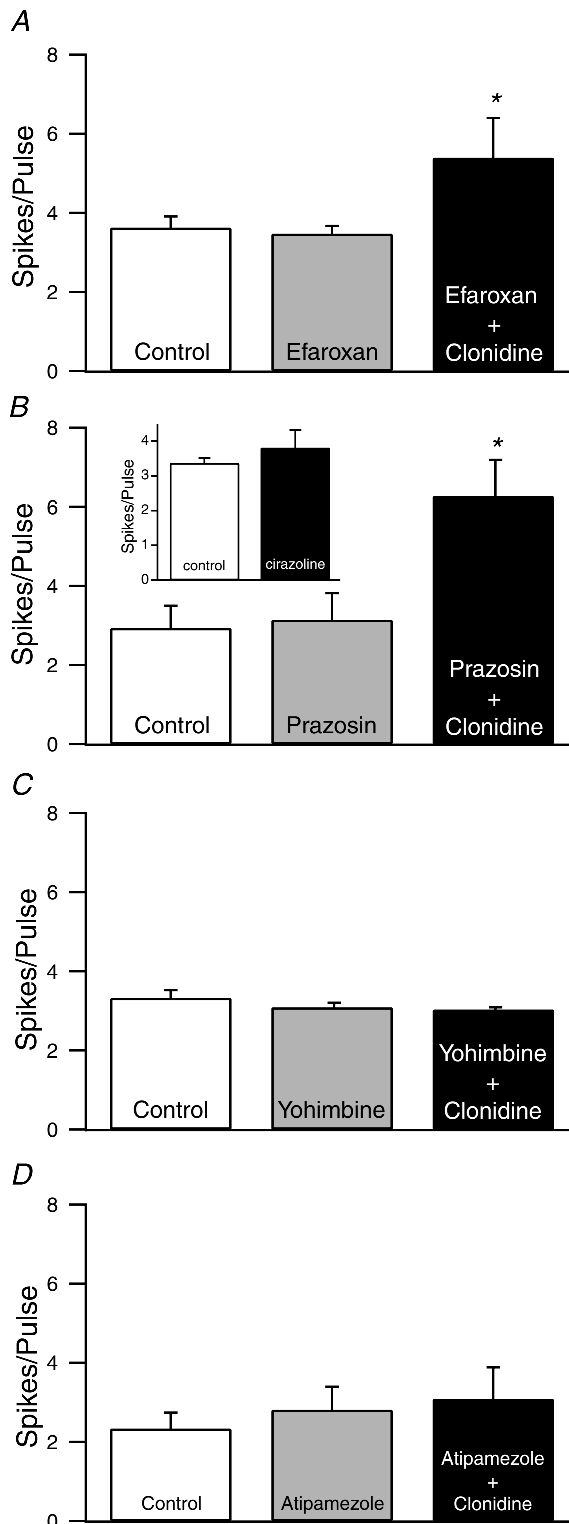

Figure 2. The effects of clonidine are mediated by α_2-NA receptors

A–B, neither the imidazole receptor antagonist eferoxan (4 nM, *A*) nor the α_1-NA receptor antagonist prazosin (1 μM, *B*) prevented the increase in spikes per pulse produced by clonidine ($n = 6$ for both groups). In addition, neither antagonist produced any changes when applied alone. *B*, inset). The selective α_1-NA receptor agonist cirazoline (10 μM) did not mimic the effect of clonidine on cell excitability ($n = 18$). *C–D*, the α_2-NA receptor antagonists yohimbine (2 μM, *C*) and atipamezole (1 μM, *D*) prevented the clonidine-mediated increases in excitability ($n = 7$ for both groups). However, neither antagonist produced any change when applied alone. $^*P < 0.05$.

The effects of clonidine are mediated by postsynaptic α_2-NA receptors

Although clonidine is a potent α_2-NA receptor agonist, it also exhibits affinity for both α_1-NA receptors and imidazole receptors. To exclude the possibility that the observed increases in excitability elicited by clonidine application were mediated via non-α_2-NA receptors, we assessed the effects of selective α_2-NA receptor, α_1-NA receptor and imidazole receptor antagonists.

To determine if the effects of clonidine are mediated by imidazole receptors or α_1-NA receptors, slices were incubated with either the imidazole antagonist efaroxan (4 nM, Haxhiu *et al.* 1994) or the α_1-NA receptor antagonist prazosin (1 μM, Crepel *et al.* 1987; Arcos *et al.* 2003) prior to application of clonidine (10 μM). Neither antagonist produced any significant effect on cell excitability when applied alone or blocked the effects of clonidine (Fig. 2*A* and *B*). Further evidence against the involvement of α_1-NA receptors in the effect of clonidine comes from the observation that the α_1-NA receptor agonist cirazoline (10 μM, Croce *et al.* 2003) did not elicit any effect on the excitability of pyramidal neurons (Fig. 2*B* inset). In contrast, the α_2-NA receptor antagonists yohimbine (2 μM) or atipamezole (1 μM) prevented the effects of clonidine on neuronal excitability, although neither antagonist produced any significant changes when applied alone (Fig. 2*C* and *D*).

In addition to their localization on postsynaptic membranes, α_2-NA receptors are also localized on noradrenergic terminals where they act as release modulating autoreceptors (Aoki *et al.* 1998). To test the hypothesis that the effects of α_2-NA receptor stimulation are due to altered presynaptic NA release, animals were injected with reserpine (5 mg kg^{-1}, I.P.) 24 h before sacrifice. Previous studies have found that this protocol reduces basal levels of catecholamines by 60–96% (Kannari *et al.* 2000; Yoshitake *et al.* 2004; Hatip-Al-Khatib *et al.* 2001). In tissue taken from these animals, clonidine still produced a significant increase in excitability, similar to the data obtained in untreated animals (baseline: 2.9 ± 0.4 spikes/pulse; clonidine: 5.7 ± 1.0 spikes/pulse, $n = 8$, $P < 0.05$) suggesting that the observed actions of clonidine are not mediated by altering presynaptic NA release.

