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Prefrontal cortex driven dopamine signals in the striatum show unique spatial and pharmacological properties

1 **Prefrontal cortex driven dopamine signals in the striatum show unique spatial and**

2 **pharmacological properties**

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27 **Abbreviated title:** Prefrontal cortex driven striatal dopamine signals

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29 **Author contributions:** M.F.A., J.H.S. performed all *in vitro* electrophysiology and voltammetry

30 experiments and analyzed data. C.Q. and S.F. performed *in vivo* microdialysis experiments.

31 J.C.L. performed histological quantification. M.F.A., J.H.S. and V.A.A. designed research and

32 wrote the paper.

33

34

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3

55   **Abstract**

56   Dopamine (DA) signals in the striatum are critical for a variety of vital processes, including

57   motivation, motor learning and reinforcement learning. Striatal DA signals can be evoked by

58   direct activation of inputs from midbrain DA neurons (DANs) as well as cortical and thalamic

59   inputs to the striatum. In this study, we show that *in vivo* optogenetic stimulation of prelimbic

60   (PrL) and infralimbic (IL) cortical afferents to the striatum triggers an increase in extracellular

61   DA concentration, which coincides with elevation of striatal acetylcholine (ACh) levels. This

62   increase is blocked by a nicotinic ACh receptor (nAChR) antagonist. Using single or dual

63   optogenetic stimulation in brain slices from male and female mice, we compared the properties

64   of these PrL/IL evoked DA signals with those evoked by stimulation from midbrain DAN axonal

65   projections. PrL/IL evoked DA signals are undistinguishable from DAN evoked DA signals in

66   their amplitudes and electrochemical properties. However, PrL/IL evoked DA signals are

67   spatially restricted and preferentially recorded in the dorsomedial striatum. PrL/IL evoked DA

68   signals also differ in their pharmacological properties, requiring activation of glutamate and

69   nicotinic ACh receptors. Thus, both *in vivo* and *in vitro* results indicate that cortical evoked DA

70   signals rely on recruitment of cholinergic interneurons (CINs), which renders DA signals less

71   able to summate during trains of stimulation and more sensitive to both cholinergic drugs and

72   temperature. In conclusion, cortical and midbrain inputs to the striatum evoke DA signals with

73   unique spatial and pharmacological properties that likely shape their functional roles and

74   behavioral relevance.

75   (248 / 250 words)

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78 **Significance Statement (120 words maximum)**

79 Dopamine signals in the striatum play a critical role in basal ganglia function such as

80 reinforcement and motor learning. Different afferents to the striatum can trigger dopamine

81 signals, but their release properties are not well understood. Further, these input-specific

82 dopamine signals have only been studied in separate animals. Here we show that optogenetic

83 stimulation of cortical glutamatergic afferents to the striatum triggers dopamine signals both *in*

84 *vivo* and *in vitro*. These afferents engage cholinergic interneurons, which drive dopamine release

85 from dopamine neuron axons by activation of nicotinic acetylcholine receptors. We also show

86 that cortically evoked dopamine signals have other unique properties, including spatial restriction

87 and sensitivity to temperature changes than dopamine signals evoked by stimulation of midbrain

88 dopamine neuron axons.

89 (120/120 words)

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Introduction (650 words maximum, including citations)

The striatum receives dense innervation from midbrain DANs, which are the main source of DA in the striatum. DA plays a critical neuromodulatory role in regulating striatal circuitry and function (Surmeier et al., 2007; Gerfen and Surmeier, 2011; Burke et al., 2017). Disruptions in striatal DA levels are associated with many neurological and psychiatric disorders, such as Parkinson's disease and substance abuse disorder (Gerfen, 2000; Luscher et al., 2020). DA is released from a fraction of the varicosities distributed along DAN axons, which contain specialized active zone-like sites (Sulzer et al., 2016; Liu et al., 2018; Liu and Kaeser, 2019). The axonal arborizations of DANs ramify extensively within the striatum and a single dopamine axon can spread over a significant area (around 3% in average) of the striatum (Matsuda et al., 2009). DA released from axonal varicosities generates a rapid increase in local extracellular DA concentration. This extracellular increase leads to the activation of multiple types of DA receptors which are localized in dendrites, somas, and presynaptic terminals. Ultimately, this impacts the network activity of the striatum and drives behavior output (Tritsch and Sabatini, 2012; Chuhma et al., 2014; Mamaligas et al., 2016; Burke et al., 2017; Shin et al., 2017; Lahiri and Bevan, 2020). This striatal DA signal is known to be triggered by two mechanisms. One mechanism involves action potential firing initiated at midbrain DAN somas, which propagates through dense axonal arborizations to reach active zone-like release sites in the striatum (Matsuda et al., 2009; Liu et al., 2018). In the other mechanism, DAN axons in the striatum are locally activated, independent of the action potential firing at DAN somas. This mode of DA release requires the activation of nAChRs expressed on DAN axons and synchronized activation of CINs, which is thought to give rise to local release of ACh (Cachope et al., 2012; Threlfell et al., 2012; Wang et al., 2014; Shin et al., 2015; Shin et al., 2017).

6

116   This local intrastriatal trigger of DA release has only recently been demonstrated and is

117   gaining attention as it represents a newly discovered means for striatal DA

118   modulation/transmission. However, little is known about the differential/unique biophysical and

119   pharmacological properties of locally evoked versus DAN-evoked DA transmission. Recent *in*

120   *vitro* studies utilizing optogenetic stimulation and fast-scan cyclic voltammetry (FSCV) show

121   that local DA signals can be triggered in the striatum by stimulation of glutamatergic inputs from

122   thalamus (Threlfell et al., 2012; Kosillo et al., 2016; Johnson et al., 2017; Cover et al., 2019),

123   motor cortex (Kosillo et al., 2016), or prefrontal cortex (PFC) (Mateo et al., 2017). These

124   findings agree with previous literature showing *in vivo* DA signals in the striatum evoked by

125   stimulation of PFC (Taber and Fibiger, 1993; Quiroz et al., 2016; Hill et al., 2018), hippocampus

126   (Tritschler et al., 2018), or amygdala (Floresco et al., 1998). Furthermore, *in vivo* intrastriatal

127   administration of glutamate and ACh was also shown to cause increases in extracellular DA

128   concentration in the rat striatum (Giorguieff et al., 1976; Giorguieff et al., 1977; Leviel et al.,

129   1990; Shimizu et al., 1990). Altogether, this evidence supports an intrastriatal mechanism for DA

130   signal generation that can be initiated by excitatory inputs to the striatum that require glutamate

131   and ACh. While cortically evoked DA signals in the striatum have been reported, identifying the

132   unique pharmacological and basic properties of these input-specific signals will allow us to

133   selectively manipulate and target them, leading to a better understanding of their functional

134   significance. In this study, we tackle this gap in knowledge using both *in vivo* microdialysis and

135   *in vitro* FSCV with optogenetic stimulation. Particulally, we set up a novel approach using dual

136   optogenetic stimulation in the same brain slices to input-specific evoke DA release and compare

137   the properties of these DA signals in the striatum.

138   (627/650 words)

141 **Materials and Methods**

142 **Animals.** All animals used in the study were maintained in accordance with the guidelines of the

143 National Institutes of Health Animal Care and the animal research procedures were approved by

144 the NIAAA Animal Care and Use Committee for mice and by the NIDA IRP Animal Care and

145 Use Committee for rats. Except for the *in vivo* microdialysis experiments, all experiments were

146 carried out using male and female mice of C57BL/6 background. Heterozygote

147 B6.SJL-*Slc6a3$^{tm1.1(cre)Bkmn}$*/J mice (Backman et al., 2006)(The Jackson Laboratory, 006660)

148 referred to as DAT$^{IRES-Cre+}$ mice and negative DAT$^{IRES-Cre-}$ littermates considered as wild-type

149 mice were used. To fluorescently label CINs, homozygote B6N.129S6(B6)-*Chat$^{tm2(cre)Lowl}$*/J mice

150 (Rossi et al., 2011) (The Jackson Laboratory, 018957) were crossed with homozygote

151 B6.Cg-*Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$*/J mice (Madisen et al., 2010) (The Jackson Laboratory,

152 007914), and the progeny is referred to as CIN-tdTomato mice. For the microdialysis

153 experiments, male Sprague-Dawley albino rats (Charles River Laboratories, Wilmington, MA)

154 were used. Rats were housed individually for the first week after intracranial injection, until

155 suture removal, after which the rats were housed 2 per cage. All animals were housed on a 12 h

156 light/dark cycle (06:30 - 18:30 light) with food and water ad libitum.

157

158 **Surgery and stereotaxic injection of AAV-ChR2 vectors.** Injections were carried out as

159 described previously (Adrover et al., 2014). Briefly, mice (5-6 weeks old) were anesthetized by

160 inhalation of isoflurane-oxygen mixture and were placed in a stereotaxic frame (David Kopf).

161 Adeno-associated virus (AAV) vectors with Cre recombinase dependent expression of

162 Channelrhodopsin2 (ChR2) protein, AAV5-EF1a-DIO-hChR2(H134R)-EYFP (4 x 10^{12} IU/ml),

163 were bilaterally injected into the ventral tegmental area/substantia nigra pars compacta

164 (VTA/SNc; AP: −3.30, ML: ±0.60, DV: −4.50) of DAT$^{IRES-Cre+}$ mice. The AAV vectors

165 AAV5-CaMKII-hChR2(H134R)-EYFP (4 x 10^{12} IU/ml) or AAV5-Syn-ChrimsonR-tdTomato

166 (1.7 x 10^{13} IU/ml), were injected in the PFC (PrL cortex, AP: +2.10, ML: ±0.35, DV: −2.30; IL

167 cortex, AP: +1.90, ML: ±0.30, DV: −3.20) of negative littermates or DAT$^{IRES-Cre+}$ mice. All

168 stereotaxic coordinates were from bregma (in mm) according to the mouse atlas by Franklin and

169 Paxinos (2007). 300-500 nl for VTA/SNc and 100-200 nl for PFC were injected at a flow rate of

170 100 nl/min. Recordings were made after a minimum of 4 weeks of incubation. For mice injected

171 in both VTA/SNc (DIO-ChR2-EYFP) and PFC (ChrimsonR-tdTomato), recordings were

172 performed after a minimum of 8 weeks of incubation. Viral vectors were purchased from Gene

173 Therapy Center Vector Core at University of North Carolina and Penn Vector Core at University

174 of Pennsylvania.

