

# Facilitation of Corticostriatal Plasticity by the Amygdala Requires $\text{Ca}^{2+}$ -Induced $\text{Ca}^{2+}$ Release in the Ventral Striatum

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**Popescu AT, Saghyan AA, Nagy FZ, Paré D.** Facilitation of corticostriatal plasticity by the amygdala requires  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in the ventral striatum. *J Neurophysiol* 104: 1673–1680, 2010. First published June 16, 2010; doi:10.1152/jn.00233.2010. Motor learning and habit formation are thought to depend on corticostriatal synaptic plasticity. Moreover, basolateral amygdala (BLA) activity facilitates consolidation of striatal-dependent memories. Accordingly, BLA stimulation *in vitro* facilitates long-term potentiation (LTP) induction at corticostriatal synapses onto medium spiny neurons (MSNs). Although these effects were found to depend on *N*-methyl-D-aspartate (NMDA) receptor activation at BLA synapses and consequent  $\text{Ca}^{2+}$  influx, it is unclear how this event can facilitate LTP at cortical synapses, even when the two inputs are not coactivated. Here, we aimed to shed light on this question, using whole cell recordings of MSNs *in vitro*. We first tested whether BLA inputs end at more proximal dendritic sites than cortical inputs. In this scenario, BLA synapses would experience stronger spike-related depolarizations and be in a strategic position to control the spread of second messengers. However, comparison of compound excitatory postsynaptic potentials and single-axon excitatory postsynaptic currents revealed that BLA and cortical synapses are intermingled. Next, we examined the sensitivity of cortical and BLA NMDA responses to ifenprodil because NR2A-containing NMDA receptors have faster kinetics than those containing NR2B subunits. However, the two inputs did not differ in this respect. Last, reasoning that propagating waves of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) could bridge temporal gaps between the two inputs, we tested the effects of CICR inhibitors on the BLA facilitation of corticostriatal LTP induction. Pharmacological interference with CICR blocked corticostriatal LTP induction. Thus our results are consistent with the notion that NMDA-dependent  $\text{Ca}^{2+}$  influx at BLA synapses initiates propagating waves of CICR, thereby biasing active corticostriatal inputs toward synaptic potentiation.

## INTRODUCTION

Much evidence indicates that in addition to motor control, the striatum plays a critical role in goal-directed behavior and stimulus–response learning (Packard and Knowlton 2002; Penartz et al. 2009). In keeping with this, the striatum receives inputs from low- and high-order sensory cortical areas (Wilson 1998), allowing it to integrate sensory information across different modalities and to select a course of action between competing ones (Balleine et al. 2007; Redgrave et al. 1999). Moreover, *N*-methyl-D-aspartate (NMDA)-dependent synaptic plasticity in the corticostriatal pathway is believed to underlie motor learning and habit formation (DeLong 2000; Lovinger 2010; Mahon et al. 2004; Penartz et al. 2009). Consistent with this, local intrastriatal infusions of NMDA receptor antagonists

impair learning of striatal-dependent tasks (Packard and Teather 1997a,b).

One subcortical structure contributing glutamatergic inputs to the striatum, the basolateral amygdala (BLA; Carlsen 1988; Kita and Kitai 1990; Krettek and Price 1978), was shown to facilitate striatal-dependent learning (Packard et al. 1994). For instance, activation or inactivation of the BLA with local drug infusions right after training on striatal-dependent tasks was found to respectively improve or impair recall tested days later in the drug-free state (Packard et al. 1994). Importantly, the same treatments performed long after training but just before testing recall had no effect (Packard and Teather 1998), indicating that the BLA is not the storage site of these memories but that it facilitates storage at a different site, likely the striatum (Packard and Cahill 2001).

Admittedly, it is possible that these manipulations of BLA excitability affected striatal-dependent memories via a third neuronal structure. However, it was recently shown that electrical stimulation of BLA inputs to the striatum *in vitro* facilitates induction of long-term potentiation (LTP) in the corticostriatal pathway, provided that activation of BLA and cortical synapses coincided with a postsynaptic spike (Popescu et al. 2007). Since like cortical inputs (Ingham et al. 1998), BLA axon terminals form asymmetric synapses with spines (Kita and Kitai 1990), it is conceivable that this effect is simply due to the increased depolarization produced by the paired stimulation of the BLA and cortical inputs. At odds with this possibility, however, activation of two different sets of cortical synapses, causing a degree of depolarization similar to that of paired BLA–cortex stimuli, failed to increase corticostriatal LTP (Popescu et al. 2007). Moreover, the same study revealed that the facilitation of corticostriatal LTP by BLA inputs required NMDA receptor activation and  $\text{Ca}^{2+}$  influx at BLA synapses onto striatal medium spiny neurons (MSNs). Last, it was found that BLA stimuli could facilitate corticostriatal LTP induction even when the cortical inputs were activated as late as 500 ms after (but not before) the BLA inputs. In contrast, cortical synapses that were not activated during LTP induction were unaffected (Popescu et al. 2007).

At present, the mechanisms underlying this facilitation of corticostriatal LTP by BLA inputs are unclear. The present study was therefore undertaken to address this question using whole cell patch recordings of MSNs in brain slices kept *in vitro*.