α_2-NA receptor stimulation inhibits HCN currents

What is the mechanism through which α_2-NA receptors enhance pyramidal cell excitability? The increase in input

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resistance by α_2-NA receptor stimulation suggests the suppression of a current that is active at the resting membrane potential (∼ −70 mV). The resting potential of PFC pyramidal cells is influenced by the balance between outward currents produced by constitutively active K^+ channels (inwardly rectifying and K^+_{leak} channels) and inward currents produced by hyperpolarization/cyclic nucleotide gated cation (HCN) channels (Day *et al.* 2005). Similar to the effect of clonidine, inhibition of resting K^+ channels would produce an increase in input resistance. However, as these channels mediate a standing outward current at −70 mV, their closure would result in membrane depolarization (Day *et al.* 2005; Taverna *et al.* 2005) rather than the hyperpolarization seen following α_2-NA receptor activation. In contrast, blockade of HCN channels produces similar effects to those observed following α_2-NA receptor stimulation (Day *et al.* 2005), suggesting that HCN channels may be an important downstream target of α_2-NA receptors. Somatic voltage clamp recordings were performed to test this hypothesis. Cells were held at −50 mV in the presence of TTX (0.5 μM) and given 500 ms voltage steps from −50 to −130 mV in 10 mV increments to evoke hyperpolarization-activated currents. ZD7288 (40 μM) was used to block HCN channels. Subtracting the currents evoked following a 5 min perfusion with ZD from control records yields the ZD-sensitive current (Fig. 3*D* and *E*), which began to activate between −50 and −60 mV and showed relatively fast activation kinetics. This observation is consistent with previous findings that HCN current is primarily carried by HCN1 channels in PFC pyramidal cells (Day *et al.* 2005). Repeating these experiments, applying clonidine (10 μM) instead of ZD revealed a clonidine-sensitive current with similar characteristics to the current blocked by ZD (Fig. 3*A*–*C*). The amplitude of this clonidine-sensitive current was significantly attenuated if clonidine was applied following a 5 min incubation in the α_2-NA receptor antagonist yohimbine (2 μM; Fig. 3*E*). The activation voltage dependence of these currents was determined by measuring the tail currents generated at the end of the hyperpolarizing pulses. The normalized tail current amplitudes were plotted as a function of voltage and fitted with a first-order Boltzmann equation (Fig. 3*E* inset). The half-activation voltage of the clonidine-sensitive current (−86.7 mV) was very similar to that of the ZD-sensitive current (−87.1 mV). It is important to note that, due to the incomplete control of dendritic membrane potential, these somatic voltage clamp data are likely to represent an underestimation of both HCN channel conductance and activation kinetics, particularly at more hyperpolarized potentials (Day *et al.* 2005). We thus sought to provide

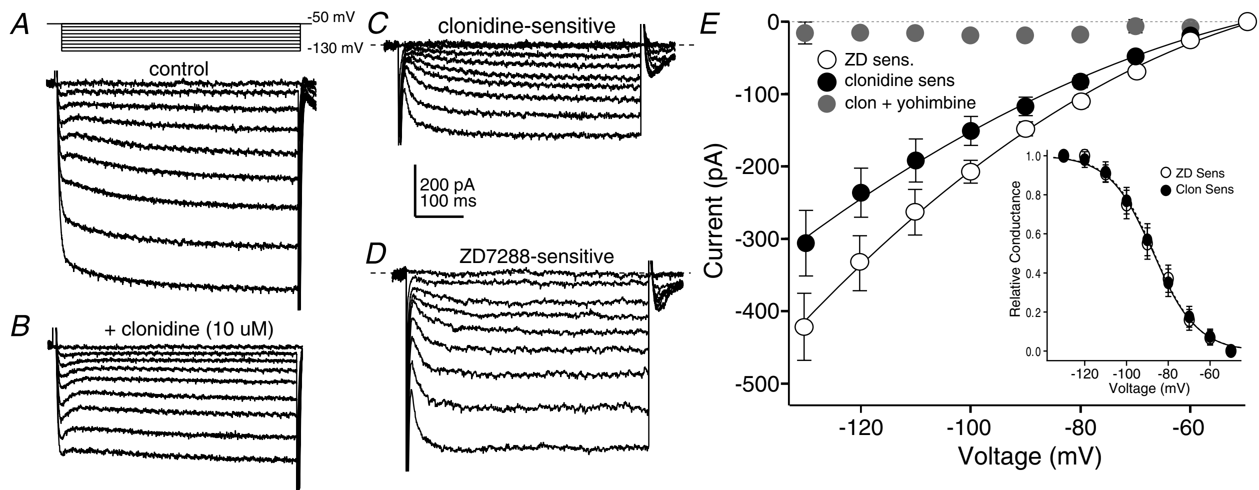

Figure 3. α_2-NA receptor stimulation inhibits hyperpolarization-activated currents in PFC pyramidal neurons

A, currents evoked by 500 ms voltage steps from −50 mV to −130 mV in 10 mV increments in the presence of TTX (0.5 μM). *B*, bath application of clonidine (10 μM, 5 min) decreased the amplitude of currents evoked by this voltage protocol without affecting holding current. *C*, subtraction of the currents in *B* from *A* yields the clonidine-sensitive current. The dashed line shows the zero current point. *D*, the ZD7288 (40 μM, 5 min)-sensitive current evoked by the same protocol in a separate neuron. *E*, summary of the current–voltage relationship of the ZD7288- (40 μM, open symbols, $n = 5$) and clonidine- (10 μM, filled symbols, $n = 6$) sensitive currents evoked by the voltage protocol shown in *A*. Points are means ± S.E.M. The clonidine-sensitive current was significantly attenuated by prior application of the α_2-NA receptor antagonist yohimbine (2 μM, grey symbols, $n = 6$). Inset, plot of the normalized amplitudes of the tail currents of the ZD- (open symbols, $n = 6$) and clonidine- (filled symbols, $n = 6$) sensitive currents. The values are fitted with a first-order Boltzmann equation (continuous line, ZD; dashed line, clonidine).

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additional pharmacologicalal and nonpharmacological evidence that the effects of clonidine are mediated via inhibition of HCN currents.