175

176 **Slice preparation.** Mice were anesthetized and rapidly decapitated. Brains were quickly

177 removed, mounted and sliced using a vibratome (VT-1200S Leica) in an ice-cold cutting solution

178 containing (in mM) 225 sucrose, 13.9 NaCl, 26.2 NaHCO$_3$, 1 NaH$_2$PO$_4$, 1.25 glucose, 2.5 KCl,

179 0.1 CaCl$_2$, 4.9 MgCl$_2$, and 3 kynurenic acid. The sagittal slices (240 μm) were incubated for 20

180 min at 33 °C in artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 1 NaH$_2$PO$_4$,

181 2.5 KCl, 1.3 MgCl$_2$, 2.5 CaCl$_2$, 20 glucose, 26.2 NaHCO$_3$, and 0.4 ascorbic acid, and kept in the

182 dark at room temperature before use. Recording chamber was perfused at 2 ml/min with ACSF

183 heated at 32°C using an in-line heater (Harvard Apparatus).

184

185 **Fast-scan cyclic voltammetry (FSCV) and amperometry.** FSCV was performed in the dorsal

186 striatum. Carbon-fiber electrodes (CFEs) were prepared with a cylindrical carbon-fiber (7 μm

10

187   diameter, ~150 μm of exposed fiber) inserted into a glass pipette. Before use, the CFEs were

188   conditioned with 8 ms long triangular voltage ramp (-0.4 to +1.2 and back to -0.4 V versus

189   Ag/AgCl reference at 400 V/s) delivered every 15 ms. CFEs showing current above 1.8 μA or

190   below 1.0 μA in response to the voltage ramp around 0.6 V were discarded. During the

191   recording, the CFEs were held at −0.4 V versus Ag/AgCl and the same triangular voltage ramp

192   was delivered every 100 ms. DA transients were evoked by electrical or optical single pulse, or 5

193   pulses at 20 Hz stimulations. Using the same CFE and location, DA signals were evoked by

194   alternating electrical and optical stimulations in some experiments. These data were combined

195   with results from other experiments where either electrical or optical stimulation was used to

196   evoke DA signals. For electrical stimulation, a glass pipette filled with ACSF was placed near

197   the tip of the carbon fiber and a rectangular pulse (0.2 ms, 100 μA) was applied every 2 min. For

198   optogenetic stimulation, a fiber optic (200 μm diameter, 0.22 NA, ThorLabs) connected to a blue

199   LED (470 nm, 1.8 mW of maximal output power measured at the tip of the fiber optics,

200   ThorLabs) was placed over the carbon fiber and light pulses (0.6 ms) were delivered every 2 min.

201   For input-output curves, the widths of light pulse were 0.1, 0.2, 0.5, 1, 2, and 5 ms. For dual

202   optogenetic recordings, two fiber optics connected to a purple LED (420 nm, 3.0 mW, ThorLabs)

203   and an orange LED (590 nm, 0.7 mW, ThorLabs) respectively, were placed over the carbon fiber.

204   Light pulses (0.6 ms for 420 nm and 0.6-2 ms for 590 nm) were delivered in an alternating

205   pattern every 2 min. Data were collected with a retrofit headstage (CB-7B/EC with 5 MΩ

206   resistor) using a Multiclamp 700B amplifier (Molecular Devices) after low-pass filter at 3 kHz

207   and digitized at 100 kHz using a DA board (NI USB-6229 BNC, National Instruments). Data

208   acquisition and analysis were performed using a custom-written software, VIGOR, in Igor Pro

209   (Wavemetrics) using mafPC (courtesy of M.A. Xu-Friedman). The current peak amplitudes of

210   the evoked DA transients were converted to DA concentration according to the post

211   experimental calibration using 1-3 µM DA. Amperometric recordings were performed using the

212   same carbon-fiber electrodes held at −0.4 V versus Ag/AgCl and a 30 s long step to +0.6 V was

213   applied. Single pulse or 5 pulses at 20 Hz stimulation were delivered at 20 s after switching to

214   +0.6 V. Since we did not find major differences between PrL and IL in evoking DA transients,

215   we combined the data obtained from two groups to represent DA transients evoked by PFC

216   inputs (PFC-oDA).

217

218   **Cell-attached recordings.** Striatal CINs from CIN-TdTomato mice injected with ChR2-EYFP in

219   the PFC were identified by fluorescence and confirmed by their characteristic spontaneous firing

220   pattern. Cell-attached recordings were performed from CINs in the striatum using glass pipette

221   electrodes with a resistance of ∼3–4 MΩ, filled with an internal solution containing (in mM) 120

222   cesium methanesulfonate, 20 CsCl, 10 HEPES, 0.2 EGTA, 10 sodium phosphocreatine, 4 Na2-

223   ATP, and 0.4 Na-GTP (pH, 7.25; 290–310 mOsm), at a holding potential of 0 mV. To estimate

224   the strength of connectivity to CINs, the lowest light intensity needed to reliably evoke action

225   potentials in CINs was determined, ranging from 0.4 mW for 'high' connectivity to 2.1 mW for

226   'low' connectivity. The data were collected using a Multiclamp 700B amplifier after low-pass

227   filter at 1 kHz and digitized at 5 kHz, using pClamp10 software (Molecular Devices). Spike

228   fidelity was calculated as percentages of the number of AP evoked from 5 trials for both single

229   pulse and trains stimulation.

230

231   *In vivo* **microdialysis and optogenetic stimulation of the cortical inputs.** Rats (80-90 g)

232   received unilateral intracranial injection of AAV-CaMKIIa-hChR2(H134R)-EYFP (titer: 10^{12}

233   IU/ml; Gene Therapy Center Vector Core at University of North Carolina) in the PrL and IL

234   cortex (AP: +3.0 mm, ML: 0.5 mm, DV: −3.5 and −5 mm with respect to bregma). Two injection

235   sites per hemisphere were used and 300 nl of viral vector solution was delivered per site via a

236   105 μm thick silica tubing injector coupled directly to a 1 μl syringe driven by an infusion pump

237   (rate = 50 nl/min) during 10 min. The injector was left in place for an additional 10 min

238   following each injection to allow for diffusion of the suspension. Ten weeks after viral vector

239   injection, rats (350-400 g) underwent surgery for probe implantation according to previously

240   published procedures (Quiroz et al., 2016). Briefly, a modified microdialysis probe with an

241   embedded light-guiding optic fiber was implanted into the nucleus accumbens (NAc) shell (AP:

242   +1.2 mm, ML: 0.5 mm, DV: -8.0 mm with respect to bregma). The probe was fixed to the skull

243   with a stainless-steel screw and glass-ionomer dental cement. All surgical procedures were

244   performed under anesthesia with 3 ml/kg of Equithesin (4.44 g of chloralhydrate, 0.972 g of Na

245   pentobarbital, 2.124 g of $MgSO_4$, 44.4 ml of propylene glycol, 12 ml of ethanol and distilled

246   H_2O up to 100 ml of final solution; NIDA Pharmacy, Baltimore, MD). To build the microdialysis

247   probe with embedded optic fiber, the tip of an optic fiber (105 μm diameter core, 0.22 Numerical

248   Aperture) was sculpted into a conical shape using a Flaming-Brown pipette puller fitted with a

249   custom platinum heating filament (Sutter Instruments, Novato, CA) to allow for a larger area of

250   stimulation. The conical optic fiber tip was embedded inside the microdialysis probe and

251   implanted. Microdialysis experiments were performed in freely moving rats 24 h after probe

252   implantation. Optical fiber was coupled to a 473 nm solid-state laser module and light

253   stimulation was driven by a Grass S88 stimulator. Light stimulation was delivered for 20 mins as

254   trains of light pulses (2 ms pulse duration; at 100 Hz for 160 ms; trains repeats once per second;

255   intensity = 5-8 mW at probe tip). ACSF containing (in mM) 144 NaCl, 4.8 KCl, 1.7 $CaCl_2$, and

13

256   1.2 MgCl$_2$, was pumped through the optogenetic-microdialysis probe (rate = 1.25 μl/min). After

257   a washout period of 90 min, dialysate samples were collected at 20 min intervals. After 80 min

258   of baseline sampling, optogenetic stimulation was applied for 20 min and samples were collected

259   for 80 additional minutes after the end of the stimulation. Samples were split and analyzed

260   separately for glutamate, DA and ACh content. ACh and glutamate contents were measured by

261   HPLC coupled to an ACh oxidase and glutamate oxidase enzyme reactors, respectively, and

262   electrochemical detection (Eicom Corporation). DA was measured by HPLC coupled with a

263   coulometric detector (5200a Coulochem III, ESA, Chelmsford, MA). At the end of the

264   microdialysis experiment, animals were deeply anesthetized with Equithesin and perfused

265   transcardially with 0.1 M phosphate buffered saline (PBS), followed by 4% formaldehyde in 0.1

266   M PBS, pH 7.4. Brains were postfixed in the same fixative for 2 h and immersed in 20%

267   sucrose/0.1 M PBS, pH 7.4, solution for 48 h at 4°C. Forty μm thick coronal sections were cut in

268   a Leica (Nussloch, Germany) CM3050S cryostat at −20°C, collected in PBS, and stored in

269   antifreeze-buffered solution (20% ethylene glycol, 10% glycerol, and 10% sucrose in PBS) at

270   −80°C until processing. Sections were then evaluated for localization of implanted probes and

271   ChR2-EYFP expression. Wide field images were acquired with a Typhoon laser scanner (GE

272   Healthcare). Confocal fluorescence microscopy images were acquired with a Zeiss microscope

273   (Examiner Z1, Zeiss, Gottingen, Germany) fitted with a confocal laser module (LSM-710,

274   Zeiss).

275

276   **Drugs.** Dihydro-β-erythroidine hydrobromide (DHβE) was purchased from Tocris. Kynurenic

277   acid   (sodium   salt),   2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide

278   (NBQX) and 3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) were purchased

279   from Abcam. All other chemicals were purchased from Sigma.