## METHODS

### *Slice preparation*

All experiments were performed using coronal brain slices obtained from Hartley guinea pigs (200–250 g; 3–4 wk old), in accordance

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with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with the approval of the Institutional Animal Care and Use Committee of Rutgers University (Newark, NJ). The subjects were anesthetized with ketamine, pentobarbital, and xylazine (80 mg/kg, 60 mg/kg, 12 mg/kg, administered intraperitoneally, respectively). The brains were extracted and cut in 400- $\mu\text{m}$ -thick slices with a vibrating microtome in ice-cold oxygenated artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM): 126 NaCl, 2.5 KCl, 1.25  $\text{NaH}_2\text{PO}_4$ , 1  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , 26  $\text{NaHCO}_3$ , and 10 glucose (pH 7.3, 300 mOsm). In some experiments,  $\text{Mg}^{2+}$  was omitted from the aCSF, to allow observation of NMDA responses from rest. Prior to the recordings, slices were kept in an oxygenated chamber at room temperature for  $\geq 1$  h. They were then transferred one at a time to a recording chamber perfused with oxygenated aCSF (7 ml/min). Before the recordings began, the temperature of the chamber was gradually increased to 32°C.

### Electrophysiology

Pairs of tungsten stimulating electrodes were placed in the BLA and at one or two cortical sites (Fig. 1A). The cortical stimulation sites were located about 0.5 mm from the lateral edge of the striatum. However, the MSNs we recorded were located along the medial edge of the striatum. One potential confound with electrical stimulation of cortex is that the activated axons might not originate from cortex. For instance, it is conceivable that current diffusion from the stimulation site led to activation of axons coursing in the striatum. However, if MSN responses to cortical stimuli depended on current diffusion to the striatum for the recruitment of glutamatergic axons, substantial overlap in the axons recruited by the two cortical sites should be observed given their position relative to the striatum. At odds with this notion, however, in our previous study (Popescu et al. 2007) occlusion tests on the responses elicited by the two cortical sites revealed negligible overlap. Moreover, in the minimal stimulation experiments described in the following text, we could routinely elicit single-axon excitatory postsynaptic currents (EPSCs) using very low stimulation

intensities, indicating that at least a proportion of activated inputs originated very close to the stimulating electrodes.

Whole cell patch recordings of MSNs in the ventral striatum were performed with pipettes (4–6 M $\Omega$ ) pulled from borosilicate glass capillaries and filled with a solution containing (in mM): 130 K-gluconate, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 10 KCl, 2  $\text{MgCl}_2$ , 2 ATP-Mg, and 0.2 GTP-tris(hydroxy-methyl)aminomethane (pH 7.2, 280 mOsm). MSNs were identified by their characteristic electrophysiological properties (Wilson 1998), including low input resistance, extremely negative resting potential, and inward rectification in the hyperpolarizing direction (see Fig. 1 of Popescu et al. 2007). Although we obtained recordings from other striatal cell types, they were excluded from the analyses. Recordings were performed in current- or voltage-clamp mode. Voltage and current signals were sampled at 10 kHz and the data were stored on a hard-drive for off-line analysis.

### Minimal stimulation experiments

In some experiments, we used minimal stimulation of BLA and cortex in control aCSF. In such cases, we used a  $\text{Cs}^+$ -based intracellular solution (in mM): 125 Cs-sulfate, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2  $\text{MgCl}_2$ , 2 ATP-Mg, and 0.2 GTP-tris(hydroxymethyl)aminomethane (pH 7.2, 280 mOsm). In voltage-clamp mode, from a holding potential of  $-50$  mV, the stimulation intensity was gradually increased at each site independently until the stimuli elicited a mixture of EPSCs and failures. Typically, the transition from failures only to a mixture of failures and EPSCs occurred abruptly and with very low intensity stimuli (10–100  $\mu\text{A}$ ; 100  $\mu\text{s}$ ). Increasing the stimulation intensity 10–30% from threshold did not increase EPSC amplitudes, consistent with the notion that these responses were elicited by the activation of single axons. The EPSCs were identified off-line on the basis of response amplitudes. The values were binned in 1 pA steps and fitted with a mixed two-Gaussian distribution using the expectation-maximization (EM) algorithm implemented in Matlab R2009a. The intersection of the two Gaussians was used as a cutoff to separate EPSCs from failures (considered noise). Typically, the distribution of failures fell in the  $\pm 10$  pA range. Here, it should be noted that due to the imperfect space clamp, fast synaptic conductances in distal dendrites were probably not well clamped. However, because BLA and cortical inputs are located at similar electrotonic distances from the soma of MSNs, this limitation likely affected data obtained at both inputs to a similar extent.

### LTP induction and monitoring

During LTP experiments, MSNs were maintained in current-clamp mode at a membrane potential of  $-90$  mV, close to their resting potential, by intracellular current injection ( $\pm 0.01$  nA). These experiments were conducted in conditions of intact GABAergic signaling (control aCSF). Input resistance was monitored throughout the experiment. Recordings with fluctuations  $> 10\%$  were excluded from the analysis. After a 10 min baseline period during which BLA and cortical inputs were activated at a low frequency (once/min), LTP was induced by simultaneously delivering electrical stimuli at two sites (cortex–cortex or BLA–cortex) and by pairing them with postsynaptic action potentials elicited by brief depolarizing current pulses (2 ms, 1.5–3 nA; Fig. 1B). The timing of the current pulse was adjusted so that the action potential peak coincided with the rising phase of the summated synaptic responses. This protocol was repeated 60 times at 2 Hz, after which responses were monitored for  $\geq 30$  min. This stimulation frequency was chosen because it matches the low spontaneous firing rates of BLA neurons *in vivo* (Paré and Gaudreau 1996; Pelletier et al. 2005). It should be noted that prior to acquiring the baseline period, the stimulation intensity at each site was adjusted so that they would evoke excitatory postsynaptic potentials (EPSPs) of about 10 mV. We monitored the initial half of the EPSP rising phase