If the actions of clonidine are mediated by inhibition of HCN channels, then blockade of these channels should mimic and occlude the effects of clonidine. To test this hypothesis, clonidine was applied in the presence of the HCN channel blockers ZD7288 (40 μM) or caesium (5 mM) (Robinson & Siegelbaum, 2003). In voltage clamp recordings the α_2-NA receptor agonist did not produce any change in the current response to 1 s voltage ramps from −50 to −130 mV when it was applied following a 5 min pretreatment with either HCN blocker (Fig. 4*A*, *B* and *E*). In addition to blocking HCN currents, caesium

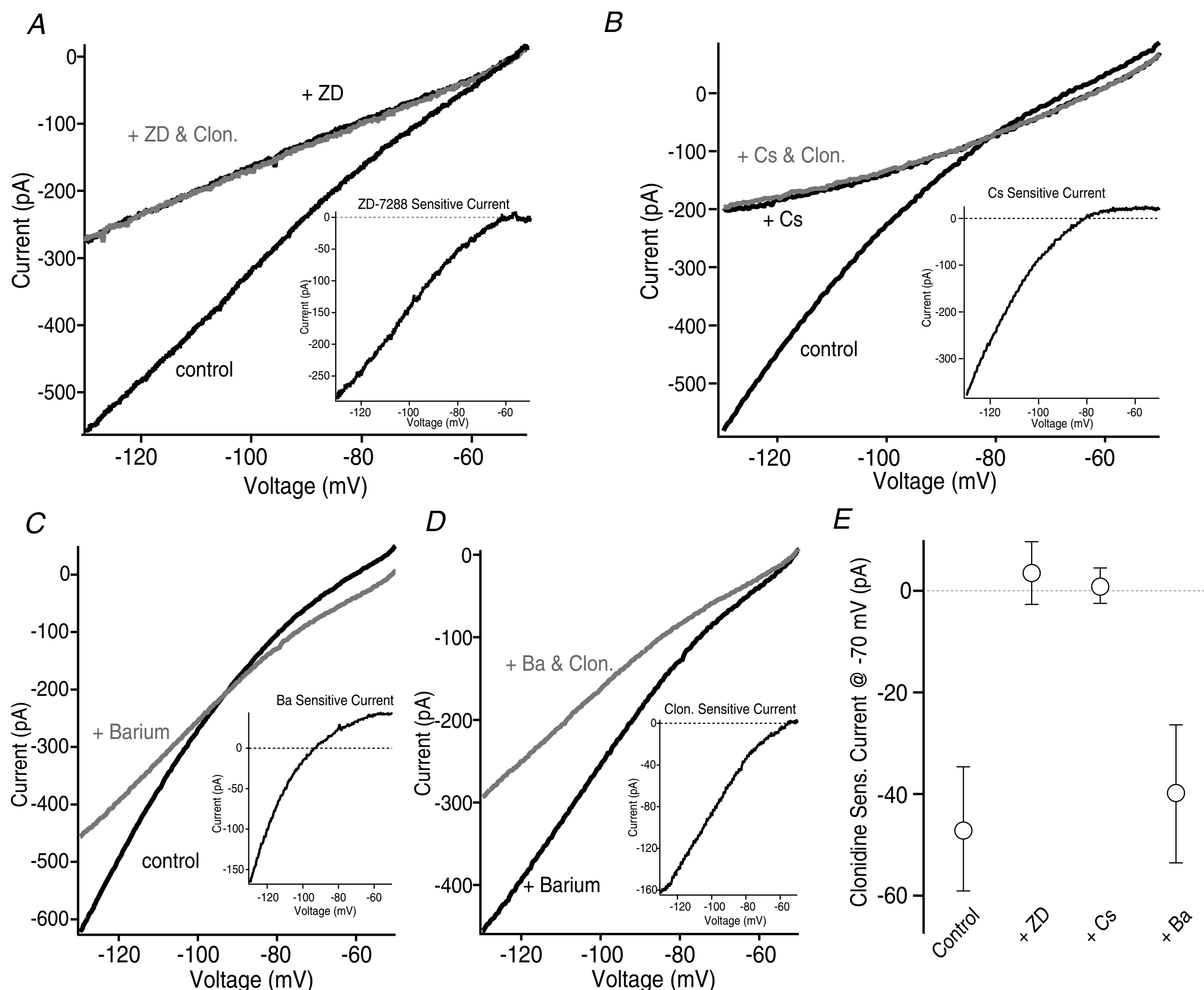

Figure 4. Block of HCN, but not Kir2, currents occludes the effects of clonidine in voltage clamp
A–D, current traces evoked by a 1 s voltage ramp from −50 to −130 mV. Each trace is the average of 5 consecutive sweeps. *A*, the HCN channel blocker ZD7288 (40 μM) produced a significant decrease in the amplitude of hyperpolarization-activated currents evoked by this voltage ramp protocol in a representative neuron. The ZD-sensitive current is shown in the inset. Subsequent addition of clonidine (10 μM, grey trace) did not produce any additional effect. *B*, clonidine (grey trace) also failed to produce any change in the hyperpolarization-activated current evoked by this voltage protocol when applied following a 5 min incubation in the HCN and Kir2 channel blocker Cs^+ (5 mM) in a separate cell. The Cs^+-sensitive current is shown in the inset. As Cs^+ blocks both HCN and Kir2 channels, the Cs^+-sensitive current reflects a mixture of both currents. *C*, the Kir2 channel blocker Ba^{2+} (100 μM, grey trace) reduced the amplitude of hyperpolarization-activated currents in a representative neuron. The Ba^{2+}-sensitive current (inset) is inwardly rectifying with a reversal potential close to the predicted K^+ reversal potential. *D*, in the same cell as *C*, subsequent addition of clonidine (10 μM, grey trace) produced an additional inhibition of hyperpolarization-activated current evoked by this voltage ramp protocol. Inset, the clonidine-sensitive current evoked in the presence of Ba^{2+} in this cell. Note the similarities to the ZD-sensitive current evoked in the cell in *A*. *E*, summary of the clonidine-sensitive current measured at −70 mV under control conditions ($n = 6$), or following incubation in ZD ($n = 6$), Cs^+ ($n = 5$) or Ba^{2+} ($n = 5$).

