Fast phasic release properties of dopamine studied with a channel biosensor

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Fast Phasic Release Properties of Dopamine Studied with a Channel Biosensor

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Few other neurotransmitters are of as intense interest to neuropsychiatry and neurology as dopamine, yet existing techniques to monitor dopamine release leave an important spatiotemporal gap in our understanding. Electrochemistry and fluorescence imaging tools have been developed to fill the gap, but these methods have important limitations. We circumvent these limitations by introducing a dopamine-gated chloride channel into rat dorsal striatal medium spiny neurons, targets of strong dopamine innervation, thereby transforming dopamine from a slow transmitter into a fast transmitter and revealing new opportunities for studying moment-to-moment regulation of dopamine release. We demonstrate pharmacological and biophysical properties of the channel that make it suitable for fast, local dopamine measurements, and we demonstrate for the first time spontaneous and evoked responses to vesicular dopamine release in the dorsal striatum. Evoked dopamine currents were separated into a fast, monosynaptic component and a slower-rising and decaying disynaptic component mediated by nicotinic receptor activation. In summary, LGC-53 represents a dopamine biosensor with properties suitable for temporal separation of distinct dopamine signals in targets of dopamine innervation.

Key words: dopamine release; ligand-gated ion channel; nicotinic; presynaptic; striatum

Introduction

Rapid, moment-to-moment changes in transmitter release underlie behavioral adaptation and short-term forms of memory (Zucker and Regehr, 2002). In the case of dopamine (DA), alteration in DA release represents a common early effect of many addictive drugs, and changes in DA release may underlie early pathology in diseases such as Parkinson’s (Garcia-Reitbock et al., 2010; Scott et al., 2010). Unfortunately, there are significant barriers to the study of short-term or rapid changes in DA release. DA activates G-protein-coupled receptors (GPCRs) that are linked indirectly to changes in membrane excitability. This typically makes endogenous GPCR-mediated signals poor sensors of amount and timing of DA release. In contrast, ligand-gated ion channels at “fast” glutamate, GABA, glycine, and peripheral nicotinic synapses (e.g., the neuromuscular junction) have high-fidelity sensors of local, synaptic transmitter release and are directly responsible for such fundamental observations as the quantal basis of release, paired-pulse facilitation/depression, and synchronous versus asynchronous transmitter release.

Limitations hamper other, non-receptor probes of DA release. Electrochemistry and microdialysis lack the spatial and temporal resolution to study rapid changes in transmitter release. They are best suited in situ to measure volumetric, spatiotemporally averaged DA transients because probes are too large to be positioned in the synapse, although amperometry can detect single vesicles when suitably placed (Staal et al., 2004). Fluorescent presynaptic reporters of DA release have been used recently (Sankaranarayanan et al., 2000; Daniel et al., 2009; Gubernator et al., 2009; Garcia-Reitbock et al., 2010; Rodriguez et al., 2013). Single presynaptic terminals can be visualized in dissociated cultures and in some cases tissue slices, but the methods do not have temporal sensitivity comparable with postsynaptic currents (PSCs; Gubernator et al., 2009; Rodriguez et al., 2013). D2-activated potassium channels have sensitivity to detect evoked (Ford et al., 2009) and quantal (Gantz et al., 2013) DA release but apparently only for specialized dendritic release within the substantia nigra/ventral tegmental area.

The above limitations have sustained an important gap in our understanding of basal DA transmission, alterations in release wrought by drugs of abuse, and changes related to pathology. To fill this gap, we introduce the use of a DA-gated chloride channel (LGC-53) from Caenorhabditis elegans (Ringstad et al., 2009) to study DA release from mammalian DA terminals in the dorsal striatum. We first examine pharmacological and biophysical properties of the receptor important in the use of LGC-53 as a biosensor of synaptic DA. By virally introducing LGC-53 into striatal medium spiny neurons, we demonstrate evoked and spontaneous phasic currents attributable to DA release. We dis-
tiguous temporally distinct components of DA release evoked by local stimulation: fast monosynaptic DA release and a surprising large and slow disynaptic component mediated by nicotinic receptor activation. We conclude that heterologous expression of LGC-53 in normal targets of DA innervation is a new tool for studying a previously poorly characterized spatiotemporal domain of DA release.

Materials and Methods

Molecular biology. A wild-type (WT) C. elegans LGC-53 DNA sequence was used for Xenopus oocyte expression studies (Ringstad et al., 2009). The WT LGC-53 sequence can be found at the National Center for Biotechnology Information as accession number NM_171812.5. For mammalian expression, the sequence was optimized for mammalian codon usage, the signal sequence was optimized to that of rat α1 GABA<sub>B</sub> receptor (GABA<sub>B</sub> R) subunit, and predicted ER retention sequences were mutated to improve surface expression (Epoch Life Sciences). These changes to the predicted retention sequences were as follows: R398A, R399A, R400A, R439A, R440A, R441A, and K442A.