280

281   **Statistical analysis.** Statistical analysis was performed with Prism (GraphPad). One-sample t-

282   test, two-tailed paired t-test, repeated measures one-way (RM1W) ANOVA with or without

283   mixed-effects models (MEM), or two-way (RM2W) ANOVA were used as specified. Tukey's or

284   Dunnett's multiple comparisons test was used for post-hoc analysis as specified. The number of

285   experiments, n, was expressed as the number of slices or cells/the number of mice or number of

286   rats for the *in vivo* microdialysis experiments.

287   **Results**

288

289   *In vivo* **stimulation of PrL/IL inputs increases striatal ACh and DA concentration, and the**

290   **increase in DA requires nAChR activation**

291   Published work *in vivo* shows that optogenetic stimulation of cortical inputs to the

292   striatum can evoke DA release (Quiroz et al., 2016). However, this study provides no evidence

293   that activation of these cortical inputs recruits striatal CINs or that the cortically evoked DA

294   release *in vivo* require activation of striatal nAChRs. To assess these matters, we used *in vivo*

295   microdialysis combined with optogenetic stimulation, as previously described (Quiroz et al.,

296   2016), and measured extracellular levels of ACh and DA in response to optogenetic stimulation

297   of cortical inputs. A modified microdialysis probe with an embedded light-guiding optic fiber

298   was implanted into the striatum of rats expressing ChR2-EYFP in the PrL/IL cortex (Figure 1A

299   and B). On the experiment day, dialysate samples were collected at baseline for 80 min before

300   stimulation started. Optogenetic stimulation of cortical axons within the striatum was delivered

301   around the microdialysis probe as trains of light pulses (16 pulses at 100 Hz every 1 s for 20

302   mins). Samples were collected for an additional 80 mins after stimulation and were split and

303   analyzed separately for DA and ACh content or DA and glutamate. This long train of *in vivo*

304   optogenetic stimulation of PrL/IL cortical axon fibers within the striatum produced an increase in

305   extracellular concentration of DA in the striatal dialysate (185.1 ± 44.0% of baseline, n = 7; F =

306   5.27, p = 0.0002, RM1W ANOVA; p = 0.0002 for 60 min vs. 80 min; Dunnett's multiple

307   comparisons test; Figure 1C), which coincided with an increase of extracellular ACh

308   concentration (196.7 ± 36.6% of baseline, n = 7; F = 3.00, p = 0.0002, RM1W ANOVA; p =

309   0.004 for 60 min vs. 80 min; Dunnett's multiple comparisons test; Figure 1D). These *in vivo*

16

310   microdialysis findings support the hypothesis that cortical stimulation recruits CINs. In addition,

311   when the *in vivo* optogenetic stimulation was performed in constant perfusion of the nAChR

312   antagonist DHβE (10 μM) delivered locally via reverse dialysis, the same stimulation protocol

313   did not cause any change in extracellular DA concentration in the striatal dialysates (96.5 ±

314   11.6% of baseline, n = 9; F = 0.64, p = 0.70, RM1W ANOVA; Figure 1E), indicating that

315   activation of nAChRs in the striatum is required for the cortically evoked DA release *in vivo*. As

316   a control, we measured glutamate concentration in these same dialysates which showed

317   increased glutamate levels and confirmed the delivery of cortical stimulation in the presence of

318   the nAChR antagonist (184.3 ± 27.3% of baseline, n = 9; $F_{6,45}$ = 4.01, p = 0.003, RM1W

319   ANOVA with MEM; p = 0.0006 for 60 min vs. 80 min; Dunnett's multiple comparisons test;

320   Figure 1F).

321

322   **Stimulation of PrL/IL inputs to striatum is sufficient to evoke local DA signals in brain**

323   **slices**

324   In order to precisely access the specific inputs and apply pharmacological agents, we

325   used transgenic mice with combination of optogenetic stimulation. DA signals were recorded in

326   the striatum using FSCV in mouse brain slice preparations. Release of DA from axonal

327   projections within the striatum were evoked by electrical stimulation and compared with DA

328   signals evoked by input-specific optogenetic stimulation. To achieve input-specific stimulation,

329   ChR2-EYFP was expressed in either midbrain DANs and their axonal projections to the striatum

330   using Cre-dependent viral vector injection in DAT[IRES-Cre+] mice (Figure 2A), or ChR2-EYFP in

331   cortical neurons of the PrL/IL cortex and their projections to the striatum using viral injection in

332   Cre-negative littermate mice (Figure 2B).

333   As expected, single brief pulses of electrical stimulation delivered via an electrode placed

334   within the striatal tissue reliably evoked DA (electrically evoked DA transient, eDA) signals in

335   striatal brain slices (Figure 2C). Also, direct optogenetic stimulation of midbrain DAN axonal

336   projections within the striatum also evoked DA signals (referred here as DAN-oDA) upon

337   delivery of a single brief pulse of blue light (0.6 ms), in agreement with previous published

338   studies (Adrover et al., 2014; Melchior et al., 2015). Lastly, similar to the *in vivo* findings in

339   Figure 1, selective optogenetic stimulation of axon projections from PrL/IL cortex into the

340   striatum was also sufficient to trigger DA signals (referred as PFC-oDA) in the striatum of in

341   slice preparation (Figure 2C). These PFC-oDA signals were also reliably evoked by brief single

342   pulses of blue light (0.6 ms) in the ventral portion of the dorsomedial striatum in sagittal brain

343   slices (Figure 2D). The viral expression around injection site was often large enough to include

344   both PrL and IL regions of the cortex. For this reason, we combined data from the two regions. It

345   is worth noting that these brain sections mainly contain the axon projections from PrL/IL

346   neurons but not their cell bodies, which are localized to more medial sagittal sections of the brain

347   (Figure 2B, inset).

348   The current-voltage plots of DA signals evoked via the three different types of

349   stimulation (electrical, optogenetic stimulation of DANs and optogenetic stimulation of PrL/IL)

350   were indistinguishable and displayed current peaks at the expected oxidation (0.56 ± 0.01 V for

351   eDA, n = 15 / 8; 0.58 ± 0.01 V for DAN-oDA, n = 14 / 6; 0.56 ± 0.01 V for PFC-oDA, n = 22 /

352   13; $F = 0.49$, $p = 0.62$, RM1W ANOVA with MEM; Figure 2E) and reduction voltages ($-0.17 \pm$

353   0.01 V for eDA, -0.14 ± 0.01 V for DAN-oDA, -0.16 ± 0.02 V for PFC-oDA; $F = 0.76$, $p =$

354   0.49, RM1W ANOVA with MEM; Figure 2E) for DA. The peak amplitude of eDA was

355   significantly higher than DAN-oDA or PFC-oDA (556 ± 42 nM for eDA; 367 ± 58 nM for

356 DAN-oDA; 325 ± 23 nM for PFC-oDA; $F_{2,12}$ = 13.430.76, $p > 0.05$, RM1W ANOVA with

357 MEM; $p = 0.01$ for eDA vs. DAN-oDA, $p = 0.0005$ for eDA vs. PFC-oDA, and $p = 0.99$ for

358 DAN-oDA vs. PFC-oDA; Tukey's multiple comparisons test; Figure 2F), and the decay time

359 constants (tau) were similarly longer with eDA (0.25 ± 0.01 s for eDA, 0.22 ± 0.01 s for DAN-

360 oDA, 0.23 ± 0.01 s for PFC-oDA; $F_{2,12}$ = 13.43, $p = 0.0009$, RM1W ANOVA with MEM; p =

361 0.004 for eDA vs. DAN-oDA, $p = 0.009$ for eDA vs. PFC-oDA, and $p = 0.83$ for DAN-oDA vs.

362 PFC-oDA; Tukey's multiple comparisons test; Figure 2G). Thus, we were able to reliable evoke

363 steady DA signals by selective optogenetic stimulation of axon projections from PrL/IL cortex in

364 the ventral portion of the dorsomedial striatum. The DA signals recorded using FSCV showed

365 indistinguishable chemical and temporal characteristics, even when evoked by different inputs.

366

367 **Unique physical and pharmacological properties across input-specific DA signals**

368   We first explored the threshold for triggering the input-specific optogenetically evoked

369 DA signals. The threshold was determined by constructing input-output curves and varying the

370 light pulse durations from 0.1 to 5 ms in brain slices from mice expressing ChR2 in midbrain

371 DANs or the PrL/IL region (Figure 3A and B). The relative amplitudes of DA transients were

372 overlapping for pulses of 0.5-5 ms duration and both DAN-oDA and PFC-oDA signals showed

373 maximal amplitude with 1-5 ms light pulse durations. However, very short light pulses (0.1 ms)

374 evoked measurable DA signals only in slices in which ChR2 was expressed in midbrain DAN

375 projections to the striatum, indicating a lower threshold for evoking DA signals with direct

376 stimulation of DA neuron axons than with stimulation of cortical inputs (duration × input, $F_{5,90}$ =

377 11.58, $p < 0.0001$, RM2W ANOVA; post hoc for the duration of 0.1 ms: 33 ± 4% for DAN-

378 oDA, n = 12 / 8, and 1.9 ± 0.7% for PFC-oDA, n = 8 / 5, $p < 0.0001$; Figure 3B). A higher

19

379 threshold for the cortically evoked DA signals could reflect technical differences like opsin

380 expression levels, or other biological factors such as the density of innervation of cortical vs

381 DAN axonal projections, difference in the release probability of the DA varicosities that respond

382 to direct stimulation of DAN axons from those responding to cortical inputs, and/or the indirect

383 and polysynaptic nature of the cortical-evoked DA signals.