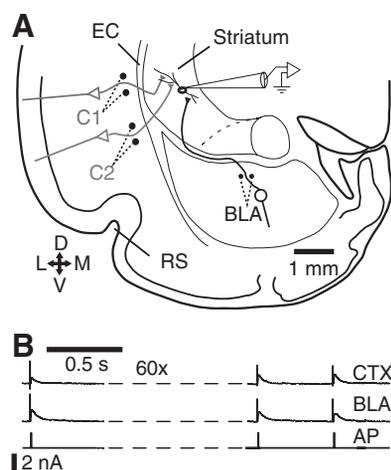


FIG. 1. Experimental set-up. *A*: scheme showing coronal brain section that includes the amygdala, temporal neocortex, and striatum. Cross indicates orientation of the slice (D, dorsal; V, ventral; L, lateral; M, medial). Whole cell patch recordings of medium spiny neurons (MSNs) were obtained under visual guidance. Stimulating electrodes were positioned at 2 cortical sites (C1, C2) as well as in the BLA. *B*: stimulation paradigm used to induce LTP. In current-clamp mode, electrical stimuli were delivered at 2 sites (BLA–cortex or cortex–cortex) and paired with postsynaptic action potentials evoked by depolarizing current injection (AP) at a frequency of 2 Hz, 60 times. The timing of the stimuli was adjusted such that the current-evoked spikes coincided with the rising phase of the excitatory postsynaptic potentials (EPSPs). AP, action potential; BLA, basolateral nucleus of the amygdala; CTX, cortex; EC, external capsule; RS, rhinal sulcus.

(approximately corresponding to the first 2 ms after response onset) for changes in slope, to avoid a potential contamination of responses by feedforward or feedback inhibition. Because all cortically or BLA-evoked inhibition is disynaptic in the striatum, the first 2 ms of evoked EPSPs should be completely free of inhibition. Consistent with this, control experiments revealed that addition of picrotoxin to the aCSF did not alter the initial slope of BLA- or cortically evoked EPSCs. Results are expressed as average  $\pm$  SE.

### Statistical analyses

To determine whether our protocols induced a significant amount of LTP, we used the Student's *t*-test analysis. The average responses recorded 20–30 min after LTP induction were calculated for each cell within a group and the distribution of these values was compared with baseline. The changes were considered significant when *P* values  $< 0.05$  were obtained. In these and other statistical comparisons, prior to using parametric tests, we verified whether the data were normally distributed using the Shapiro–Wilk test for normality (Matlab implementation of this test, found at <http://www.mathworks.com/matlabcentral/fileexchange/13964>) with *P*  $\leq 0.05$  threshold for rejecting the null hypothesis. All data sets listed in the following text passed the normality test, with values of *P*  $\geq 0.2$ .

## RESULTS

### Amplitude–slope relationship of BLA- and cortically evoked compound EPSPs

Recently we reported that repeated coactivation of cortical and BLA inputs enhances corticostriatal LTP in MSNs (Popescu et al. 2007). However, the mechanisms allowing BLA synapses to facilitate plasticity at cortical inputs remained unknown. One possibility is that BLA synapses end at more proximal levels than cortical inputs on the dendrites of MSNs. Consequently, BLA inputs would be more strongly affected by backpropagating somatic action potentials, allowing them to exert a determining influence on the dendritic spread of second messengers. Indeed, it was previously reported that dendritic propagation of somatic spikes occurs unreliably in MSNs expressing D1 receptors (Day et al. 2008). In these cells, single somatic spikes fail to elicit detectable  $\text{Ca}^{2+}$  transient at dendritic sites  $> 60 \mu\text{m}$  from the soma. This effect was not due to a declining dendritic  $\text{Ca}^{2+}$  channel density with distance from soma, since  $\text{Ca}^{2+}$  transients could be elicited in the distal processes of all MSNs with repetitive spiking or by single spikes in the presence of intracellular  $\text{Cs}^+$  (Day et al. 2008).

To determine the termination site of BLA inputs, we first analyzed the relation between EPSP slopes and amplitudes evoked by electrical activation of BLA and cortical inputs

converging onto the same MSNs. To avoid confounds associated with the potentially differing relative contributions of NMDA and non-NMDA glutamate receptors at the two inputs, we limited these analyses to the non-NMDA component of the EPSPs by adding  $100 \mu\text{M}$  D-2-amino-5-phosphopentanoic acid (D-AP5) to the aCSF. These tests were carried out in current-clamp mode, at a membrane potential of  $-90 \text{ mV}$ , as determined by intracellular current injection. Very little current had to be injected to maintain the cells at this potential because it is close to rest in MSNs ( $-89.8 \pm 0.50 \text{ mV}$ ). In each MSN ( $n = 10$ ), multiple cortical and BLA stimuli (0.2 ms) were applied at a low frequency (0.05 Hz). The stimulation intensity (0.5–0.8 mA) was adjusted such that, on average, these stimuli elicited EPSPs of about 10 mV. However, marked trial-to-trial variation in response amplitudes were observed, allowing us to compare amplitude–slope relations at the two inputs. EPSP slopes were estimated during the first half of the EPSP rising phase while their amplitude was measured at the peak of each response.