Solutions. Bath solutions for the various experiments are referred to by letter below. Solution A (oocyte culture) contained 96 mM NaCl, 1 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 10 mM HEPES at pH 7.4, supplemented with 5 mM pyruvate, 100 μM penicillin, 100 μg/ml streptomycin, and 30 μg/ml gentamycin. Solution B (oocyte recording) contained the following (in mM): 96 NaCl, 1 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, and 10 HEPES at pH 7.4. Solution C (culture recording) contained the following (in mM): 96 NaCl, 1 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, and 10 HEPES at pH 7.4. Solution D contained the following (in mM): 87 NaCl, 75 sucrose, 25 NaHCO<sub>3</sub>, 1.25 Na<sub>2</sub>PO<sub>4</sub>, 2.5 KCl, 0.5 CaCl<sub>2</sub>, 3 MgCl<sub>2</sub>, and 25 glucose. Solution E contained the following (in mM): 92 choline chloride, 2.5 CaCl<sub>2</sub>, 2.5 KCl, 30 NaHCO<sub>3</sub>, 1.25 Na<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 25 glucose, 20 HEPES, 5 sodium ascorbate, 2 thiourea, and 3 sodium pyruvate. Solution F contained the following (in mM): 125 NaCl, 25 NaHCO<sub>3</sub>, 1.25 Na<sub>2</sub>PO<sub>4</sub>, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 25 glucose.

Standard whole-cell pipette solutions included the following (in mM): 130 potassium gluconate, 4 NaCl, 5 EGTA, 0.5 CaCl<sub>2</sub>, and 10 HEPES, pH 7.3. For some experiments, when indicated, cesium gluconate, cesium methanesulfonate, cesium chloride, or potassium chloride substituted for potassium gluconate. For slice studies, 4 mM QX-314 was included in the pipette solution.

Xenopus oocytes. Oocytes were harvested and prepared by standard methods. Briefly, stage V–VI oocytes were harvested from sexually mature female Xenopus laevis (Xenopus One) under 0.1% tricaine (3-aminobenzoic acid ethyl ester) anesthesia, according to protocols approved by the Washington University Animal Studies Committee. Oocytes were dechorionated by shaking for 20 min at 37°C in collagenase (2 mg/ml) dissolved in calcium-free solution containing the following (in mM): 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, and 5 HEPES at pH 7.4. Constructs were prepared in pGEMHE plasmids containing Xenopus β-globin 5’ and 3’ UTRs to stabilize mRNA. Capped mRNA encoding WT LGC-53 was transcribed in vitro using the mMESSAGE mMACHINE Kit (Ambion) from linearized vectors containing receptor coding regions. Subunit transcripts were injected in a volume of up to 20–50 nl and 10–50 ng of total RNA 16–24 h after dechorionation. Oocytes were incubated up to 5 at 18°C in solution A. Xenopus oocytes were recorded using standard two-electrode voltage-clamp recordings. Oocytes were cultured in bath solution A and recorded in bath solution B using pipettes filled with 3 mM KCl.

Two-electrode voltage-clamp experiments were performed with an OC725 amplifier (Warner Instruments) 2–5 d after RNA injection. The extracellular recording solution was solution B. Intracellular recording pipettes were filled with 3 mM KCl and had open tip resistances of ~1 MΩ. DA and modulators were applied from a common tip via a gravity-driven multibarrel delivery system. Except when indicated, cells were voltage clamped at −70 mV, and the peak current or the current at the end of 30 s was measured as indicated. Nicotinic antagonists were preapplied for 5 min before challenge with coapplied DA and antagonist.

Cultures. HEK 293T and Neuro 2A (N2a) cells were cultured under standard conditions and transiently transfected with pcDNA3.1 vector encoding LGC-53. RFP or GFP was cotransfected as a marker of expression. Primary neuronal culture details have been published previously. Briefly, postnatal rat striatal neurons were seeded onto a monolayer or island of astrocytes prepared as described previously (Moulder et al., 2007) and grown for 10–14 d before use in synaptic studies.

For transfected cell lines and primary neurons, whole-cell recordings were performed in recording solution C. To isolate LGC-53 PSCs, we used 1–5 μM NBQX, 25–50 μM D-APV, and 5–10 μM gabazine. In some experiments, 100 μM picrotoxin was also added as described.