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also blocks inwardly rectifying K^+ (Kir2) channels in this voltage range (Stanfield *et al.* 2002). To test whether α_2-NA receptor stimulation inhibits Kir2 channels, clonidine was applied following a 5 min application in barium at a concentration (100 μM) which blocks Kir2 channels while leaving HCN currents intact (Stanfield *et al.* 2002; Robinson & Siegelbaum, 2003). Consistent with previous observations (Day *et al.* 2005; Carr & Surmeier, 2007), Ba^{2+} blocked an inwardly rectifying current that reversed close to the predicted K^+ equilibrium potential (Fig. 4*C*). However, contrary to the results obtained in the presence of ZD or Cs^+, clonidine still produced a significant reduction in hyperpolarization-activated current when applied in the presence of Ba^{2+} (Fig. 4*D* and *E*).

ZD also mimicked and occluded the actions of clonidine on evoked firing in current clamp recordings. Application of ZD produced a significant increase in evoked firing, but subsequent addition of clonidine did not produce any additional change (Fig. 5*A*). If the amplitude of the depolarizing test pulse was reduced so that the cell only fired three to four spikes in the presence of ZD, subsequent addition of clonidine still did not produce any significant change in excitability (Fig. 5*A*) arguing that the lack of a clonidine effect in the presence of ZD was not because the cell had reached its maximal firing rate in the presence of ZD. Repeating these experiments utilizing Cs^+ rather than ZD yielded similar effects on evoked firing (Fig. 5*B*). As Cs^+ also blocks Kir2 channels, whose closure leads to depolarization and tonic firing (Day *et al.* 2005), the membrane potential of cells was held at −70 mV using direct current (DC) injection during these experiments. Similar to the data obtained using ZD, Cs^+ produced a significant increase in evoked firing, but subsequent addition of clonidine did not produce any additional change (Fig. 5*B*).

The slow kinetics of HCN currents are responsible for the voltage sag seen in cortical pyramidal cells in response to hyperpolarizing current pulses as well as the depolarizing voltage overshoot following the end of the pulse (Pape, 1996). To obtain additional evidence that α_2-NA receptor stimulation produces a reduction in HCN current, the voltage sag ratio (peak voltage change/voltage change at steady state) and voltage overshoot ratio (voltage overshoot/voltage change at steady state) were determined under control conditions and in the presence of clonidine (Fig. 6). Figure 6*A* shows the effects of clonidine (10 μM) on the response to hyperpolarizing current pulses in a representative cell. For each cell tested, the resting potential was maintained at ∼−70 mV with DC injection and the amplitude of the current was adjusted to yield peak hyperpolarizing voltage deflections of 10, 20 and 30 mV. Under these conditions both the voltage sag ratio (Fig. 6*A* and *B*) and the normalized voltage overshoot (Fig. 6*A* and *C*) significantly decreased in the presence of clonidine.

α_2-NA receptor activation inhibits HCN currents via a PLC-PKC signalling pathway

How does activation of α_2-NA receptors lead to inhibition of HCN currents? HCN channels are sensitive to intracellular levels of cAMP, with a reduction in cAMP resulting in a hyperpolarizing shift in activation voltage dependence (Robinson & Siegelbaum, 2003). As α_2-NA receptors are classically coupled to inhibition of adenylate cyclase via $G_{\alpha i}$ we tested the hypothesis that the observed

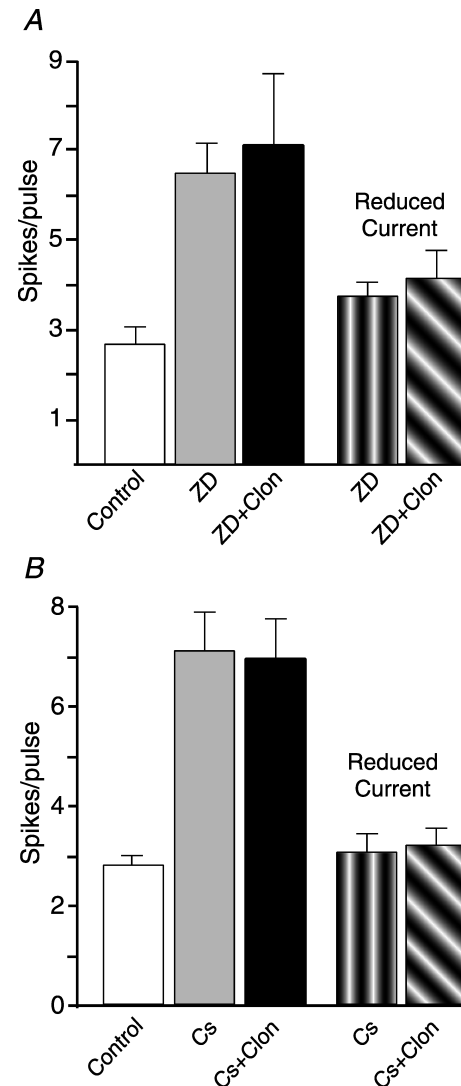

Figure 5. Blockade of HCN channels mimics and occludes the effects of clonidine on evoked firing
A, in current clamp, ZD7288 (40 μM, grey bar) significantly increased the average number of evoked spikes per pulse (see text for protocol). Subsequent application of clonidine (10 μM, black bar) did not produce any additional change in cell excitability. If the amplitude of the current pulse was reduced following ZD application (vertically hatched bar), subsequent addition of clonidine still did not produce an increase in cell excitability (diagonally hatched bar). $n = 5$ for each condition. *B*, repeating the experiment in *A* using Cs^+ (5 mM) instead of ZD yielded similar results. $n = 5$ for each condition.

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effects of α_2-NA receptor stimulation are mediated via this signalling pathway. In current clamp, recorded neurons were dialysed with the adenylate cyclase inhibitor SQ22536 (1 mM in pipette, Rosenkranz & Johnston, 2006). However, in the presence of this adenylate cyclase inhibitor, clonidine still continued to produce a significant increase in evoked firing (Fig. 7*A*). Similar results were also obtained in experiments utilizing two other adenylate cyclase inhibitors, MDL-12 330A (0.5 mM in pipette) and 2′,5′-dideoxyadenosine (0.1 mM in pipette, Pape, 1992; Jiang *et al.* 1993; Fig. 7*A*). Dialysis with SQ22536 also failed to prevent the effect of clonidine on hyperpolarization-activated currents evoked in voltage clamp (Fig. 7*B*). Taken together, these data do not support the hypothesis that the α_2-NA receptor mediated inhibition of HCN channels is mediated by inhibition of adenylate cyclase.