Figure 2A plots individual responses obtained in all cells, revealing a linear slope–amplitude relationship for BLA and cortical inputs, with no overall difference between the two. Examination of cell-by-cell variations in amplitude–slope ratio (Fig. 2B1) revealed heterogeneity in our sample, with some cells showing higher ratios at BLA than cortical inputs and others, the opposite. However, when the data were averaged across all cells (Fig. 2B2), no significant difference was found between the two sites (*t*-test, *P* = 0.78), suggesting that the two inputs are similarly distributed in the dendrites of MSNs.

### Properties of EPSCs elicited by minimal electrical stimuli delivered in BLA and cortex

In the preceding experiment, it is possible that the compound EPSPs elicited by the relatively high intensity BLA and cortical stimuli recruited voltage-gated conductances, potentially obscuring differences between the two inputs. To circumvent this confound, we next used minimal stimulation to analyze responses elicited by one or just a few synapses in voltage-clamp mode from a membrane potential of  $-50 \text{ mV}$ . To this end, a lower stimulus duration was used (0.1 ms) than before and the stimulation intensity at each site (BLA and cortex) was gradually increased (in 0.05 mA steps) until a mixture of failures and responses could be recorded and separated (see METHODS). These tests were carried out in 16 MSNs and the two inputs were studied in the same cells in most cases (14 of 16). In all cases, the threshold intensity was  $\leq 0.2 \text{ mA}$ .

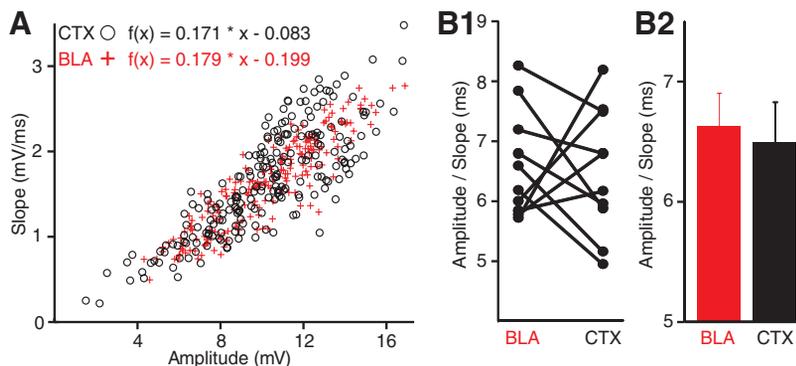


FIG. 2. Slope–amplitude relationship of compound EPSPs evoked by BLA and cortical stimuli. *A*: graph plotting EPSP slope (y-axis; measured during the first half of the EPSP rising phase) as a function of their amplitude (x-axis) for cortical (black circles) and BLA (red crosses) inputs in 10 MSNs. Prior to conducting these tests, the stimulation intensity at each site was adjusted such that, on average, the shocks elicited EPSPs of about 10 mV in amplitude. The range of EPSP amplitudes seen in *A* thus reflects trial-to-trial variations. *B*: EPSP amplitude to slope ratios (y-axis) in response to BLA and cortical stimuli in individual cells (*B1*) or averaged across all cells (*B2*).

Evoked responses were examined before versus after addition of D-AP5 (100  $\mu$ M) to the aCSF to determine the relative contribution of NMDA and non-NMDA receptors to evoked responses.

Figure 3 shows examples of responses elicited in an MSN by minimal stimuli delivered in BLA (Fig. 3A) and cortex (Fig. 3B). Across our sample of MSNs, there was no difference in the average slope of the pharmacologically isolated non-NMDA component between the two inputs (Fig. 3C1; *t*-test,  $P = 0.56$ ), again suggesting that the two inputs are similarly distributed in the dendrites of MSNs. Also, release probability, as estimated from the proportion of failure trials, was nearly identical at both inputs (Fig. 3C2; *t*-test,  $P = 0.74$ ). However, the NMDA to non-NMDA ratio was significantly higher at BLA than cortical inputs (Fig. 3C3; *t*-test,  $P = 0.017$ ). This difference resulted from two factors: the NMDA component at BLA inputs was significantly higher than that at cortical synapses (Fig. 3D1; *t*-test,  $P = 0.038$ ), whereas the non-NMDA component was significantly higher at cortical inputs than that at BLA synapses (Fig. 3D2; *t*-test,  $P = 0.002$ ). Nevertheless, there was no overall difference in the peak amplitude of BLA- and cortically evoked EPSCs (Fig. 3D3). Overall, these results suggest that the two inputs are similarly distributed in the dendrites of MSNs, although it remains possible that they end on different dendritic branches.

#### Sensitivity of BLA- and cortically evoked responses to ifenprodil

The results of the preceding experiments suggest that BLA and cortical synapses have a similar pattern of termination in the dendrites of MSNs but that the relative contributions of NMDA and non-NMDA glutamate receptors at the two inputs differ. To test whether this contrasting profile of ionotropic glutamate receptor expression is paralleled by the presence of different NR2 subunits at the two inputs, we next studied the sensitivity of pharmacologically isolated NMDA responses to

ifenprodil (5  $\mu$ M), a NR2B antagonist. Indeed, depending on the NR2 subunit expressed, the kinetics of NMDA-mediated EPSCs vary, with receptors containing NR2A subunits having faster offset kinetics than that of receptors containing NR2B subunits (reviewed in Yamakura and Shimoji 1999). Therefore, the facilitation of corticostriatal LTP by BLA inputs might also depend on a prevalent expression of NR2B subunits at BLA compared with cortical inputs, allowing them to trigger longer-lasting  $Ca^{2+}$  transients than cortical synapses.