Lentiviruses injections and striatal slices. Lentivirus particles were prepared by the Hope Center Viral Vector Core at Washington University. Constructs used the FCIV vector, a lentiviral expression vector that drives gene expression under control of the ubiquitin C promoter (Li et al., 2010). In some cases, lentivirus was prepared in a vector in which expression was under the control of the synapsin I promoter, which was more effective than FCIV when injected into striatum of more mature animals (older than postnatal week 3). Coexpression of Venus fluorescent protein was controlled by an IRES sequence. Lentiviruses were produced in HEK 293T cells. Viral particles were stereotactically injected into P5 rat pups (FCIV vector) or postnatal week 3 pups (Synapsin I promoter), followed by slice harvest at P20–P22 or P30–P40 for recording. Coronal or horizontal slices were prepared with a Leica 1200S tissue slicer in low-sodium buffer D (see above). Slices were stored for 30 min at 34°C in a low-sodium choline solution E (see above); thereafter, slices were transferred to oxygenated solution F stored at −25°C. Slices were perfused at 2–4 ml/min with oxygenated saline F at −25°C with antagonists added as indicated. Whole-cell recordings from slices were performed using pipette solutions of the compositions given above.

Stimulation of evoked striatal PSCs was achieved with a whole-cell pipette filled with bath solution and connected to a stimulus isolator, with stimulus amplitudes of 5–300 μA at 100–150 μs. A 500 ms paired-pulse interval was used for the paired-pulse experiments, with a sweep interval of 60 s. For the study of nicotinic acetylcholine receptor (nAChR) and D<sub>2</sub> pharmacology in slices, a nearly maximal PSC was elicited before nAChR antagonist or D<sub>2</sub> agonist application by varying the stimulus intensity up to 250 μA with a 150 μs pulse width. This helped to ensure the presence of the early PSC. For matched-amplitude comparisons of early and late PSCs, stimulus strength was adjusted to produce a 1000 ± 150 pA PSC amplitude in the presence of 1 μM dihyd ro-β-erythroidine (DHβE) (early PSC) or without antagonist (compound PSC). The standard holding potential was −70 to −80 mV unless otherwise indicated. Voltages, including the current–voltage relations used in the figures, were not corrected for liquid junction potentials.

Imaging. Images were acquired on an upright Nikon microscope equipped with infrared differential interference contrast optics, a QImaging camera, and MetaMorph software (Molecular Devices). Images were adjusted in NIH Image and Adobe Photoshop (Adobe Systems) for brightness, contrast, and pseudocoloring.

Data analysis. Electrophysiology data files were analyzed with Clampfit (versions 9.2 and 10.4; Molecular Devices) or imported into Igor (version 6.3; WaveMetrics) for analysis with custom-written routines or the plug-in NeuroMatic. Graphs were constructed with SigmaPlot (version 12.5). PSC amplitudes were measured from the baseline 5 ms before the stimulation. For biphasic responses, the first component (peak within 17 ms from the stimulus onset) was termed the early PSC, with the latter component termed the late PSC. Paired-pulse ratios were calculated after subtracting baseline current 5 ms before each stimulus. The half-decay was calculated from the peak PSC amplitude to the 50% amplitude. Statistical comparisons were performed in GraphPad Prism (version 6; GraphPad Software) using tests as indicated. All data in figures and text are presented as mean ± SE.

Results

Pharmacological and biophysical properties relevant to fast biosensor To serve as a sensor of quasi-synaptic or synaptic DA release rather than ambient, low-level or volumetric transmitter, LGC-53 should
have a relatively low agonist affinity. Consistent with a previous report (Ringstad et al., 2009), we found that LGC-53 peak currents were activated by DA with an EC$_{50}$ of 6.4 µM (Fig. 1A,B). This value is comparable with receptors for fast transmitters: a slightly higher EC$_{50}$ than glutamate gating NMDARs and slightly lower than glutamate gating AMPARs or GABA acting at GABA$\text{A}$Rs (Patneau and Mayer, 1990; Mortensen et al., 2010). The EC$_{50}$ is much higher than mammalian DA GPCRs, with $K_i$ values ranging from 1 to 30 nm (Seeman and Van Tol, 1994). Although under the non-steady-state conditions of synaptic release in intact cells high DA concentrations may be needed to activate GPCRs (Ford et al., 2009), a high EC$_{50}$ for LGC-53 suggests that detection of low, submicromolar ambient concentrations will be minimized and that detection of high, synaptic concentrations will be favored.