384   To determine whether PrL/IL activation elicits striatal DA release engage the CINs

385 dependent mechanism as shown in *in vivo* microdialysis experiment (Figure 1), we then studied

386 the basic pharmacological characteristic of DA signal evoked by stimulation of PFC inputs and

387 compared them with those evoked by electrical and DAN inputs stimulation. In striatal brain

388 slices, PFC-oDA signals were completely abolished by a mixture of AMPA and NMDA

389 receptors antagonists (NBQX/CPP), while eDA and DAN-oDA signals were unaffected

390 (amplitude after the antagonists = $0.3 \pm 1.6\%$ of baseline for PFC-oDA, n = 6 / 5; $107.3 \pm 3.0\%$

391 for eDA, n = 6 / 4; $99.8 \pm 1.4\%$ for DAN-oDA, n = 6 / 4; $F_{2,3} = 694.8$, $p < 0.0001$, RM1W

392 ANOVA with MEM; $p = 0.14$ for eDA vs. DAN-oDA, *p's* < 0.0001 for PFC-oDA vs. eDA or

393 PFC-oDA vs. DAN-oDA; Tukey's multiple comparisons test; Figure 3C and D). These findings

394 indicate a requirement for ionotropic glutamate transmission for PrL/IL inputs to evoke local DA

395 signals, which is in agreement with previous reports on the requirement of glutamate

396 transmission for DA signals evoked by motor cortex and thalamic inputs to the striatum

397 (Threlfell et al., 2012; Kosillo et al., 2016; Cover et al., 2019). Further, similar to our *in vivo* data

398 (Figure 1D), DHβE also abolished PFC-oDA signals, and significantly depressed eDA signals,

399 while having no effect on DAN-oDA signals ($1.9 \pm 1.4\%$ of baseline for PFC-oDA, n = 11 / 7;

400 $21 \pm 3\%$ for eDA, n = 10 / 5; $99 \pm 3\%$ for DAN-oDA, n = 5 / 2; $F_{2,7} = 395.2$, $p < 0.0001$, RM1W

401 ANOVA with MEM; $p < 0.0001$ for eDA vs. DAN-oDA, $p = 0.0006$ for eDA vs. PFC-oDA, and

20

402   *p* < 0.0001 for DAN-oDA vs. PFC-oDA; Tukey's multiple comparisons test; Figure 3E and F).

403   Taken together, the pharmacology results support the hypothesis that PFC-oDA signals are

404   mediated by activation of glutamate synapses from PrL/IL cortex to striatal CINs, which excites

405   them to fire and trigger ACh release, thereby driving DA release via activation of nAChRs on

406   DAN axon fibers within the striatum.

407   ACh is rapidly cleared by enzymatic degradation by acetylcholinesterase (Quinn, 1987).

408   The extremely dense presence of acetylcholinesterase in the striatum (Zhou et al., 2001) assures

409   the termination of cholinergic action. We also have previously shown the influence of the

410   acetylcholinesterase on the DA transmission in the striatum (Shin et al., 2015; Shin et al., 2017).

411   As acetylcholinesterase activity shows temperature dependence (Vidal et al., 1987), we then

412   tested the temperature dependence of the input-specific DA transients by varying the temperature

413   of the bath solution. The amplitude of both DAN-oDA and PFC-oDA signals increased linearly

414   as the temperature was lowered from the standard 32°C to 25°C and both showed a tight

415   negative regression with similar slopes significantly different from zero ($-10.8 \pm 0.6\%$ per °C, n

416   = 8 / 3, for DAN-oDA, and $-10.8 \pm 2.0\%$ per °C, n = 6 / 3, for PFC-oDA; *p's* < 0.0001; Figure

417   3G and H). The temperature dependence of the DA signal amplitude likely reflects the change in

418   rate of DA transporter activity with temperature. DA transporter activity was reported to have a

419   temperature coefficient Q_{10} ranging from 1.39 to 2.95 (Zhu and Hexum, 1992), from which we

420   estimate a Q_{10} of around 2.3 for the DA transporter for 24 - 37°C range. Our estimate predicts

421   that large changes in the rate of DA reuptake by the transporter would be around the tested and

422   physiological temperatures. Indeed, the decay of the DA transients, which is a parameter that

423   reflects the clearance of extracellular DA by the transporter, was also affected as the temperature

424   was lowered. The decay time constant of both DAN-oDA and PFC-oDA increased linearly with

21

425   the decrease of the temperature (DAN-oDA: −0.017 ± 0.001 s/°C; PFC-oDA: −0.025 ± 0.001

426   s/°C; Figure 3I), which corresponded with the linear increase in amplitude. However, raising the

427   temperature from 33°C to 35°C caused a dramatic and selective drop in the amplitude of the

428   PFC-oDA signals, compared to DAN-oDA signals (Figure 3G and H). The slope for PFC-oDA

429   signals increased 3-fold from −11% to −31% per °C.

430   Vidal et al. (1987) have reported on a high dependence with temperature of the activity of

431   acetylcholinesterase from rat brain, which reaches maximal rate around 35°C. Our *in vivo* and *in*

432   *vitro* pharmacology experiments showed that PFC-oDA signals require activation of nAChR.

433   Therefore, we speculate that the observed steep temperature dependence of the signals is in large

434   part mediated by rate increase in acetylcholinesterase activity. Furthermore, at physiological

435   temperatures the PFC-evoked DA signals may be very localized near ACh release sites,

436   presumably overlapping with nAChR expression in DAN axons.

437

438   **Activity dependent summation of the midbrain and cortical DA signals**

439   Trains of stimulation pulses has been shown to produce sublinear summation in the

440   amplitude of DA signals evoked by electrical and optogenetic stimulation of DAN axon

441   projections in the striatum *in vitro* (Zhang and Sulzer, 2004; Threlfell et al., 2010; Melchior et

442   al., 2015; Shin et al., 2017). We then tested the degree of summation of the DA signals in

443   response to short trains of stimulation pulses (5 pulses at 20 Hz) and compared it with the

444   transients evoked by a single pulse (Figure 4A and B). This pattern of train stimulation was

445   chosen based on burst firing patterns recorded *in vivo* for DANs during behavior (Schultz et al.,

446   1993; Hyland et al., 2002). Indeed, we found that trains of 5 pulses at 20 Hz evoke DA transients

447   50% larger than those evoked by single pulse stimulation for eDA signals and almost double for

448   DAN-oDA (5p/1p: 1.44 ± 0.06, n = 5 / 3, for eDA; 1.92 ± 0.19, n = 5 / 2, for DAN-oDA; 1.00 ±

449   0.02, n = 10 / 7; $F_{2,2}$ = 110.6, p = 0.009, RM1W ANOVA with MEM; p = 0.04 for eDA vs.

450   DAN-oDA, p = 0.03 for eDA vs. PFC-oDA, and p = 0.008 for DAN-oDA vs. PFC-oDA;

451   Tukey's multiple comparisons test; Figure 4A and B). On the contrary, PFC-oDA transients

452   evoked by this train stimulation had similar amplitudes compared to those evoked by a single

453   pulse, indicating no summation of DA signals evoked by PrL/IL inputs. In order to measure DA

454   signals with better temporal resolution, we performed amperometric recordings and again

455   compared DA signals evoked by single pulse and trains (Figure 4C). In line with the FSCV

456   results, amperometric recordings showed that DAN-oDA signals display sublinear summation

457   whereas PFC-oDA signals are indistinguishable between single pulse or trains of 5 pulses at 20

458   Hz. The lack of summation in the PFC-oDA signals in response to trains resembles the findings

459   obtained with DA signals evoked by synchronized activation of CINs (Threlfell et al., 2012; Shin

460   et al., 2017) and further supports the idea that the PFC evoked local DA signals are mediated by

461   synchronized action of CINs in response to stimulation of PrL/IL inputs. Note that the large

462   transient current deflection in response to each pulse of stimulation (n = 5 / 4; Figure 4C, right) is

463   suggestive of the action potential firing evoked in CINs and other striatal neurons by the

464   optogenetic stimulation of PrL/IL inputs. The fact that every pulse of the train stimulation

465   triggers a current deflection reflects the ability of PrL/IL inputs to evoke action potentials firing

466   in downstream neurons and supports the idea that the lack of summation in DA signals is not

467   likely due to the failure of action potential firing by CINs. To directly test the contribution of

468   failure of action potential firing by CINs to the lack of summation in PFC-oDA signals, we

469   carried out cell-attached recordings from CINs and measured action potential fidelity in response

470   to a single pulse and trains of 5 pulses at 20 Hz (single pulse: 96.5 ± 2.7%, n = 23 / 4, t = 1.28, p

471   = 0.21; trains of 5: 81.5 ± 7.7%, n = 10 / 4, *t* = 2.42, *p* = 0.04; both with one sample t-test to

472   100% value; Figure 4D and E). Although there was a small but significant percentage of failure,

473   the totality of the data indicate that the lack of summation is not likely caused by action potential

474   failure, but instead can be attributed to other downstream mechanisms such as desensitization of

475   nAChRs (Threlfell et al., 2012; Shin et al., 2017).

476

477   **Input-specific DA signals are evoked by dual optogenetic stimulation in the same brain**

478   **slice**

479   Thus far, DA signals evoked by the different inputs were recorded in brain slices from

480   different mice that expressed ChR2 in either midbrain DANs or in PrL/IL cortex. In order to

481   strictly test the segregation/distinction of these two pathways, it was important to examine these

482   two inputs and compare the input-specific DA signals in the slice preparation from the same

483   mice. We took advantage of opsins activated by different light wavelengths, specifically, ChR2

484   and the red-shifted opsin ChrimsonR (Klapoetke et al., 2014). We first set up the conditions for

485   selective stimulation of each opsin without cross-activation. In brain slices expressing only ChR2

486   in midbrain DANs, pulses of purple light (420 nm) evoked reliable DA signal while similar and

487   longer pulses of orange light (590 nm) did not trigger any detectable signals (Figure 5A).

488   Conversely, in slices expressing only ChrimsonR in the PrL/IL cortex, brief orange light pulses

489   evoked DA signal, but not purple light (Figure 5B). Thus, under these experimental conditions,

490   ChR2 and ChrimsonR can be used in combination to selectively stimulate two different inputs by

491   delivering pulses of two different light wavelengths without any detectable cross- activation.