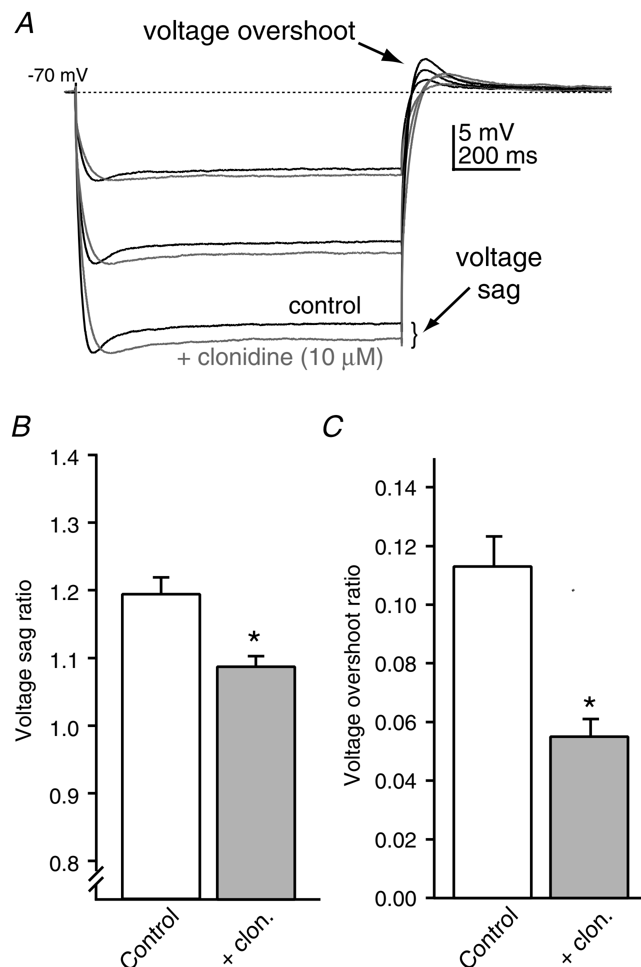

Figure 6. Clonidine alters the voltage response to hyperpolarizing current pulses
A, responses in a representative neuron to hyperpolarizing current steps. Cells were held at −70 mV using DC injection and the amplitude of the current pulses adjusted for each condition to yield peak voltage deflections of −10, −20 and −30 mV. Clonidine (10 μM, grey traces) reduced the amplitude of the voltage sag as well as the depolarizing overshoot at the end of the current pulse. *B*, summary of the voltage sag ratio (see text for definition) under control conditions and in the presence of clonidine ($n = 7$). *C*, summary of the voltage overshoot ratio (see text for definition) under control conditions and in the presence of clonidine ($n = 7$). $^*P < 0.05$. The data presented in *B* and *C* are from −10 mV peak amplitude hyperpolarizations for each cell.

HCN currents have also been reported to be inhibited by receptors coupled to increased phosphoinositide turnover and stimulation of PKC (Cathala & Paupardin-Tritsch, 1997). Although it is classically coupled to inhibition of adenylate cyclase via $G_{\alpha i}$, α_2-NA receptors have also been linked to increased phosphoinositide turnover via $G_{\beta\gamma}$ stimulation of phospholipase C (PLC) and subsequent activation of PKC (Boehm *et al.* 1996; Gesek, 1996; Dorn *et al.* 1997; Talaia *et al.* 2006). Consistent with the hypothesis that the observed effects of α_2-NA receptor activation are mediated via this signalling pathway, the effects of clonidine in current clamp were prevented by either intracellular perfusion with the PLC inhibitor U73122 (10 μM, Fig. 7*C*) or pretreatment with the PKC inhibitors chelerythrine chloride (1 μM in bath, Fig. 7*C*) or calphostin C (1 μM in pipette, Fig. 7*C*). Pretreatment with chelerythrine chloride also blocked the effect of clonidine on hyperpolarization-activated currents when tested in voltage clamp (Fig. 7*D*).

α_2-NA receptor stimulation enhances temporal integration of distal EPSPs evoked by layer I stimulation

HCN channels have been shown to play an important role in the temporal integration of distal synaptic inputs onto the apical dendrites of pyramidal cells (Magee, 1998; Berger *et al.* 2001; Day *et al.* 2005; Rosenkranz & Johnston, 2006). Deactivation of HCN channels by distal EPSPs results in a net outward current that opposes subsequent EPSPs, resulting in sublinear summation during EPSP trains. To determine if the inhibition of HCN currents by α_2-NA receptors influences synaptic integration, distal EPSPs were generated by a bipolar stimulating electrode placed in layer I, ∼500 μm dorsal to the recorded cell in layer V (Fig. 8*A* inset). The temperature of the aCSF was maintained at 33 ± 1°C. Under control conditions, a 40 Hz train of five stimuli produced EPSPs that showed sublinear temporal summation (Fig. 8*A*). The ratio of the amplitude of the fifth to the first EPSP in the train ($EPSP_5/EPSP_1$) had a median value of 2.4 ($n = 7$; Fig. 8*B* inset). In the presence of clonidine (10 μM), this ratio increased significantly to a median value of 4.4 ($n = 7$; $P < 0.05$, Wilcoxon's signed rank test; Fig. 8*B* inset). Moreover, although clonidine typically produced a 2–4 mV hyperpolarization of the resting membrane potential, the concurrent enhancement in temporal summation resulted in a larger absolute