Previous work has suggested that LGC-53 is an anion channel. To confirm this, we compared reversal potentials of LGC-53, gated by DA, with GABA$\text{A}$Rs, gated by GABA and well-characterized anion channels (Bormann et al., 1987). LGC-53 expressed in Xenopus oocytes exhibited a reversal potential near −20 mV and was statistically indistinguishable from the reversal potential of GABA acting at $\alpha\beta\gamma\delta$ GABA$\text{A}$R subunits in the same batches of oocytes (data not shown). However, a difference in anion permeability compared with GABA$\text{A}$Rs was revealed in whole-cell recordings from transfected mammalian cells, in which alternative intracellular anions substituted for chloride. GABA-gated GABA$\text{A}$ channels exhibited weak gluconate and methanesulfonate permeability; these anions led to indistinguishable, negative reversal potentials, consistent with classic studies on GABA$\text{A}$Rs and glycine receptors (Bormann et al., 1987). However, DA-gated currents from LGC-53 exhibited significantly more positive reversal potentials when methanesulfonate was used as an anion than when gluconate was used (Fig. 1C,D). This result suggests atypical anion permeability for LGC-53 relative to other cys-loop chloride channels, such as the GABA$\text{A}$R (Fig. 1D). The observation has practical implications for the choice of pipette solutions in whole-cell recordings to optimize driving force on DA-activated LGC-53 currents. When LGC-53-transfected cells were filled with CsCl, DA-gated currents exhibited the expected reversal potential near 0 mV (data not shown).

Some transmitter receptors may exhibit limited sensitivity as a result of desensitization properties (Trussell and Fischbach, 1989; Zorumski et al., 1996; Overstreet et al., 2000). In striatal neurons ($n = 4$) and HEK 293T cells ($n = 4$) transfected with LGC-53, 30 µM DA elicited characteristic biexponential macroscopic desensitization to application of 30 µM DA ($\tau_{fast} = 128 ± 12$ ms, $\tau_{slow} =$...
GABAARs. We started with compounds typically used for phar- 
tiate (allopregnanolone, 3

3tently and allosterically inhibit (pregnenolone sulfate) or poten-
tion; histamine, and GABA failed to gate detectable LGC-53 
respond to the alternate agonists (100 

mM DA to oocytes expressing LGC-53. 
Figure 2B, C). The antagonism 
expected with increased agonist concentration, 
and GABA failed to gate detectable LGC-53 
strongly inhibited LGC-53 at concentrations typically used 
to block GABA transmission (Fig. 2B, C). The antagonism 
was essentially overcome with increased agonist concentration, 
suggesting at least a partly competitive mechanism (Fig. 2C). 
other antagonists of GABAARs had weaker effects (Fig. 2B), 
and GABA also exhibited mild sensitivity to neurotransmitters that po-
tently and allosterically inhibit (pregnenolone sulfate) or potent-
tate (allopregnanolone, 3α5αP) GABAAR function (Eisenman et 
observations suggest that caution is 
warranted when choosing the pharmacological tools used to iso-
late LGC-53 responses in native tissue.

Previous results suggested that LGC-53 obeys a pharmacology 
that is most similar to D2-type mammalian receptors (Ringstad et 
We screened LGC-53 against modulators of GluRs and 
GABAARs. We started with compounds typically used for 
pharmacological isolation (NBQX to block AMPARs and bicuculline, 
picrotoxin, and gabazine to block GABAARs; Fig. 2B). LGC-53 
exhibited unexpected sensitivity to antagonists of GABAARs. Bi-
cuculline strongly inhibited LGC-53 at concentrations typically 
used to block GABA transmission (Fig. 2B, C). The antagonism 
was essentially overcome with increased agonist concentration, 
suggesting at least a partly competitive mechanism (Fig. 2C). 
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Previous results suggested that LGC-53 obeys a pharmacology 
that is most similar to D2-type mammalian receptors (Ringstad et 
We confirmed this observation by screening various 
ligands at 50 μM against currents generated by 10 μM DA. We 
found that, among D3 ligands tested, spiperone was the most 
effective antagonist (Fig. 3A, B). However, the concentration 
of spiperone required to substantially inhibit LGC-53 responses was 
significantly higher than the Kᵢ₃ at mammalian receptors, ~0.06 
and 0.08 nM Kᵢ₃ at D₃ and D₄ receptors, respectively (Seeman 
reported competitive, because its primary effect was 
to shift the concentration–response profile of DA to the right 
(Fig. 3C). Sulpiride (10 μM), used in ensuing pharmacology ex-
periments, weakly suppressed 5 μM DA currents (35 ± 4% 
and 4). Nemonapride and haloperidol weakly 
antagonized 5 μM DA responses (Fig. 3A, B). When tested in 
LGC-53-transfected striatal neurons, several additional D₂ li-
gands tested had weak or no activity at 50 μM (respiridon, 66 ± 
4% inhibition; raclopride, 24 ± 15% potentiation; eticlopride, 
19 ± 12% inhibition; n = 4 cells each). The D₂ ligand SKF38393 
(2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzepine 
HCl) also had little effect at 50 μM (Fig. 3A, B).
DA-gated phasic currents in dissociated culture and brain slices