To address this question, in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20  $\mu$ M) and picrotoxin (100  $\mu$ M), in an aCSF nominally free of  $Mg^{2+}$  ions, the intensities of BLA and cortical stimuli were adjusted to evoke responses of similar amplitudes ( $\sim 5$  mV). Then, we obtained a baseline period of 5 min, during which the two inputs were activated independently at a low frequency (0.1 Hz; Fig. 4A). Ifenprodil (5  $\mu$ M) was then added to the aCSF. After stabilization of the EPSP amplitudes, we tested whether the residual responses were mediated by NMDA receptors by adding D-AP5 (100  $\mu$ M) to the extracellular solution.

Figure 4A shows a representative example of such an experiment. The average results obtained in nine such experiments are shown in Fig. 4B. Ifenprodil produced a small but statistically significant reduction of BLA- and cortically evoked NMDA responses (cortex,  $-45 \pm 7\%$ , paired *t*-test,  $P = 0.0005$ ; BLA,  $-33 \pm 8\%$ , paired *t*-test,  $P = 0.0002$ ). However, the magnitude of this reduction was similar at the two inputs ( $n = 9$ ; *t*-test,  $P = 0.88$ ).

#### Effects of antagonists of CICR on corticostriatal LTP

As mentioned earlier, it was previously shown that BLA inputs can facilitate corticostriatal LTP, even when BLA inputs preceded cortical impulses by as much as 500 ms (Popescu et al. 2007). These results raise the question of what intracellular signal could bridge the spatial and temporal gaps between the two inputs. One possibility is that

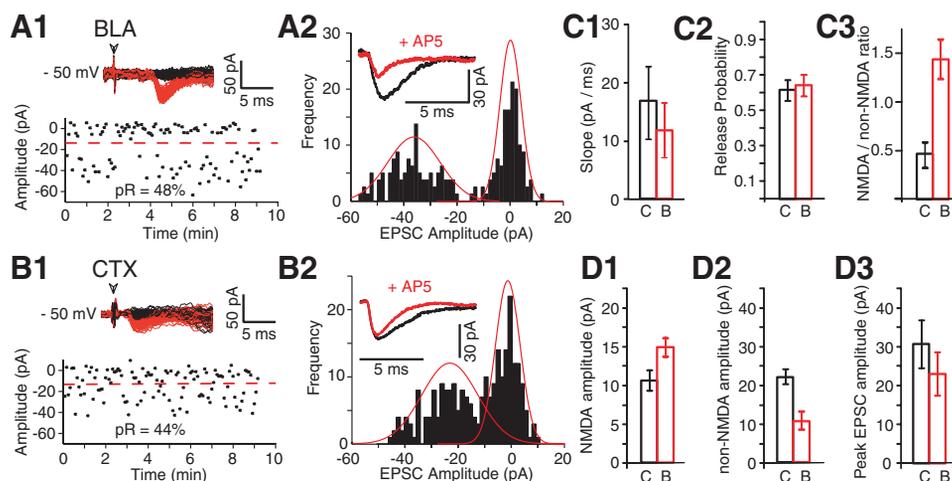


FIG. 3. Properties of excitatory postsynaptic currents (EPSCs) triggered by minimal electrical stimuli delivered in the BLA and cortex. Examples of EPSCs elicited by BLA (A) and cortical (B) minimal stimuli. *Top of A1 and B1*: superimposed trials with failures (black) and responses (red). *Bottom of A1 and B1*: EPSC amplitude (y-axis; same data as above) plotted as a function of time (x-axis). *A2 and B2*: frequency distribution of response amplitudes (1 pA bins). The data were used as a cutoff to separate EPSCs from failures (red dashed line in *A1* and *B1*). *Inset in A2 and B2*: average EPSCs in the presence (red trace) and absence (black trace) of D-2-amino-5-phosphonopentanoic acid (D-AP5). *C*: histograms comparing EPSC slope (*C1*), release probability (*C2*), and NMDA to non-NMDA ratio (*C3*) for EPSCs elicited by cortical (C, black) or BLA (B, red) stimuli. *D*: histograms comparing the amplitude of the NMDA (*D1*) or non-NMDA components (*D2*), as well as the peak amplitude (*D3*) of EPSCs triggered by cortical (C) or BLA (B) stimuli.