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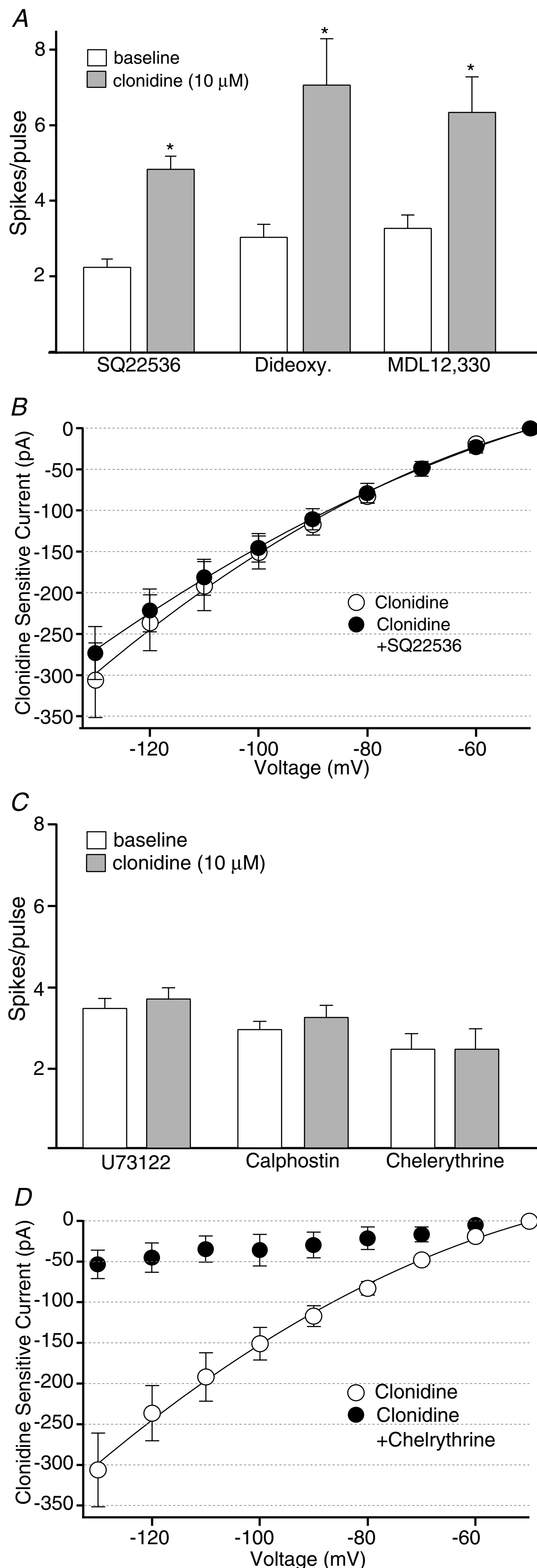

depolarization by the end of the train (Fig. 8*A*). This increase in temporal summation was not due to the hyperpolarization of the membrane potential as it persisted when V_m was subsequently adjusted to control levels by DC injection (median $EPSP_5/EPSP_1$ value: 4.5; $n = 7$; data not shown).

To determine if these effects of α_2-NA receptor activation on temporal summation were mediated through inhibition of HCN channels, the experiment was repeated in the presence of the HCN channel blocker ZD7288 (40 μM). Consistent with previous observations (Magee, 1998; Berger *et al.* 2001; Day *et al.* 2005; Rosenkranz & Johnston, 2006), ZD produced a significant increase in temporal summation during the EPSP train (Fig. 8*C* and *D*). When clonidine (10 μM) was subsequently applied in the presence of ZD, there was no further enhancement in the $EPSP_5/EPSP_1$ ratio (Fig. 8*C* and *D*), suggesting that inhibition of HCN channels is the primary mechanism through which α_2-NA receptor stimulation enhances temporal synaptic summation.

Discussion

The studies reported here indicate that α_2-NA receptor activation results in the inhibition of HCN channels, which are active at resting membrane potentials in PFC pyramidal neurons (Day *et al.* 2005). Closure of these HCN channels produces a hyperpolarization of the resting membrane potential, but a significant increase in evoked spiking due to an increase in input resistance. The inhibition of HCN channels also produces a significant enhancement in the temporal integration of distal excitatory inputs. These combined effects result in an enhancement in the gain function of the pyramidal neuron's integrate and fire properties and may thus be an important cellular mechanism through which NA enhances task related activity while suppressing background firing during the performance of working memory tasks.

Figure 7. α_2-NA receptor stimulation inhibits HCN currents via a PLC–PKC signalling pathway

A, dialysis with the adenylate cyclase inhibitors SQ22536 (1 mM, $n = 5$), 2′,5′-dideoxyadenosine (0.1 mM, $n = 5$), or MDL 12 330 (0.5 mM, $n = 5$) did not prevent the increase in evoked spikes per pulse produced by clonidine (10 μM) in current clamp. *B*, summary of the current–voltage relationship of the clonidine-sensitive current under control conditions (○, $n = 6$, data from Fig. 3*E*) or from cells dialysed with SQ22536 (1 mM, ●, $n = 6$). Points are means ± S.E.M. *C*, the effects of clonidine on evoked firing were blocked by the PLC inhibitor U73122 (10 μM in pipette, $n = 5$). The PKC antagonists calphostin C (1 μM in pipette, $n = 6$) and chelerythrine (1 μM in bath, $n = 7$) also prevented the increase in evoked spikes per pulse produced by clonidine in current clamp. *D*, summary of the current–voltage relationship of the clonidine-sensitive current under control conditions (○, $n = 6$, data from Fig. 3*E*) or in the presence of chelerythrine (●, $n = 6$). **P*-value < 0.05.