Aided by biophysical and pharmacological information about the advantages and limitations of LGC-53, we next evaluated the utility of LGC-53 as a biosensor of vesicular DA release. Potential challenges to this utility include expression levels and localization of LGC-53. As a test of principle, we first sparsely transfected primary striatal cultures with LGC-53 and loaded DA as a false transmitter into striatal presynaptic terminals. Using previously published protocols, we incubated cultures with DA during de-polarization. Under these conditions, DA enters vesicles through a combination of passive mechanisms and through reloading endocytosing vesicles (Kim et al., 2000; Zhang and Zhou, 2002). After removal of extracellular DA, we established whole-cell recordings from transfected or nontransfected striatal neurons in the presence of antagonists of GluRs and GABAARs. Depolarization of the surrounding network elicited rapid, PSC-like currents in transfected neurons but never in nontransfected neurons or in neurons not previously loaded with DA (Fig. 4). These results suggest that LGC-53 can be heterologously expressed at levels sufficient to detect vesicular DA, albeit from an unknown vesicular concentration.

To determine the time course of LGC-53-mediated currents achieved at bona fide synaptic connections with single action potential stimuli, we extended the false-transmitter loading pro-

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**Figure 3.** LGC-53 is sensitive to D2 ligands, which can positively or negatively modulate. A, Response of DA alone (5 μM, black trace) and in the presence of 50 μM of the indicated D1- and D2 receptor ligands from an oocyte expressing LGC-53. B, Summary of antagonist pharmacology from four to six oocytes. Antagonists were preapplied and then coapplied with DA, and peak concentrations above 10 μM, L741,626 slowed macroscopic desensitization of LGC-53 currents and enhanced steady-state DA responses (Fig. 3D–H). L741,626 also slowed deactivation of DA-gated currents and directly gated channels at >10 μM (Fig. 3E–H).

Although these observations suggest a potentially rich pharmacology for LGC-53, we were concerned that ligands that modulate LGC-53 may have off-target effects at the concentrations required to modulate channel function (∼50 μM), orders of magnitude higher than concentrations used to modulate D2 receptors. To address this concern, we tested the most active negative and positive modulators, spiperone and L717,626, at 50 μM on evoked PSCs from hippocampal autaptic synapses, which use glutamate as a neurotransmitter. Both compounds depressed evoked AMPAR EPSCs (34 ± 10% for 50 μM spiperone, 55 ± 9% for 50 μM L717,626; n = 3 and 6). In both cases, this depression appeared to arise primarily presynaptically, because it was associated with reversible suppression (25 ± 6%) of voltage-gated sodium current. Similar glutamate EPSC suppression was observed in acutely prepared brain slices containing striatum (50 μM spiperone, 60 ± 8% suppression; n = 5). Thus, at concentrations required to modulate LGC-53, spiperone and L717,626 have significant off-target effects.
sufficient LGC-53 is trafficked to synapses to mediate synaptic-like PSCs under conditions in which synaptic junctions are hijacked to release DA cargo.

To extend the biosensor approach to a bona fide DA circuit that includes both morphologically synaptic and volumetric junctions (Descaries et al., 1996), we injected lentiviral particles encoding LGC-53 and the fluorescent protein Venus into the dorsal striatum of P5 rat pups. Infected striatal neurons were identified in brain slices 2 weeks after injection by fluorescence and targeted for whole-cell recording (Fig. 5A, B). Presumably as a result of restricted lentiviral expression (Fig. 5A), during normal handling of animals before tissue harvest, we noticed no obvious behavioral abnormalities in LGC-53-infected animals. However, systematic behavioral testing was not performed. We investigated the properties of electrically evoked currents resistant to GluR and GABAR blockers. In nontransduced neurons filled with cesium gluconate pipette solution, evoked PSCs mainly attributable to glutamate were observed at −70 mV. Evoked PSCs attributable to GABA were evoked at +20 mV. PSCs were entirely eliminated by the combination of 1 μM NBQX, 25–50 μM d-APV, and 5 μM gabazine (Fig. 5C; 1.0 ± 0% of baseline; n = 7). In contrast, transduced neurons often exhibited an evoked outward current at +20 mV resistant to GABA, R and GluR blockers (Fig. 5D). Residual current in 10 transduced neurons was 39 ± 14% of the baseline PSC amplitude (p < 0.05 compared with nontransduced controls). Compared with GABA, R IPSCs in the same neuron (Fig. 5D), putative LGC-53-mediated currents had a similar latency but rose more slowly (rise time of evoked GABA IPSCs, 5.7 ± 0.8 ms vs 23.5 ± 3 ms for residual currents after stimulation; Fig. 5D, inset; p < 0.05). Many residual PSC-like currents exhibited an inflection on the rising phase (Fig. 5D, inset), reflecting temporally distinct components of DA release explored further below.