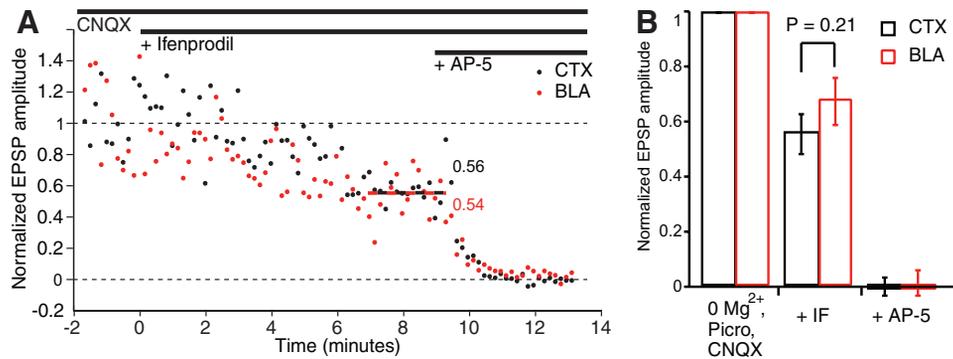


FIG. 4. Effect of ifenprodil on pharmacologically isolated NMDA-dependent EPSPs elicited by BLA and cortical stimulation. Experiments were performed in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20  $\mu$ M) and picrotoxin (100  $\mu$ M) in an artificial cerebrospinal fluid (aCSF) nominally free of  $Mg^{2+}$  ions. *A*: graph plotting the normalized amplitude of EPSPs (y-axis) elicited by BLA (red) and cortical (black) stimuli as a function of time (x-axis) in a representative MSN. The dashed line indicates average response values after application of ifenprodil. The red and black numbers indicate the residual response (as a proportion of baseline) for BLA and cortically stimuli, respectively. *B*: average results ( $\pm$ SE) obtained in a subset of 7 cells where we tested BLA and cortical stimuli. The *P* value reported in the figure was therefore obtained with a paired *t*-test.

BLA stimuli elicit intracellular waves of  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR). Indeed, ryanodine receptors are expressed by the soma and dendrites of MSNs (Martone et al. 1997) and work in other systems has revealed that  $Ca^{2+}$  influx through NMDA receptors can trigger CICR (Alford et al. 1993; Emptage et al. 1999) and that CICR has a significant impact on synaptic plasticity and homeostasis (Futatsugi et al. 1999; Reyes and Stanton 1996; Royer and Paré 2003). Moreover, activation of inositol trisphosphate (IP3) receptors can also trigger  $Ca^{2+}$  release from intracellular stores and initiate waves of CICR (Berridge et al. 2003; Marchant et al. 1999). CICR depends on the activation of ryanodine receptors on the surface of the smooth endoplasmic reticulum by increases in intracellular  $Ca^{2+}$  concentration (reviewed in Berridge et al. 2003).  $Ca^{2+}$  ions released from intracellular stores then move away from their release site by passive diffusion, activating other ryanodine receptors and therefore initiating a propagating wave of intracellular  $Ca^{2+}$  that rapidly spreads from its initiation site (Boulware and Marchant 2008; Marchant et al. 1999). Moreover, the rise in intracellular  $Ca^{2+}$  concentration that results from CICR decays slowly (Pozzan et al. 1994), which fits well with our requirements.

Therefore NMDA-mediated  $Ca^{2+}$  entry at BLA synapses could raise the intracellular  $Ca^{2+}$  concentration at distant locations where cortical synapses end, well beyond the limits of passive  $Ca^{2+}$  diffusion (Barbara 2002). To test this idea, we examined the effects of two drugs interfering with CICR on corticostriatal LTP: ruthenium red (RR), a drug that blocks ryanodine receptors (Xu et al. 1999), and cyclopiazonic acid (CPA), a  $Ca^{2+}$ -ATPase inhibitor that depletes intracellular  $Ca^{2+}$  stores (Demaurex et al. 1992). Both drugs were applied intracellularly, by adding them to the pipette solution (RR, 50  $\mu$ M; CPA, 15  $\mu$ M).

We first attempted to replicate the findings of our earlier study (Popescu et al. 2007) by comparing the amount of corticostriatal LTP induced by paired activation of two cortical sites versus paired activation of a BLA and a cortical site. As in our previous study, after a 10 min baseline during which the two inputs were activated at a low frequency (once/min), LTP was induced by delivering electrical stimuli at two sites (cortex–cortex or BLA–cortex) simultaneously 60 times at 2 Hz

and by pairing them with current-evoked postsynaptic action potentials (see METHODS for details).

Consistent with our earlier observations (Popescu et al. 2007), pairing two cortical sites caused a small but significant enhancement in the slope of cortically evoked EPSPs that averaged  $122.0 \pm 3.6\%$  of baseline (Fig. 5*A*,  $n = 8$ ). In contrast, BLA–cortex pairing led to a significantly larger enhancement (*t*-test,  $P = 0.0035$ ) in the slope of cortically evoked responses (average increase of  $160.1 \pm 6.7$ ;  $n = 6$ ; Fig. 5*B*), with no significant change in the slope of BLA-evoked EPSPs ( $94.3 \pm 6.5\%$ ,  $P = 0.41$ ).

In interleaved experiments, we examined the effect of RR (Fig. 5*B1*) or CPA (Fig. 5*B2*) on the BLA-induced facilitation of corticostriatal LTP. Identical results were obtained with both drugs: a complete abolition of corticostriatal LTP (average change from baseline: RR,  $98.8 \pm 4.7\%$ ,  $n = 5$ , *t*-test,  $P = 0.73$ ; CPA,  $98.0 \pm 2.2\%$ ,  $n = 6$ , *t*-test,  $P = 0.39$ ). With respect to the control experiments, the slope of BLA-evoked EPSPs remained unchanged ( $102.5 \pm 3.1\%$ ,  $P = 0.86$ ).

## DISCUSSION

Previous pharmacobehavioral studies have shown that BLA activity facilitates the consolidation of striatal-dependent memories (Packard and Cahill 2001; Packard and Teather 1998; Packard et al. 1994). Consistent with this and previous findings implicating NMDA-dependent corticostriatal plasticity in learning (DeLong 2000; Lovinger 2010; Mahon et al. 2004; Pennartz et al. 2009), it was later found that activation of BLA inputs in vitro could facilitate induction of corticostriatal LTP (Popescu et al. 2007). This effect could be seen, provided the two inputs coincided with a strong postsynaptic depolarization and cortical synapses were activated  $\leq 500$  ms after BLA inputs (Popescu et al. 2007). Furthermore, this study revealed that the facilitation of corticostriatal LTP by BLA inputs depended on NMDA receptor activation at both sets of synapses and consequent  $Ca^{2+}$  influx. However, it remained unclear how BLA inputs could facilitate LTP at cortical synapses, even when the two inputs were separated by a long temporal gap. The present study was undertaken to shed light on this question.