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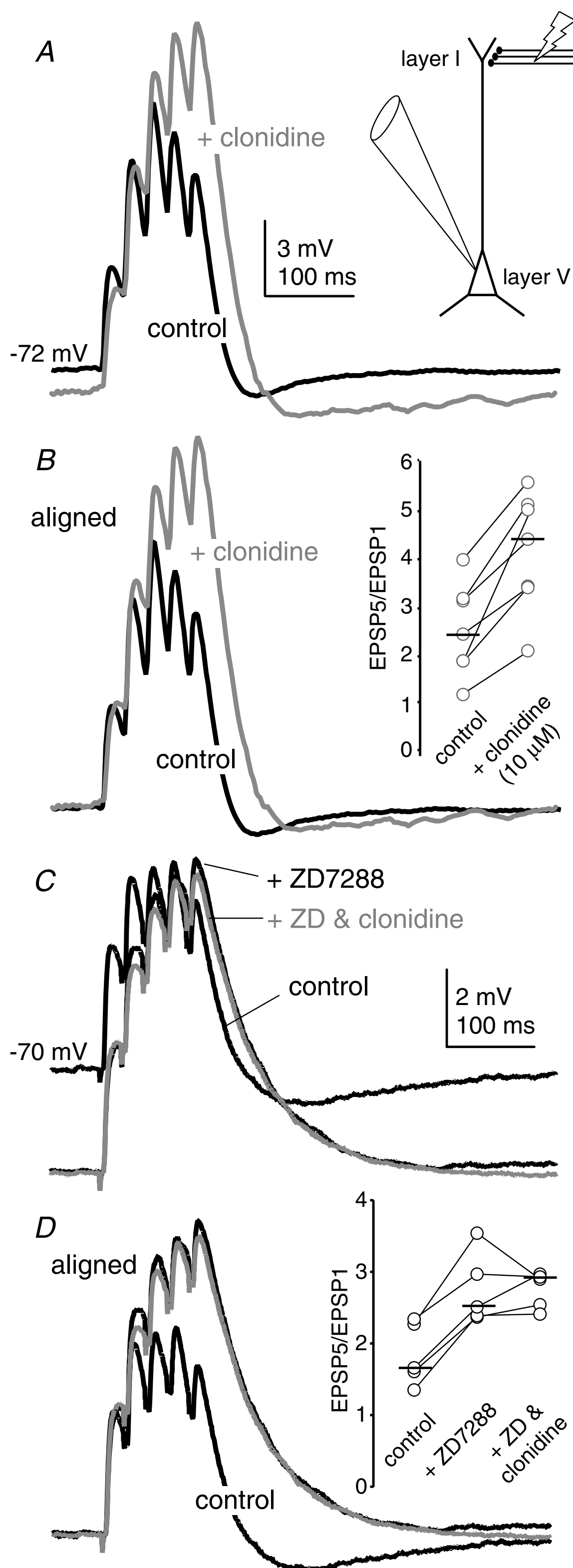

Figure 8. α_2-NA receptor stimulation enhances temporal integration of EPSPs evoked by layer I stimulation
A, current clamp recordings from a representative layer V PFC pyramidal neuron during a 40 Hz train of 5 EPSPs generated via a stimulating electrode (50 μA stim.) placed in layer I, ~500 μm dorsal to the recorded neuron (inset). Each record is the average of 10 consecutive sweeps under control conditions (black trace), or in the presence of clonidine (10 μM, grey trace). *B*, the traces in *A* are aligned to the resting membrane potential. Inset, summary of the change in the $EPSP_5/EPSP_1$ ratio produced by clonidine ($n = 7$). The horizontal bars represent the median value for each group. *C*, in a separate neuron, application of the HCN blocker ZD7288 (40 μM) hyperpolarizes the membrane potential, but enhances the temporal summation during the 40 Hz, 5 pulse train. Subsequent addition of clonidine (grey trace) did not produce any additional effect on either membrane potential or EPSP summation. *D*, the traces in *C* are aligned to the resting membrane potential. Inset, summary of the change in the $EPSP_5/EPSP_1$ ratio produced by ZD7288. Subsequent addition of clonidine did not produce any additional change in the $EPSP_5/EPSP_1$ ratio ($n = 5$).

α_2-NA receptors target HCN channels in PFC pyramidal cells

The effects of clonidine in current clamp were blocked by antagonists of α_2-NA, but not other adrenergic or imidazole receptors. Both α_{2A}- and α_{2C}-NA receptor subtypes are expressed by PFC pyramidal neurons (Scheinin *et al.* 1994; Aoki *et al.* 1998). Of these subtypes, α_{2A} displays the highest level of expression (Scheinin *et al.* 1994; Aoki *et al.* 1998) and likely mediates the observed effects of clonidine. This contention is based on the inability to block the effects of clonidine with prazosin, which shows considerable affinity for α_{2C}, but not α_{2A} receptors (Renouard *et al.* 1994). This finding is of note as the improvement in working memory performance produced by α_2-NA receptor agonists is lost in α_{2A} but not in α_{2C} receptor knockout mice (Tanila *et al.* 1999; Franowicz *et al.* 2002). In addition, a recent report indicates that α_{2A} receptors are colocalized with HCN1 subunits in dendritic spines of PFC pyramidal cells (Wang *et al.* 2007) further supporting a link between the two.

Our conclusion that the effects of α_2-NA receptor stimulation are mediated by the inhibition of HCN channels is based on several observations. First, the effects of clonidine in current clamp recordings (hyperpolarization, increased input resistance, increased temporal integration, decreased voltage sag, decreased voltage overshoot) are very similar to the effects of HCN blockade using Cs^+ or ZD7288 (Magee, 1998; Berger *et al.* 2001; Day *et al.* 2005). Second, the clonidine-sensitive current evoked by hyperpolarizing steps in voltage clamp exhibits similar kinetics and voltage dependence to the current that is blocked by the HCN channel blocker ZD7288. Most significantly, blockade of HCN channels with either ZD or Cs^+ occluded any additional effects of clonidine in either current clamp or voltage clamp recordings. Thus, while we do not exclude additional downstream targets of α_2-NA receptor stimulation, our data suggest that the observed effects of clonidine are mediated primarily through inhibition of HCN channels. Inhibition of HCN currents by α_2-NA receptor stimulation

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has also been observed in hypoglossal motoneurons (Parkis & Berger, 1997) and dorsal root ganglion neurons (Yagi & Sumino, 1998).