To verify that residual PSCs in transduced neurons had characteristics of LGC-53, we examined reversal potentials of the PSCs using gluconate-based versus methanesulfonate-based pipette solutions. Recordings obtained with a methanesulfonate pipette solution exhibited a reversal characteristically more positive than recordings with a gluconate pipette solution (Fig. 5E, F). In contrast, GABA, R IPSCs in nontransduced neurons exhibited a similar reversal potential regardless of whether gluconate or methanesulfonate was used as the major intracellular anion (Fig. 5E, F). The dopaminergic nature of PSCs was further verified by sensitivity of the PSC decays to the DA transporter inhibitor GBR12909 (1-[2-[bis(4-fluorophenyl)-methoxy]ethyl]-4-[3-phenylpropyl]piperazine; 1 μM; Fig. 5G, H).

We investigated whether LGC-53 imparted sensitivity to detect unitary phasic DA release by examining spontaneous events at −70 mV in chloride-filled cells. Under baseline conditions, spontaneous transmission was evident in both transduced and nontransduced neurons. In both cell types, this was mainly attributable to GABA and glutamate transmission, because most spontaneous events were blocked by a mixture of ionotropic GluR and GABAR blockers (Fig. 5I, J). However, in transduced neurons (n = 9 of 11 cells) but not nontransduced neurons (0 of 7 cells from the same cohort), infrequent events remained that exhibited a time course characteristic of synaptic currents (Fig. 5I, amplitude range, −10 to −50 pA). Average decay time constant of these spontaneous events was 20 ± 3 ms (n = 9 neurons, 92 events in 40 min of total summated recording time). This decay time constant was significantly faster than decay of evoked responses to even the weakest stimuli presented (best-fit single-exponential time constant for evoked phasic currents, 62 ± 7 ms;
We more closely examined the slow, asynchronous rise times of LGC-53-mediated PSCs. We wondered whether the temporally distinct components could reflect direct and indirect components of DA release, which have not heretofore been temporally distinguished. With strong optogenetic stimulation of the population of cholinergic interneurons, nicotinic-driven DA release has been demonstrated (Cachope et al., 2012; Threlfell et al., 2012; Nelson et al., 2014), occurring presumably through activation of nAChRs on DA fibers (Jones et al., 2001). We examined whether the temporally distinct components might involve direct (monosynaptic) and indirect (disynaptic) nicotinic receptor (nAChR)-driven components of DA release by applying nAChR antagonists. Both mecamylamine (10 μM) and the β2-selective antagonist DHβE (1 μM) reduced the late component of the PSC and left the rapidly rising early component (Fig. 6A,B). The effect of nicotinic antagonists did not result from a direct pharmacological effect of the drugs on LGC-53. In oocytes, LGC-53 currents were not significantly affected by up to 5 min preapplication of 10 μM mecamylamine or 1 μM DHβE (Fig. 6C).

The early PSC component in striatal neurons was not a breakthrough glutamate EPSC because its reversal potential was indistinguishable from that of the late component, either when part of the compound PSC or when pharmacologically isolated (Fig. 6D,E). Furthermore, the early component was not a breakthrough GABA current because it persisted in 100 μM picrotoxin added to the omnipresent 10 μM gabazine (Fig. 6F). The early component was almost invariably smaller than the large component and often was visible only with stronger stimulation intensities. We conclude that local electrical stimulation elicits a compound LGC-53-mediated PSC in striatal targets composed of temporally distinct direct (monosynaptic) and a larger indirect (disynaptic), nAChR-driven component of DA release.

The temporal distinction between nicotinic nAChR-evoked and directly evoked release has not been observed previously and highlights the utility of LGC-53. We looked more closely at the temporal characteristics of the two components and relative modulation by paired stimulation (Fig. 7). In the absence of nAChR antagonist, the late PSC peak persisted even when stimulus amplitude was reduced to simulate the overall suppressive effect of nAChR antagonists (Fig. 7A). With weak stimulation, PSCs also decayed more rapidly, consistent with the idea that DA overflow contributes to the decay of large PSCs (Fig. 7A, C). Conversely, PSCs in the presence of nAChR antagonists rose faster and decayed faster than the compound PSC, even when compound and nAChR-antagonized PSCs were matched for amplifi-
Profound paired-pulse depression (overflow and receptor desensitization, because decreasing stimulation of the compound PSC did not primarily result from DA counteraction as suggested by discussion, the profound depression of LGC-53 responses exhibited profound depression. Direct release. Presynaptic D2 receptors are best known as autoreceptors on DA terminals. However, D2 receptors are also localized to axons and axon terminals of cholinergic fibers in the striatum (Alcantara et al., 2003). The relative effect of D2 receptor stimulation on nicotinic-driven DA release versus direct DA release is unknown. We probed the relative contribution by examining the effect of the D2 receptor agonist quinpirole (2 μM) on the two components of evoked DA release. To our surprise, D2 stimulation profoundly depressed the late PSC with a milder effect on the early PSC (Fig. 7A, C). The quinpirole effect was reversed by coaddition of the D2 receptor antagonist sulpiride (10 μM; Fig. 7B, D). As expected from the effect of the D2 agonist on both the direct and indirect components of DA release, the paired-pulse ratio was strongly elevated by quinpirole (Fig. 7A, D). We conclude that D2 activation has a disproportionate effect on the indirect, nAChr-driven component of release, in contrast to canonical views of a primarily autoreceptor function for D2 receptors.