492   Next, in the same DAT$^{IRES-Cre+}$ mice, ChR2-YFP was expressed in midbrain DANs with a

493   Cre-dependent expression vector and ChrimsonR-TdTomato was expressed in PrL/IL cortex.

24

494   Figure 5C shows examples of the fluorescent expression pattern of ChR2-YFP (green) and

495   ChrimsonR (red) in the same sagittal brain slice from this double-injected mouse. Figure 5D

496   shows the experimental arrangement for the dual input recordings with the placement of the

497   carbon fiber and the two fiber-optics for delivering the purple and orange light pulses that were

498   used to activate ChR2 and ChrimsonR, respectively. In these slices with dual opsin expression,

499   brief pulses of either purple or orange light evoked DA signals with indistinguishable current-

500   voltage plots showing the characteristic peaks for DA oxidation and reduction (Figure 5E). The

501   DA concentration transients had comparable peak amplitudes with means of 407 ± 32 nM for

502   DAN-oDA and 420 ± 36 nM for PFC-oDA (n = 43 / 13, $t = 0.47$, $df = 42$, $p = 0.64$, paired t-test;

503   Figure 5F) and overlapping time courses as measured at 10 Hz sample rate of FSCV, in

504   agreement with the results shown in Figure 2. The decay time constant of the DA transients were

505   similar (0.28 ± 0.01 s for DAN-oDA and 0.28 ± 0.01 s for PFC-oDA, n = 43 / 13, $t = 0.41$, $df =$

506   41, $p = 0.68$ from paired t-test; Figure 5F).

507   Then, we tested the pharmacological properties of DAN-oDA and PFC-oDA by

508   delivering alternating purple and orange light pulses and recording from a single carbon fiber.

509   Application of the glutamate receptor antagonists NBQX/CPP ($99.2 \pm 3.1\%$ of the baseline for

510   DAN-oDA and $1.6 \pm 1.1\%$ for PFC-oDA, n = 5 / 3; $t = 25.3$, $df = 4$, $p < 0.0001$, two-tailed paired

511   t-test; Figure 5G) or the nAChR antagonist DHβE ($101.9 \pm 0.6\%$ of the baseline for DAN-oDA

512   and $0.8 \pm 0.8\%$ for PFC-oDA, n = 5 / 3; $t = 79.13$, $df = 4$, $p < 0.0001$, two-tailed paired t-test;

513   Figure 5H) completely abolished PFC-oDA signals while leaving DAN-oDA signals in the same

514   location intact. These results confirm the findings from Figure 3, which use antagonists and

515   single input optogenetic stimulation to evoke either DAN-oDA or PFC-oDA in separate

516   slices/mice. These results also validate the experimental approach by showing input specificity

517   with no apparent crossover in the optogenetic stimulation of ChR2 and ChrimsonR with the

518   described wavelengths. More importantly, these pharmacological findings highlight the different

519   mechanisms and the unique nature of the input-specific DA signals evoked by PFC and midbrain

520   inputs.

521

522   **Cortically evoked signals are spatially restricted**

523   As shown in Figure 2D, DA signals evoked by selective stimulation of PrL/IL inputs

524   were preferentially detected between the nucleus accumbens core and dorsal striatum. We then

525   set out to investigate in more detail the spatial distribution of the input-specific DA signals using

526   the dual-opsin expression approach by sampling the amplitude of DA signals across 18 different

527   striatal areas of the sagittal brain slice while evoking responses with purple and orange light.

528   While DAN-oDA signals were detected throughout the whole striatum, PFC-oDA signals were

529   spatially restricted (Figure 6A and B). Both DAN-oDA and PFC-oDA signals showed significant

530   correlations with the fluorescent intensity of the projections ($p = 0.03$ and $r^2 = 0.28$ for DAN-

531   oDA; $p = 0.01$, $r^2 = 0.37$ for PFC-oDA; Figure 6B). However, the slope of the linear regression

532   was larger for DAN-oDA signals than PFC-oDA signals (10 vs 6 nM/AU, respectively; Figure

533   6B). Thus, the intensity of the DAN labeled fibers was a good predictor of the magnitude of

534   DAN-oDA signals. On the other hand, the presence of cortical axon fluorescence was not always

535   a predictor of PFC-oDA signals, likely due to the larger density of passing cortical axons that are

536   not synaptic terminal run through the striatum.

537   Averaging the area responses in 5 brain slices from 3 mice, we found that the mean

538   amplitude of the PFC-oDA signals was largest in the ventral part of the dorsal striatum, which

539   again maps in gross terms with the location of the brightest intensity of ChR2-EYFP labeled

26

540   projections from PrL/IL cortex (Figure 6C and D). However, the correlation between

541   fluorescence intensity and amplitude of PFC-oDA signals is not always strong and, for example,

542   the more intensely labeled caudal areas near the globus pallidus show almost no PFC-oDA signal

543   (Figure 6C and D). The evidence of small or no PFC-oDA signals in the more caudal striatum

544   could possibly reflect greater fiber density rather than innervation of the caudal portion of the

545   striatum by the axonal projections from PrL/IL cortex.

546   The number of CINs is less than 1% of the total striatal neurons and they are sparsely

547   distributed throughout the striatum (Burke et al., 2017). We then quantified the density of CINs

548   from the same area to further determine whether the distribution of CINs contribute to the spatial

549   restriction of PFC-oDA signals. For this purpose, we took advantage of CIN-tdTomato mouse

550   line which fluorescently labels CINs throughout the striatal slices (Figure 6E). The quantification

551   analysis of cell numbers in 400×400 μm^2 showed that the density of CINs was also highest in

552   the middle part of the dorsomedial striatum (6 slices from 3 mice), suggesting that an uneven

553   distribution of CINs could also contribute to the spatial restriction of the local DA signals evoked

554   by PrL/IL cortical inputs to the ventral portion of the dorsomedial striatum. To further test

555   whether the spatial profile of PFC-oDA signals is in part determined by the strength of the

556   innervation of CINs by PrL/IL cortical neurons, we performed cell-attached recordings from the

557   same CINs of CIN-TdTomato mice and measured the ability of PFC optogenetic stimulation to

558   evoke action potentials in CINs throughout the striatum (46 CINs from 3 mice). We found that

559   the connectivity followed a similar pattern to the PFC-oDA profile, and the probability that CINs

560   will fire an action potential in response to PFC stimulation was higher in the central part of the

561   striatum than in the nucleus accumbens and dorsal regions of the dorsal striatum (Figure 6F).

562   In conclusion, this study offers a series of *in vitro* and *in vivo* evidence that stimulation of

563   cortical inputs from the PrL/IL cortex can evoke DA release in the striatum via a local

564   mechanism that recruits CINs and requires activation of nAChRs. Since these cortically evoked

565   DA signals are spatially restricted and have different properties, we speculate that they are

566   engaged in distinctive striatal functions from those assigned for DA signals evoked by midbrain

567   DANs.

568

569

Discussion

This study provides strong evidence that DA signals in the striatum can occur both *in vivo* and *in vitro* in response to stimulation of PrL/IL cortical inputs. These PrL/IL evoked DA signals have distinctive pharmacological and physiological properties, compared to the DA signals evoked by DAN inputs. The *in vitro* experiments also introduce a dual optogenetic stimulation approach with which we can activate two different inputs to the striatum in the same brain slice, without apparent cross-activation. This study further reveals the spatial localization of the DA signals evoked by PFC inputs.

We showed that the PrL/IL evoked DA signals require activation of glutamate and nicotinic ACh receptors, in line of a series of elegant published work. Using *in vitro* radioactive assays and *in vivo* microdialysis, it was first shown that locally administered ACh and glutamate can trigger DA release in the striatum (Giorguieff et al., 1976; Giorguieff et al., 1977). Giorguieff and coauthors (1976) showed that an nAChR antagonist blocked ACh-evoked DA signals, and speculated that "…the release of DA from dopaminergic terminals can be regulated by cholinergic presynaptic receptors exhibiting nicotinic characteristics." To their credit, their conclusions agree with our interpretations of the findings from the current study as well as other recent optogenetic studies where it was shown that selective stimulation of CINs is sufficient to evoke DA signals (Cachope et al., 2012; Threlfell et al., 2012; Wang et al., 2014; Shin et al., 2017). The involvement of CINs in the glutamate-dependent DA signals was also suggested by early work by Taber and Fibiger (1994) who showed that electrical stimulation of PFC can increase levels of ACh in the striatum and further supported by more recent optogenetic studies (Threlfell et al., 2012; Kosillo et al., 2016; Johnson et al., 2017; Mateo et al., 2017; Cover et al., 2019).