HCN channels play important roles in regulating resting membrane potential, synaptic integration and synaptic plasticity (Pape, 1996; Magee, 1999; Nolan *et al.* 2004; Day *et al.* 2005). Given the importance of HCN channels in shaping neural activity, it is perhaps not surprising that they are the target of many neuromodulatory systems including dopamine (Jiang *et al.* 1993; Rosenkranz & Johnston, 2006), serotonin (Bobker & Williams, 1989; Pape & McCormick, 1989), and noradrenaline (Pape & McCormick, 1989; Parkis & Berger, 1997; Yagi & Sumino, 1998). The most well characterized signalling pathway for altering HCN channel function is the depolarizing shift in activation voltage dependence produced by elevations in cAMP (Robinson & Siegelbaum, 2003). As α_2-NA receptors are classically coupled to inhibition of adenylate cyclase via $G_{\alpha i}$, one might predict that the inhibition of HCN currents by this receptor is mediated via this signalling pathway. However, intracellular dialysis with three different adenylate cyclase inhibitors failed to block the effects of clonidine in current and voltage clamp recordings. In addition, if the effects of α_2-NA receptor stimulation were mediated primarily through a hyperpolarizing shift in activation voltage dependence, one would expect that clonidine would produce a much smaller effect on HCN current amplitude at very hyperpolarized potentials where conductance is maximal. However, this was not the case for the clonidine-sensitive current. Although these observations were contrary to our original hypothesis, they are consistent with the finding that HCN currents in PFC pyramidal cells are primarily carried by HCN1 channels (Day *et al.* 2005), which are considerably less sensitive to changes in cAMP than HCN2 channels (Chen *et al.* 2001). However, it is also possible that basal AC activity is low under our recording conditions, precluding any additional reduction by $G_{\alpha i}$.

Our finding that the α_2-NA receptor-mediated inhibition of HCN channels requires activation of a PLC–PKC pathway is consistent with other reports that α_2-NA receptors are linked to increased phosphoinositide turnover via $G_{\beta\gamma}$ stimulation of PLC and subsequent activation of PKC (Boehm *et al.* 1996; Gesek, 1996; Dorn *et al.* 1997; Talaia *et al.* 2006). Our findings are also consistent with a previous report that PKC activation produced an inhibition of HCN currents in substantia nigra dopamine neurons (Cathala & Paupardin-Tritsch, 1997). North and colleagues (Jiang *et al.* 1993) have also shown that stimulation of another $G_{i/o}$ linked receptor (dopamine D_2) in ventral tegmental area neurons produces a cAMP-independent decrease in the maximal conductance of HCN currents without changing the activation voltage dependence. Although the involvement of PKC was not tested in this paper, D_2, like other $G_{i/o}$ coupled receptors (Selbie & Hill, 1998), has been shown to couple to PLC-linked signalling cascades in other neurons (Hernández-López *et al.* 2000; Maurice *et al.* 2004). Although our data indicate that the α_2-NA receptor inhibition of HCN current requires PKC activation, it is not known if PKC acts directly on HCN channels or via an intermediary, such as the transactivation of a protein tyrosine kinase (Shah & Catt, 2004).

In addition to their regulation by cAMP and PKC, recent data have demonstrated that HCN channels are also regulated by a host of other factors, including p38 MAP kinase (Poolos *et al.* 2006), Src kinase (Zong *et al.* 2005), membrane phosphoinositides (Zolles *et al.* 2006), and elevations in intracellular Ca^{2+} produced by neural activity (van Welie *et al.* 2004). Thus HCN channels are potentially under the dynamic control of a host of different intracellular signalling pathways. One important task for future studies will be to determine how these different signalling pathways interact to regulate the activity of HCN channels in native cells.

α_2-NA receptor modulation of HCN channels enhances temporal integration

HCN channels are predominantly expressed within the dendrites of pyramidal neurons where they influence the temporal integration of distally generated synaptic inputs (Magee, 1998; Berger *et al.* 2001; Day *et al.* 2005). The depolarization produced by trains of EPSPs deactivates HCN channels, generating a net outward current that opposes further depolarization and leads to sublinear summation. Inhibition of these channels prevents this sublinear summation and increases the probability that a train of excitatory input will bring the neuron to spike threshold. Inhibition of HCN channels may thus be an important cellular mechanism for enhancing the gain of a neuron's response to excitatory synaptic input. Due to the relatively slow deactivation kinetics of HCN channels, their influence on the amplitude of individual EPSPs is minimal (Magee, 1998; Berger *et al.* 2001; Day *et al.* 2005). However, because inhibition of HCN channels hyperpolarizes the resting potential away from spike threshold, the net effect of an isolated EPSP is reduced. In contrast, by preventing sublinear summation, inhibition of HCN channels enhances the amplitude and duration of the response to a burst of excitatory synaptic input. Thus the overall effect of HCN channel inhibition is to suppress the response to isolated excitatory inputs while enhancing the response to a coherent burst of synaptic activity.

By enhancing the response to trains of excitatory input, it is likely that α_2-NA receptor-mediated inhibition of HCN channels plays a significant role in the maintenance of delay activity within a recurrent network of PFC neurons during the performance of a working memory

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task. For example, previous work in behaving primates has shown that intra-PFC administration of an α_2-NA agonist improved working memory performance (Franowicz & Arnsten, 1999) while selectively increasing delay-related activity in PFC neurons (Li *et al.* 1999; Wang *et al.* 2007). In contrast, administration of an α_2-NA antagonist impairs working memory performance (Li & Mei, 1994) as well as selectively reducing delay-related activity in PFC neurons (Sawaguchi, 1998; Li *et al.* 1999; Wang *et al.* 2007). Evidence that these α_2-NA effects are mediated by inhibition of HCN channels is provided by a recent report by Arnsten and colleagues (Wang *et al.* 2007). These authors report that iontophoresis of ZD7288 enhanced the delay related firing of PFC neurons during a working memory task, similar to the application of the α_2-NA agonist guanfascine. In addition, the improvement in working memory performance produced by intra-PFC administration of α_2-NA agonists (Franowicz & Arnsten, 1999) could be mimicked by either intra-PFC injections of ZD or RNAi induced down-regulation of HCN1 expression (Wang *et al.* 2007).

Deficits in executive functioning are found in a number of neuropsychiatric disorders, including schizophrenia, depression, and attention deficit hyperactivity disorder. It is hoped that better understanding of the cellular mechanisms that mediate these executive functions of the PFC, combined with the knowledge of how these mechanisms are influenced by neuromodulators and intracellular signalling pathways, will highlight novel targets in the search for new treatments to enhance cognitive function in these patient populations.

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