**Discussion**

Here we introduce a simple method to transform a slow transmitter into a fast transmitter, thereby facilitating study of rapid modulation of DA release, a transmitter important to reward, volitional movement, addiction, attention, and motivation. Unlike nACh, GABA, glycine, or glutamate synapses, nature does not endow mammalian DA target cells with receptors that can be readily exploited to study fundamental features of spike-driven and spontaneous transmitter release. By equipping DA target cells with a fast neurotransmitter receptor, we revealed direct evidence for combined synaptic and volumetric transmission. For the first time, we temporally dissociate di- and monosynaptic components of DA transmission and demonstrate their differential modulation.

Our method has a number of advantages that make it readily applicable. Unlike GABAARs and GluRs, LGC-53 is homomeric and thus requires transduction/transfection of only a single subunit. The low affinity and steady-state desensitization properties render the receptor relatively insensitive to tonic DA levels but...
sensitive to transient levels of neurotransmitter that exceed $\sim 1 \mu M$. Desensitization properties are intermediate between AMPA-type GluRs on one hand (desensitization time constant of $\sim 10$ ms) and NMDARs and GABAARs on the other hand (several hundred milliseconds desensitization time constant) and therefore do not hinder sensitivity to synaptic-like pulses of transmitter. LGC-53 currents can be pharmacologically distinguished from glutamate and GABA PSCs. The unique permeability characteristics of LGC-53 suggest that, under carefully chosen experimental conditions, LGC-53 responses can also be distinguished from endogenous glutamate and GABA PSCs on the basis of differential reversal potentials (Figs. 1C,D, 5E,F). Although introduction of a foreign gene has risks of inducing unanticipated

Figure 7. Different temporal characteristics of early and late components of the compound PSC. A, Effect of reduced stimulus amplitude (red trace) on PSC time course and paired-pulse depression. The insets show the two PSCs after scaling the first PSC peak. B, Responses to 500 ms paired stimuli in a neuron recorded in the absence (black) and another in the presence (blue) of $1 \mu M$ DH$\beta$E, matched in amplitude for the size of the PSC to highlight temporal differences. Insets, Difference in time-to-peak between the antagonist-isolated early component and the late component (absence of antagonist) PSC1 and development of PSC2 after nAChR antagonism (blue). Note the profound depression of PSC2 before nAChR block. C, Summary plot of the difference in the time-to-peak and half-decay for both the antagonist-isolated early PSC1 ($-1037 \pm 22$ pA) and late PSC1 ($-1000 \pm 22$ pA). D, Summary plot of paired-pulse ratio after nAChR antagonism (blue) ($n = 8$ without antagonist, $n = 11$ with antagonist; *$p < 0.05$).

Figure 8. Preferential modulation of disynaptic DA release by exogenous D$_2$ receptor stimulation. A, Paired-pulse response before (black) and after application of a D$_2$ agonist (red; 2 $\mu M$ quinpirole). Insets show the disappearance of the late PSC1 after quinpirole (red) and a more modest amplitude decrement in the early PSC1 response. Inset shows the development of PSC2 after elevated D$_2$ receptor stimulation (red). PSC2 is displayed after subtracting current 5 ms before stimulation. B, Addition of the D$_2$ antagonist sulpiride (10 $\mu M$, gray) blocked the effect of quinpirole. In the inset, the PSC is displayed after subtracting current 5 ms before stimulation. C, Summary of the change in PSC amplitudes with D$_2$ receptor stimulation ($n = 4$ (red), $n = 3$ (gray); *$p < 0.05$). D, Increase in the paired-pulse ratio after elevated D$_2$ receptor stimulation can be occluded with a D$_2$ antagonist (*$p < 0.05$). quin, Quinpirole; sulp, sulpiride.
functional changes and adaptations, it can be argued that our strategy is less invasive than amperometry, microdialysis, or even optical probes, which can introduce tissue disruption and photodynamic alterations.