29

593   Here, our experiments confirmed the requirement for ionotropic glutamate receptor

594   activation in the PFC-evoked DA signals as previously shown (Mateo et al., 2017). We also

595   show that nAChRs are required for the PrL/IL evoked DA signals *in vitro,* similar to other

596   findings when stimulating inputs from other cortical (Kosillo et al., 2016) and thalamic areas

597   (Threlfell et al., 2012; Kosillo et al., 2016; Cover et al., 2019) and indirectly *in vivo* (Mateo et al.,

598   2017). Our results from the *in vivo* microdialysis experiments reproduce the original findings

599   from Quiroz et al. (2016) showing that optogenetic stimulation of PFC inputs increases striatal

600   levels of DA *in vivo* (and glutamate, as expected, Figure 1). More importantly, we showed that

601   stimulation of PrL/IL inputs to the striatum also elevates striatal levels of ACh *in vivo* and that

602   this cortically evoked DA signals are blocked when a nAChR antagonist is perfused (Figure 1C

603   and D). Thus, these findings support that idea PrL/IL inputs form excitatory synapses on striatal

604   CINs and can activate then to evoke ACh release (Figure 1C). In agreement with this conclusion,

605   recent work showed that optogenetic stimulation of either M1 motor cortex or parafascicular

606   nucleus of the thalamus can induce release of ACh that was detected using exogenous G-protein

607   coupled inward rectifying K-current expressed in medium spiny neurons (Mamaligas et al.,

608   2019). Taken together, our findings from both *in vitro* and *in vivo* strongly support the

609   hypothesis of a local mechanism for evoking DA release in the striatum, in addition to the more

610   conventional mechanism based on midbrain DAN firing. This local mechanism engages striatal

611   CINs, the targets of inputs from PFC, which have an essential role in evoking DA release from

612   DAN fibers.

613   DA signals evoked by PrL/IL inputs and those evoked by DAN fibers stimulation share

614   common properties such as overlapping electrochemical profiles of the voltammetric currents

615   and similar concentration range, time course and decay time constant of the signals (Figure 2,

616 Figure 5E and F). However, there are also several differences between the input-specific DA

617 signals. First, DANs evoked DA signals do not require activation of either ionotropic glutamate

618 receptors or nAChRs (Figure 3C-F, Figure 5G and H), confirming the direct nature of this

619 mechanism that is triggered by ChR2 evoked action potentials in DAN axon fibers. Second,

620 PrL/IL evoked DA signals display different temperature sensitivity and a higher threshold

621 compared to DAN-oDA requiring a longer duration of light stimulation as determined in the

622 input-output relationship (Figure 3 and 4). This finding may reflect the poly-synaptic nature of

623 the cortically evoked signals and the requirement of synchronized activation of CINs in order to

624 trigger DA release by this local mechanism (Threlfell et al., 2012; Kosillo et al., 2016; Liu et al.,

625 2018). Third, the amplitude of DANs evoked DA signals increases as the number of stimulation

626 pulses increases, indicating some summation in the DA signals evoked by a train of stimulation

627 pulses (Figure 4A and B). In contrast, the amplitude of DA signals in response to a train of

628 pulses of cortical input stimulation is similar to the amplitudes obtained in response to a single

629 pulse of stimulation, indicating no summation under this condition for the local mechanism

630 (Figure 4A and B). ChR2 displays use-dependent inactivation when stimulated at high frequency

631 (Hass and Glickfeld, 2016). However, the lack of summation by the train stimulation is unlikely

632 due to this use-dependent inactivation of ChR2 since 20 Hz train stimulation was still able to

633 evoke action potentials in CINs recorded in cell attached mode (Figure 4D and E) and

634 amperometry recording showed large deflections in response to train stimulation (Figure 4C).

635 Anecdotally, these current deflections disappeared when the ionotropic glutamate receptor

636 antagonists were applied, also supporting the idea that firing in CINs and other downstream

637 neurons occurs in response to 20 Hz train stimulation. The lack of summation was also reported

638 in DA signals evoked by direct optogenetic stimulation of CINs in the dorsal striatum (Threlfell

31

639 et al., 2012; Shin et al., 2017), which was suggested to be due to nAChRs desensitization. We

640 speculate then that the lack of summation we report here by 20 Hz trains in PrL/IL evoked DA

641 signals, which also require nAChRs activation, is also due to desensitization.

642   To directly study and compare the input-specific DA signals in the same animal, we

643 established dual optogenetic stimulation using a red-shifted opsin, ChrimsonR, in combination

644 with the blue-light activated ChR2. Two wavelengths for excitation were chosen based on the

645 excitation spectrum of each opsin (Klapoetke et al., 2014), 590 nm for ChrimsonR and 420 nm

646 for ChR2, which showed no cross-activation (Figure 5A and B). This dual optogenetic approach

647 was further validated using pharmacology in animals expressing ChR2 in midbrain DANs and

648 ChrimsonR in PrL/IL neurons. We found that PrL/IL evoked DA signals were completely

649 blocked by antagonists of either ionotropic glutamate receptors or nAChRs, without affecting

650 DAN-oDA signals in the same slice (Figure 5G and H). Therefore, we determined that the two

651 inputs can be activated independently using this dual optogenetic approach in brain slices from

652 the same animal.

653   Interestingly, using this dual optogenetic approach, an intriguing spatial pattern was

654 revealed for the cortically evoked PrL/IL DA signals. While DAN-oDA signals were recorded

655 throughout the striatum, PrL/IL oDA signals were spatially restricted to a "hot-spot" region

656 around the boundary between the nucleus accumbens core and dorsal medial striatum (Figure 6).

657 This hot-spot area for the PrL/IL oDA signals corresponds to the same area where we found the

658 highest density of CINs in our analysis. Thus, CIN density could contribute in part to the

659 generation of this hot-spot area for the PrL/IL evoked DA signals. Further, we also found that

660 this area has the highest intensity of fluorescently labeled PrL/IL fibers, as well as the highest

661 degree of synaptic connections to CINs, which is also in agreement with reported patterns of

32

662   cortical connectivity (Voorn et al., 2004). Our initial interest was to study the PFC projections to

663   the accumbens. However, since PrL/IL evoked DA signals were largest and more reliable in the

664   ventral DS (Figure 6), recordings were performed in this area. Altogether, these findings suggest

665   that PrL/IL inputs and the synaptic innervation to CINs are preferentially localized to the

666   dorsomedial subregion, giving rise to the largest cortically evoked DA signals.

667   From the mechanism described here for this PrL/IL evoked DA signals, we propose that

668   any input to the striatum that strongly activates CINs (cortical, thalamic, or any other brain

669   region) can in theory evoke local DA signals in the striatum. We also speculate, based on ours

670   and other groups' works, that these glutamate-driven DA signals can similarly happen in other

671   striatal subregions. The selective localization of PrL/IL evoked DA signals suggests the

672   existence of a "topographic map" for the local DA signals where inputs from different cortical

673   subregions can trigger local DA signals at the striatal subregion which they innervate. Spatial

674   segregation of input-specific DA signals in the striatum is a new concept introduced here that is

675   rarely considered in the field. This concept may become helpful in understanding how DA

676   signals in the striatum can be involved in so many diverse processes ranging from reinforcement

677   learning and motivation to motor output and action selection.

678   (1499/1500 words)

679

680   **Figure legends**

681

682   **Figure 1. Effect of the nAChR antagonist DHβE on the extracellular DA level evoked by**

683   **local optogenetic stimulation of PFC fibers *in vivo*.**

684   **A.** A diagram showing the injection of ChR2-EYFP in the PrL and IL cortices (green circle) and

685   projection to the striatum with the microdialysis-optogenetics probe in a sagittal brain view. **B.**

686   Left, optogenetic-microdialysis probe schematics; a. liquid inlet, b. dialysis membrane, c. liquid

687   outlet, d. sculpted optic fiber. Middle, probe tip detail. Scale bar: 0.2 mm. Right, a picture of the

688   optogenetic-microdialysis probe showing light spread pattern at the probe tip. Scale bar: 1 mm.

689   **C-D.** Time course of extracellular concentrations of **(C)** DA (red) and **(D)** ACh (black) in the

690   NAc. **E-F.** Time course of extracellular concentrations of **(E)** DA (red) and **(F)** glutamate (Glu,

691   green) in the NAc with constant perfusion (reverse dialysis) of the nAChR antagonist DHβE (10

692   µM). Time 0-60 represents the values of samples before stimulation; the period of optogenetic

693   stimulation (20 min) is represented as a train of blue vertical lines. Results are expressed as mean

694   ± SEM of percentage of the average of three values before stimulation. * $p < 0.05$, ** $p < 0.01$,

695   *** $p < 0.001$.

696

697   **Figure 2. Striatal DA signals evoked by midbrain and cortical inputs.**

698   **A.** Example of the fluorescence pattern (bottom) observed in a sagittal brain slice from a

699   DAT$^{IRES-Cre+}$ mice injected with DIO-ChR2-EYFP in the midbrain (top). **B.** Example of the

700   fluorescence pattern (bottom) observed in a sagittal brain slice from a C57Bl6/J (DAT$^{IRES-Cre-}$)

701   mice injected with ChR2-EYFP in the PrL or IL cortex (top). The inset shows a more medial

702   slice with the site of injection. **C.** Representative DA transients, current-voltage (CV) plots and

703   color voltammograms when evoked by electrical stimulation (eDA, left), optogenetic stimulation

704   of DAN fibers (DAN-oDA, middle), or optogenetic stimulation of PFC inputs (PFC-oDA, right).

705   All three CV plots show the electrochemical profile of DA oxidation. Scalebars: 100 nM. **D.** A

706   sagittal brain section modified from The Mouse Brain Atlas (Franklin and Paxinos, 2007)

707   showing the FSCV recording sites from mice injected with ChR2-EYFP in the PrL (filled) and

708   IL (empty) cortex. **E.** The oxidation (top) and reduction (bottom) voltages from the CV plots of

709   eDA (left), DAN-oDA (middle), and PFC-oDA (right) were plotted as averages with SEMs. The

710   open and filled circles represent individual values. **F-G. (F)** DA peak concentrations and **(G)**

711   decay time constants of eDA (left), DAN-oDA (middle), and PFC-oDA (right) were plotted as

712   averages with SEMs. The open circles represent individual values. For PFC-oDAs, the same

713   color code was applied as in (D) for PrL and IL according to injection location. Data points in E-

714   G include experiments where electrical and optogenetic stimulation were delivered alternatingly

715   in the same slice or in different slices (see Materials and Methods). ** $p < 0.01$, *** $p < 0.001$.

716   LV: lateral ventricle, DS: dorsal striatum, ac: anterior commissure, AcC: accumbens core, AcSh:

717   accumbens shell, VP: ventral pallidum, PBP: parabrachial pigmented nucleus, SNR: sbstantia

718   nigra reticulata, Th: thalamus, Tu: olfactory tubercle.

719

720   **Figure 3. DAN-oDA and PFC-oDA show different physical and pharmacological**

721   **properties.**

722   **A.** Representative DAN-oDA (left) and PFC-oDA (right) transients evoked with different light

723   pulse duration (in ms). Scalebars: 100 nM, 0.5 s. **B.** oDA amplitudes normalized to their

724   maximum response were averaged and plotted as a function of the stimulus duration. **C.**

725   Representative traces of eDA, DAN-oDA, and PFC-oDA transients before and after bath

726 application of the glutamate receptor antagonists, NBQX and CPP (both 5 µM). The dotted line

727 (upper) represents the amplitude of DA transients before the drugs. Scalebars: 200 nM, 2 s. **D.**

728 Averages with SEM of DA amplitude after NBQX and CPP were plotted. The open circles

729 represent individual value. **E.** Representative traces of eDA, DAN-oDA, and PFC-oDA

730 transients before and after bath application of the β2-contatining nAChR antagonist, DHβE (1

731 µM). Scalebars: 200 nM, 2 s. **F.** Averages with SEM of DA amplitude after DHβE were plotted.