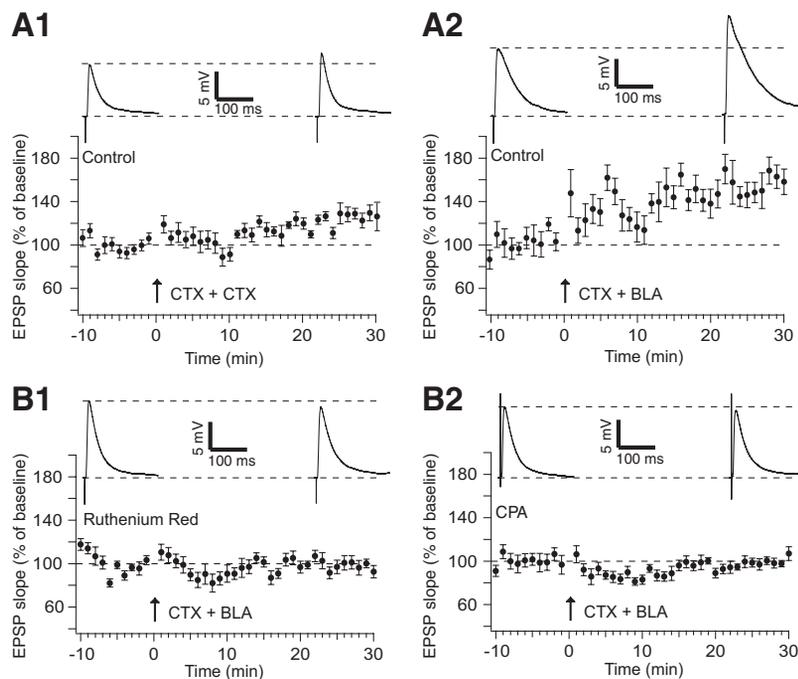


FIG. 5. Effect of drugs blocking  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) on the facilitation of corticostriatal long-term potentiation (LTP) by BLA inputs. *A* and *B*: graphs plotting the normalized slope of EPSPs (y-axis) elicited by cortical stimuli as a function of time (x-axis). The *top insets* show the average of the first 10 (*left*, preinduction) and last 10 (*right*, postinduction) cortically evoked responses in representative cells. *A*: LTP induction in control conditions. LTP was induced by pairing the activation of either 2 cortical sites (*A1*) or a BLA and a cortical site (*A2*) with a current-evoked postsynaptic spike 60 times (arrow). *B*: LTP induction in the presence of blockers of CICR in the pipette solution (*B1*, ruthenium red; *B2*, cyclopiazonic acid [CPA]). In both cases, BLA–cortex pairings were used (arrow).

*BLA and cortical synapses are similarly distributed in the dendrites of MSNs and predominantly express NR2A subunits*

Earlier ultrastructural studies have shown that the articulation of BLA and cortical inputs with MSNs is indistinguishable, both forming asymmetric axospinous synapses (Ingham et al. 1998; Kita and Kitai 1990). However, it was unknown whether the two inputs ended at similar electrotonic distances from the somata of MSNs. If BLA inputs ended at more proximal dendritic levels than cortical synapses, spike-related depolarizations would affect BLA inputs more strongly, potentially allowing them to control the recruitment and spread of second messengers more efficiently. To test this possibility, we therefore compared the slope of compound EPSPs and single-axon EPSCs evoked by BLA and cortical stimuli. However, the two inputs did not differ in this respect, suggesting that they have a similar pattern of distribution in the dendrites of MSNs. Nevertheless, these analyses revealed that the relative contribution of NMDA and non-NMDA glutamate receptors at the two inputs differs, with BLA inputs triggering significantly larger NMDA-mediated EPSCs than cortical synapses, whereas non-NMDA responses were more important at cortical than at BLA inputs.

The more important contribution of NMDA receptors at BLA than cortical inputs led us to examine whether the subunit composition of NMDA receptors at the two inputs differs. Indeed, earlier results revealed that the offset kinetics of NMDA responses is faster in receptors containing NR2A subunits compared with those containing NR2B subunits (reviewed in Yamakura and Shimoji 1999). Therefore a preferential expression of NMDA receptors containing NR2B subunits at BLA inputs would allow them to trigger larger and longer-lasting  $\text{Ca}^{2+}$  transients than cortical synapses, potentially explaining the larger NMDA currents observed at BLA inputs. To test this idea, we compared the sensitivity of pharmacologically isolated NMDA responses to ifenprodil, a

relatively selective antagonist of NMDA receptors containing NR2B subunits. However, the NMDA responses triggered by the two inputs were similarly affected by ifenprodil. Thus these results suggest that BLA synapses onto MSNs express more NMDA receptors and fewer  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors than cortical synapses.