However, the LGC-53 expression strategy suffers from some disadvantages. Selectivity for DA is high, but care is needed because of the low sensitivity to NE and epinephrine. Fortunately, NE release in the striatum seems unlikely to be a serious confound (Doucet et al., 1986). Furthermore, overlap in GABA, R and LGC-53 antagonist pharmacology and off-target effects of DA ligands at concentrations needed to modulate LGC-53 are limitations. Surface LGC-53 expression levels can vary. Although some of this variability could result from differences among target cells in the degree of DA innervation, surface expression variability likely also participates.

Are the LGC-53 responses from striatal neurons synaptic? Spontaneous events appear to be closest to true synaptic events, representing single-fiber and possibly even quantal transmission. It seems likely that these events arise from a subset of the ~30% of true synaptic appositions in striatum (Descarries et al., 1996). Based on the decay time course, most evoked events involve a component of volumetric spillover, with disynaptic nAChR responses exhibiting the strongest volumetric component. This strong volumetric component likely results from the large spatial coverage of the axon collaterals of the cholinergic interneurons (Lehmann and Langer, 1983; Contant et al., 1996). A single local electrical stimulus appears to activate widespread indirect release of DA, probably extending well beyond DA terminals near the site of stimulation. The combined overflow from these terminals is sensed by the target, recorded neuron. In contrast, the same stimulus directly evokes release from a smaller subset of DA terminals (Fig. 6). Even these smaller direct responses likely contain a volumetric component, because a large majority of striatal DA terminals are morphologically nonsynaptic (Descarries et al., 1996). In comparison, only 4% of terminals of the conventional fast transmitter glutamate in the hippocampal CA1 region exhibit a nonsynaptic morphology (Harris and Weinberg, 2012). Thus, although LGC-53 is capable of detecting fast, quasi-synaptic responses (Fig. 4) with estimated "true" synaptic rise times of <5 ms and decay times of ~20 ms given by false-transmitter experiments, the microanatomy and physiology of DA connections ensure mixed volumetric and quasi-synaptic responses.

Both direct and nAChR-indirect DA release exhibited strong paired-pulse depression, implying a surprisingly high DA vesicular release probability. This is in contrast to views of DA release properties studied recently with optical techniques in dissociated cultures (Daniel et al., 2009; but see Cragg, 2003; Adrover et al., 2014). High release probability is often associated with fast synapses that require highly reliable postsynaptic activation (Zucker and Regehr, 2002). The profound, nearly complete depression of compound DA responses (Fig. 7) is consistent with the idea that nAChR activation promotes a very high release probability in terminals, thus usurping and occluding direct release (Threlfell et al., 2012). Because of our use of a ligand-gated ion channel that exhibits desensitization, we cannot exclude a role for desensitization in the paired-pulse depression observed (Figs. 7, 8). However, a very recent study using amperometry, a technique not subject to desensitization, also found evidence for strong paired-pulse depression of DA responses in rodent striatum (Wang et al., 2014). This result validates our conclusion that high presynaptic release probability and vesicle depletion are the main contributors to paired-pulse depression detected by LGC-53.

Our studies also reveal surprising differential modulation of monosynaptic and disynaptic components by D2 receptor activation. DA is known to regulate cholinergic interneuron firing in complex ways. An important effect of D2 receptor activation on cholinergic interneurons is to decrease the high tonic firing rate (Ding et al., 2010). D2 receptors are also present on cholinergic presynaptic elements (Pisani et al., 2000; Alcantara et al., 2003). However, the functionality of these receptors is unclear. Previous results demonstrated that quinpirole reduces nAChR-driven DA release, but the location of the D2 receptors remained unclear (Threlfell et al., 2012). Our results suggest that D2 activation on the ACh fibers themselves dramatically decreases cholinergic output, resulting in nearly complete elimination of the indirect DA response (Fig. 8). The weaker suppression of direct DA release suggests that the quinpirole effect cannot be accounted for solely by D2 autoreceptors on the DA terminals. Thus, direct presynaptic suppression can be added to the mechanisms of negative feedback regulation between the DA and cholinergic systems within the striatum.

Our studies reveal temporal facets of DA release in the dorsal striatum. We temporally distinguish direct DA release from stronger, nAChR-mediated release with longer-lived actions. We demonstrate the differential modulation of monosynaptic and disynaptic components by paired-pulse stimulation and an unexpectedly strong effect of D2 receptor activation on the nAChR-driven component of DA release. We conclude that heterologous LGC-53 expression offers opportunities for studying DA in a spatiotemporal domain that has been limited previously.

References