732 The open circles represent individual value. *** vs. DAN-oDA, ### vs. eDA. **G.** Representative

733 traces of DAN-oDA (left) and PFC-oDA (right) transients at 25, 32, and 35°C. The dotted line

734 (upper) represents the oDA amplitude at 32°C. Scalebars: 200 nM, 2 s. **H.** Average oDA peak

735 amplitudes normalized to 32°C and **I.** average oDA decay time constants were plotted as a

736 function of temperature. * $p < 0.05$, *** and ### $p < 0.001$.

737

738 **Figure 4. PFC-oDA shows no summation by train stimulations.**

739 **A.** Representative DA traces of eDA, DAN-oDA, and PFC-oDA transients evoked by single

740 pulse (1p, thin traces) or train of 5 pulses at 20 Hz (5p, thick traces). Scale bars: 200 nM, 2 s. **B.**

741 Averages with SEM of the DA amplitude ratio (5p/1p) for eDA, DAN-oDA, and PFC-oDA

742 transients were plotted. The open circles represent individual values. **C.** Representative

743 amperometric traces for DAN-oDA (left) and PFC-oDA (right) transients evoked by single pulse

744 (grey traces) or train of 5 pulses at 20 Hz (color traces). PFC-oDA amperometric transients

745 evoked by 50 at 20 Hz were indistinguishable from 1p stimulation, except the large deflection at

746 the stimulation time for 5p pulses. Scale bars: 200 pA, 100 ms. **D.** Representative cell-attached

747 recordings from CINs with single (top, grey) or train of 5 pulses at 20 Hz (bottom, green). Scale

36

748   bars: 20 pA, 100 ms. **E.** Averages with SEM of the action potential fidelity were plotted for the

749   single pulse and train stimulation. The open circles represent individual values. **** $p < 0.0001$.

750

751   **Figure 5. Dual opsin expression to evoke cortical and midbrain DA signals in the same**

752   **brain slices.**

753   **A, B.** Left, Representative traces of DA signals evoked by either 420 nm or 590 nm light pulses

754   from mice expressing **(A)** only ChR2 in midbrain DANs or **(B)** only ChrimsonR in PrL/IL

755   cortex. Right, oDA amplitudes were plotted as pairs for each wavelength. **C.** Example of the

756   fluorescence patterns with filter set for yellow signal (left) or red signal (right) from a sagittal

757   brain slice of a DAT$^{IRES-Cre+}$ mice injected with ChrimsonR-TdTomato in the PFC and DIO-

758   ChR2-EYFP in the midbrain. **D.** Configuration to the FSCV DA recording using a carbon fiber

759   and two fiber optics delivering 420 nm and 590 nm, respectively. **E.** Representative DA

760   transients, CV plots and color voltammograms of DAN-oDA and PFC-oDA. **F.** Left, Amplitudes

761   were plotted as pairs for DAN-oDA and PFC-oDA recorded from same slices. Right, Averages

762   with SEM of decay time constant were plotted for DAN-oDA and PFC-oDA. The dots represent

763   individual values. **G.** Left, Representative traces of DAN-oDA (top) and PFC-oDA (bottom)

764   before and after the application of glutamate receptor antagonists NBQX and CPP. Right,

765   Averages with SEM of DAN-oDA and PFC-oDA were plotted as a function of time as

766   NBQX/CPP was applied. **H.** Left, Representative traces of DAN-oDA (top) and PFC-oDA

767   (bottom) before and after the application of nAChR antagonists DHβE. Right, Averages with

768   SEM of DAN-oDA and PFC-oDA were plotted as a function of time as DHβE was applied.

769

770   **Figure 6. Cortically evoked DA signals are spatially restricted.**

771   **A.** The locations of carbon fiber and two fiber optics were adjusted to measure DAN-oDA (left)

772   and PFC-oDA (right) from each location where the corresponding oDA's were superimposed on

773   the fluorescence patterns. Scale bars: 500 µm. **B.** Peak amplitudes of DAN-oDA (top) and PFC-

774   oDA (bottom) were plotted as a function of florescence intensity at each location shown in **(A)**.

775   The dotted lines represent linear regression between the oDA amplitudes and the fluorescence

776   intensities. **C.** PFC-oDAs were measured from 74 locations (around between 1.0 and 1.2 mm in

777   ML coordinate) of mice injected with ChR2-EYFP in PrL/IL cortex and color-coded according

778   to their peak DA concentrations. The average PFC-oDA amplitudes were calculated and color-

779   coded for the three sub-regions. **D.** The PFC inputs fluorescence intensities were color-coded for

780   the same 74 locations in **(C)**. The averages from the three sub-regions were calculated and color-

781   coded. **E**. Left, Fluorescence image of striatal CINs labeled with td-Tomato. (Inset) Examples of

782   identified CINs (red) from the area with the dotted yellow line. Right, the average numbers of

783   CINs per 400 x 400 μm^2 (area as shown in the inset) were calculated and shown for the three sub

784   regions. **F.** Using cell-attached patch recording, action potentials evoked by PFC stimulation

785   were observed from a total of 46 CINs. For each CIN, a minimum light intensity to evoke action

786   potentials was determined to score the connectivity. The white circles with a thicker line

787   represent CINs which did not show any evoked action potentials even with the maximum light

788   intensity.

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935

A dialysate samples

LASER

PrL/IL
cortex

B

C

250
200
150
100
50
0

% of baseline

*

DA

0   40   80   120
Time (min)

D

250
200
150
100
50
0

% of baseline

***

ACh

0   40   80   120
Time (min)

E

250
200
150
100
50
0

% of baseline

DA   + DHβE

0   40   80   120
Time (min)

F

250
200
150
100
50
0

% of baseline

**

Glu   + DHβE

0   40   80   120
Time (min)

Figure 1 of Adrover et al.
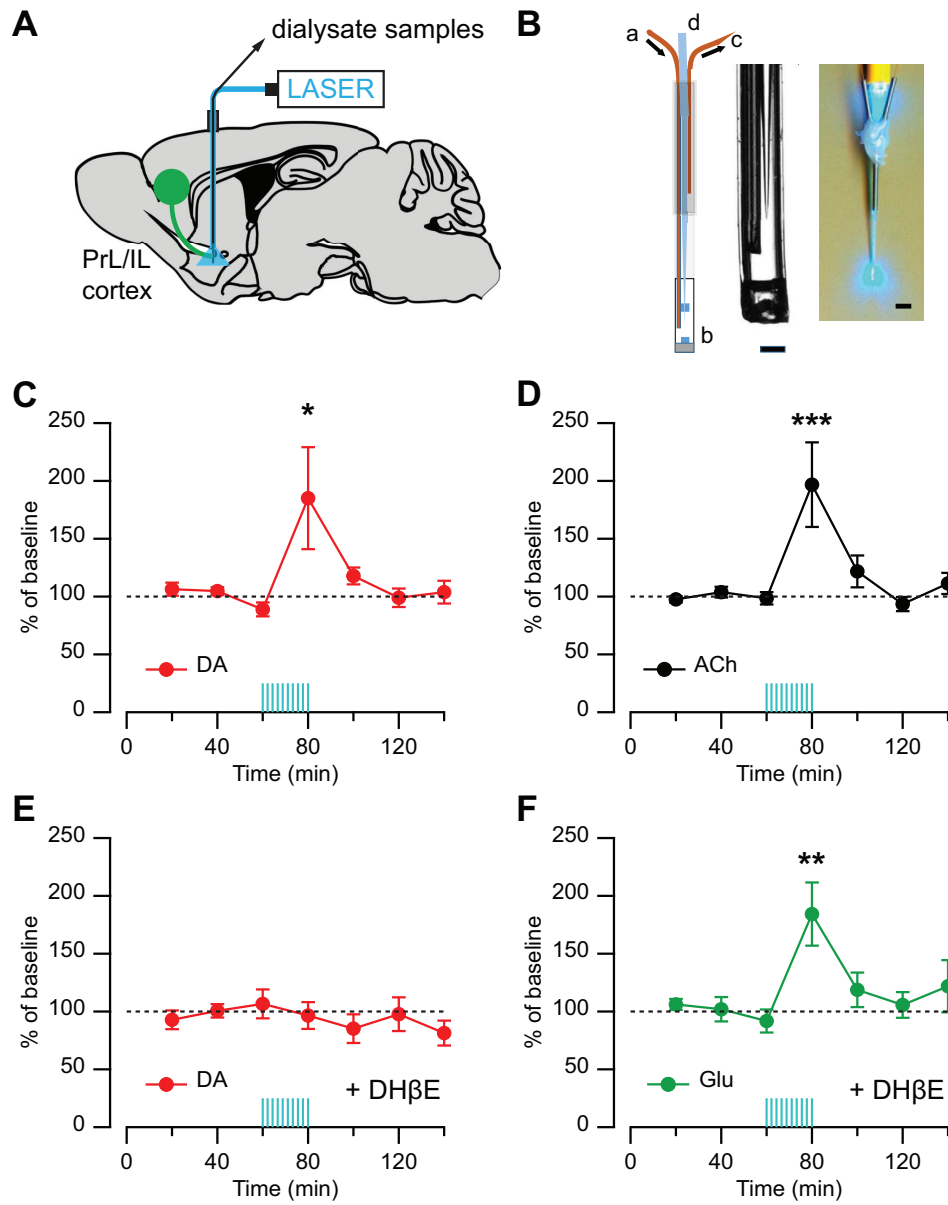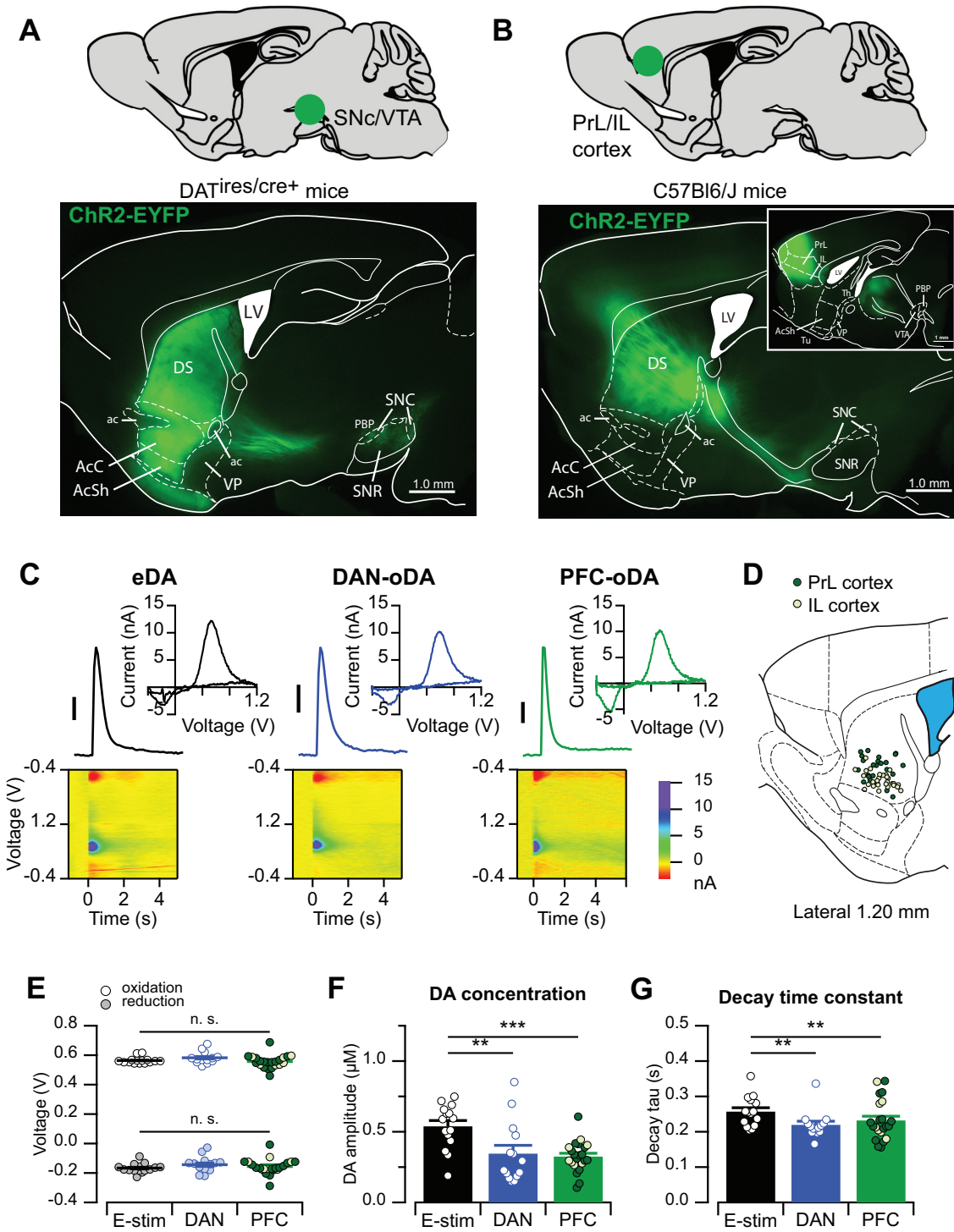
Figure 2 of Adrover et al.