It is possible that extrasynaptic receptors are involved in mediating the responses we observed. Indeed, there is physiological evidence of glutamate spillover outside the synaptic cleft in the striatum (Zang and Sulzer 2003) and some glutamatergic receptors have an extrasynaptic location (Mitrano et al. 2010). However, given the contrasting properties of BLA-versus cortically evoked single axons EPSCs coupled to the differences in activity-dependent plasticity at two inputs, it would appear that different populations of extrasynaptic NMDA receptors are recruited by our BLA and cortical stimuli. In other words, assuming that glutamate spillover leads to the recruitment of extrasynaptic NMDA receptors, there should be minimal overlap between the extrasynaptic receptors recruited by BLA and cortical synapses. Otherwise, no differences in the two inputs would have been observed, either in terms of NMDA to non-NMDA ratio or in properties of activity-dependent plasticity.

*Corticostriatal LTP facilitation by BLA inputs depends on  $\text{Ca}^{2+}$  entry at NMDA receptors and subsequent CICR*

In our previous study (Popescu et al. 2007), we showed that BLA inputs could facilitate corticostriatal LTP provided that cortical inputs were activated  $\leq 500$  ms after the BLA stimuli and the two inputs were paired with postsynaptic spikes. Several lines of evidence support the notion that this facilitation of corticostriatal LTP by the amygdala is critically dependent on  $\text{Ca}^{2+}$  entry at the NMDA receptors expressed by BLA synapses. First, our minimal stimulation experiments indicate that the NMDA to non-NMDA ratio is much higher at BLA than that at cortical inputs. Second, we previously showed that

selective blockade of NMDA receptors at BLA inputs only (with MK-801) prevents the facilitation of corticostriatal LTP (Popescu et al. 2007). In the latter experiments, prior to attempting LTP induction, the amplitude of BLA-evoked stimuli was adjusted to evoke the same amount of depolarization as that in control experiments. This implies that  $\text{Ca}^{2+}$  entry at NMDA receptors, not only depolarization, is the key factor behind the facilitation of corticostriatal LTP by BLA inputs (Popescu et al. 2007). Although the depolarization produced by BLA inputs may have activated voltage-gated  $\text{Ca}^{2+}$  currents, this effect, although perhaps required, is not sufficient for the facilitation of corticostriatal LTP by BLA inputs. This statement is supported by the fact that the LTP magnitude seen after pairing two cortical inputs with postsynaptic spikes is much lower than that after pairing BLA and cortical inputs, even though in both cases MSNs experience comparable levels of depolarization during LTP induction. Moreover, a previous  $\text{Ca}^{2+}$  imaging study reported that the  $\text{Ca}^{2+}$  transients produced in MSNs by paired synaptic inputs and action potentials are abolished by NMDA receptor blockade (Kerr and Plenz 2004).

However, given that the electrical stimuli we used recruited only a minority of inputs to MSNs, it is likely that most cortical and BLA synapses were at significant distances from each other in our LTP experiments. In addition, the facilitation of corticostriatal LTP by BLA inputs develops even when cortical inputs are activated as late as 500 ms after BLA synapses (Popescu et al. 2007). These observations raise the question of what intracellular signal could bridge the spatial and temporal gaps between  $\text{Ca}^{2+}$  entry at BLA synapses and other relatively distant cortical inputs.

Indeed, previous  $\text{Ca}^{2+}$  imaging studies in MSNs have revealed that  $\text{Ca}^{2+}$  influx resulting from single glutamatergic synaptic inputs is restricted to spines with only slight changes in  $\text{Ca}^{2+}$  concentration in adjacent dendritic shafts (Carter and Sabatini 2004; Carter et al. 2007). Although input identity was not determined in these studies, given the prevalence of cortical afferents to the striatum, it is likely that most had a cortical origin. Importantly, minimal interactions were seen to occur between distant synaptic inputs or when neighboring spines onto MSNs were activated asynchronously (Carter et al. 2007). Nonlinear boosting of  $\text{Ca}^{2+}$  transients was observed only when clusters of immediately neighboring spines were repetitively activated in a synchronized manner from a depolarized membrane potential (Carter et al. 2007).

These observations highlight the fact that in most conditions, the spines contacted by cortical axon terminals act as independent compartments in MSNs. Thus following  $\text{Ca}^{2+}$  entry, an additional signal is required to bridge the  $\text{Ca}^{2+}$  transients at cortical and BLA inputs. We propose that CICR fulfills this role. In support of this idea, the present study revealed that intracellular application of drugs that prevent CICR abolishes the facilitation of corticostriatal LTP by BLA inputs.

Overall, these results support a model in which  $\text{Ca}^{2+}$  entry through NMDA receptors at BLA synapses triggers propagating waves of CICR in MSNs, thereby facilitating the induction of corticostriatal LTP by enhancing the  $\text{Ca}^{2+}$  transients in spines postsynaptic to cortical axon terminals. However, a surprising observation to emerge from these experiments was that blockers of CICR not only prevented the facilitation of corticostriatal LTP by BLA inputs, but also abolished the low levels of LTP normally seen following repeated pairings of

cortical inputs with postsynaptic spikes. Although the meaning of this observation is currently unclear, a possible explanation is that in normal conditions, NMDA-mediated  $\text{Ca}^{2+}$  entry at spines postsynaptic to cortical axon terminals triggers local  $\text{Ca}^{2+}$  release from the spine apparatus. Although small and local, this  $\text{Ca}^{2+}$  release would be critical for LTP induction. The abolition of corticostriatal LTP by CPA and RR would therefore result from interference with this boosting  $\text{Ca}^{2+}$  signal. Therefore an important challenge for future  $\text{Ca}^{2+}$  imaging experiments will be to test the impact of CICR on synaptically evoked  $\text{Ca}^{2+}$  transients in MSNs.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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