Figure 3 of Adrover et al.

A **Single vs. train**

— 1 pulse
— 5 pulses @20 Hz

eDA   DAN-oDA   PFC-oDA

B

oDA ratio (5p/1p)

****

E-stim   DAN   PFC

C **Amperometry**

DAN-oDA   PFC-oDA

D **Cell-attached from CIN**

1 pulse

5 pulses @20 Hz

E

AP fidelity (%)

1p   5p

Figure 4 of Adrover et al.

A Only ChR2 in midbrain DAN

420 nm   590 nm

oDA amplitude (µM)

420 nm   590 nm

B Only ChrimsonR in PrL/IL cortex

420 nm   590 nm

oDA amplitude (µM)

420 nm   590 nm

ChR2-EYFP in midbrain DAN + ChrimsonR-TdTomato in PrL/IL cortex

C

LV
DS
ac
AcC
AcSh   VP
ac
SNC
SNR
1.0 mm

LV
DS
ac
AcC
AcSh   VP
ac
SNC
SNR
1 mm

IR image of brain slice

D

420 nm
Carbon
Fiber
590 nm
ac
200 µm

E   DAN-oDA

Current (nA)
-5   1.2
Voltage (V)

-0.4
1.2
0   2   4
Time (s)
Voltage (V)
15 nA
10
5
0

PFC-oDA

Current (nA)
-5   1.2
Voltage (V)

-0.4
1.2
0   2   4
Time (s)
15 nA
10
5
0

F   oDA peak (nM)

DAN   PFC

oDA tau (sec)

DAN   PFC

G   ACSF   NBQX/CPP

NBQX/CPP
oDA amplitude (%bsln)
DAN
PFC
0   10   20   30
Time (min)

H   ACSF   DHβE

DHβE
oDA amplitude (%bsln)
DAN
PFC
0   10   20   30
Time (min)

Figure 5 of Adrover et al.

Sagital slice with dual opsins

A ChR2 in DAN fibers   ChrimsonR in PFC fibers

DAN-oDA   PFC-oDA

B DAN-oDA

PFC-oDA

Peak Amp (µM)

Fluo. intensity (a.u.)

C   PFC-oDA amplitude

1200
800
400
0
DA transient (nM)

152 nM
200 nM
8 nM

D   PFC input density

1.0
0.5
0.0
Fluo. intensity (a. u.)

0.43
0.73
0.14

E   CIN density

6.0
5.0
4.0
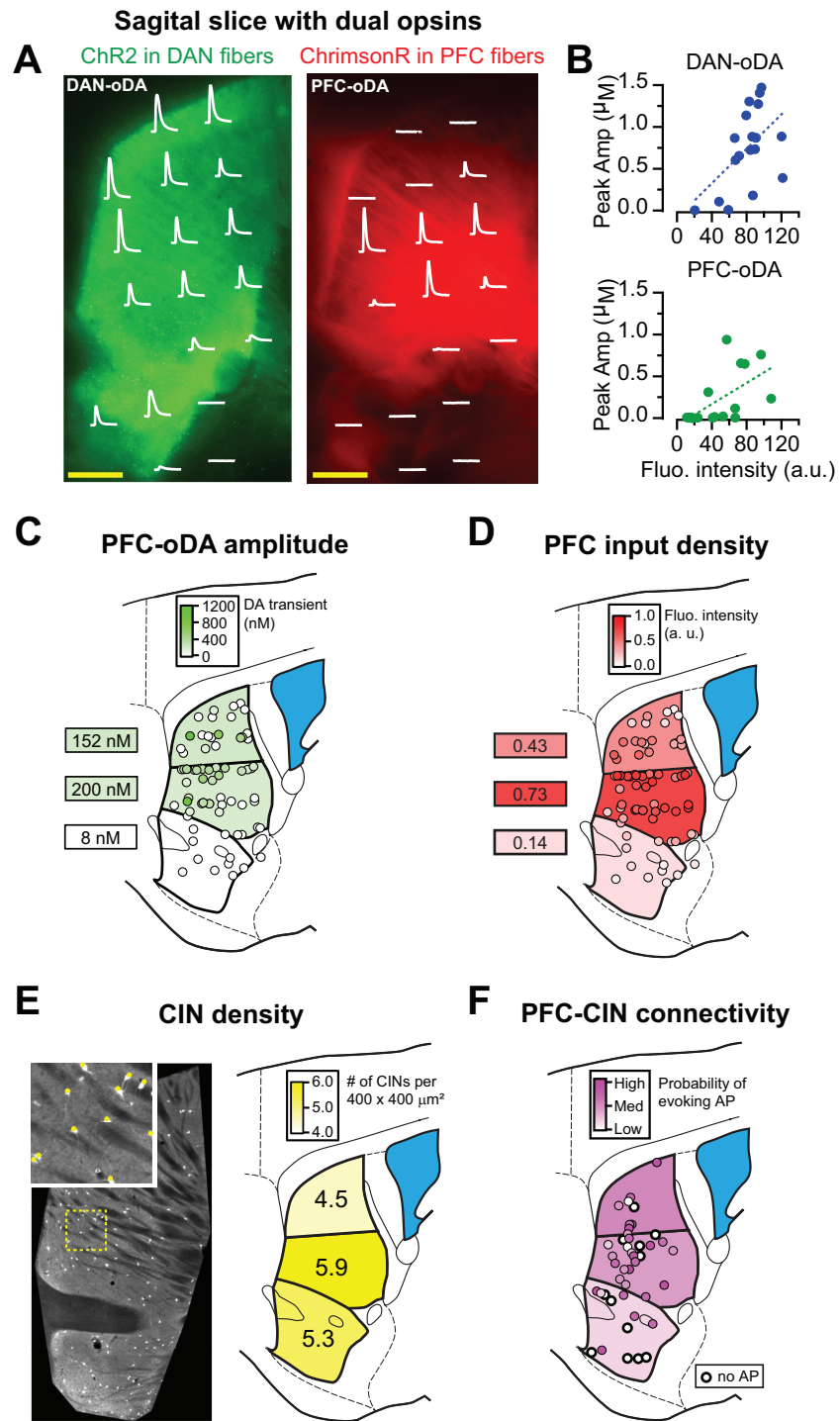# of CINs per 400 x 400 µm²

4.5
5.9
5.3

F   PFC-CIN connectivity

High
Med
Low
Probability of evoking AP

no AP

Figure 6 of Adrover et al.