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Research Articles: Systems/Circuits

Inhibition of nigrostriatal dopamine release by striatal $GABA_A$ and $GABA_B$ receptors

Emanuel F. Lopes[1], Bradley M. Roberts[1], Ruth E. Siddorn[1], Michael A. Clements[1] and Stephanie J. Cragg[1,2]

[1]*Centre for Integrative Neuroscience, Department of Physiology, Anatomy and Genetics, University of Oxford, OX1 3PT, UK*

[2]*Oxford Parkinson's Disease Centre, Oxford, OX1 3PT.*

https://doi.org/10.1523/JNEUROSCI.2028-18.2018

Received: 8 August 2018

Revised: 7 November 2018

Accepted: 15 November 2018

Published: 12 December 2018

Author contributions: E.L., B.R., M.C., and S.J.C. designed research; E.L., B.R., R.S., and M.C. performed research; E.L., B.R., R.S., and M.C. analyzed data; E.L. and S.J.C. wrote the first draft of the paper; B.R. and S.J.C. edited the paper; S.J.C. wrote the paper.

Conflict of Interest: The authors declare no competing financial interests.

Supported by Parkinson's UK (G-1504), Oxford Clarendon Fund, and the BBSRC.

Corresponding author: Professor Stephanie Cragg, Department of Physiology, Anatomy and Genetics, University of Oxford, OX1 3PT UK. Stephanie.cragg@dpag.ox.ac.uk

Cite as: J. Neurosci 2018; 10.1523/JNEUROSCI.2028-18.2018

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1 # Inhibition of nigrostriatal dopamine release by striatal $GABA_A$ and
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3 Emanuel F. Lopes[1†], Bradley M. Roberts[1†], Ruth E. Siddorn[1], Michael A. Clements[1], Stephanie J.
4 Cragg[1,2*]

5 [1]Centre for Integrative Neuroscience, Department of Physiology, Anatomy and Genetics, University
6 of Oxford, OX1 3PT, UK; [2]Oxford Parkinson's Disease Centre, Oxford, OX1 3PT.

7 **Abbreviated Title:** Striatal GABA receptors inhibit dopamine release

8 ***Corresponding author:** Professor Stephanie Cragg, Department of Physiology, Anatomy and
9 Genetics, University of Oxford, OX1 3PT UK. Stephanie.cragg@dpag.ox.ac.uk

10 [†]These authors contributed equally to this work.

11 **Number of pages**: 20
12 **Number of**: Figures: 4
13 Tables: 0
14 Multimedia: 0
15 3D models: 0
16 **Number of words** in: Abstract: 245
17 Introduction: 637
18 Discussion: 1246

19 **Conflicts of Interest:** The authors declare no competing financial interests.

20 **Acknowledgements:** Supported by Parkinson's UK (G-1504), Oxford Clarendon Fund, and the
21 BBSRC.

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22 **Abstract**

23 Nigrostriatal dopamine (DA) is critical to action selection and learning. Axonal DA release is
24 locally influenced by striatal neurotransmitters. Striatal neurons are comprised principally of
25 GABAergic neurons, projection neurons and interneurons, while a minority are cholinergic
26 interneurons (ChIs). ChIs strongly gate striatal DA release via identified nicotinic receptors
27 (nAChRs) on DA axons. Striatal GABA is thought to modulate DA, but GABA receptors have not
28 been documented conclusively on DA axons. However, ChIs express GABA receptors, and are
29 therefore key candidates for potential mediators of GABA regulation of DA. We addressed whether
30 striatal GABA and its receptors can modulate DA release directly, independently from ChI
31 regulation, by detecting DA in striatal slices from male mice using fast-scan cyclic voltammetry in
32 the absence of nAChR activation.

33 DA release evoked by single electrical pulses in the presence of nAChR antagonist dihydro-
34 β-erythroidine DHβE, was reduced by GABA or agonists at $GABA_A$ or $GABA_B$ receptors, with
35 effects prevented by selective GABA receptor antagonists. GABA agonists slightly modified the
36 frequency sensitivity of DA release during short stimulus trains. GABA agonists also suppressed
37 DA release evoked by optogenetic stimulation of DA axons. Furthermore, GABA receptor
38 antagonists significantly enhanced DA release, evoked by either optogenetic or electrical stimuli.
39 These results indicate that striatal GABA can inhibit DA release through $GABA_A$ and $GABA_B$
40 receptors, and that these actions are not mediated by cholinergic circuits. Furthermore, these data
41 reveal that there is a tonic inhibition of DA release by GABA, operating through predominantly
42 $GABA_B$ receptors.

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43 ## Significance statement

44 The principal inhibitory transmitter in the mammalian striatum, GABA, is thought to modulate
45 striatal dopamine (DA) release, but definitive evidence for GABA receptors on DA axons is
46 lacking. Striatal cholinergic interneurons regulate DA release via axonal nicotinic receptors
47 (nAChRs) and also express GABA receptors, but they have not been eliminated as potentially
48 critical mediators of DA regulation by GABA. Here, we find that $GABA_A$ and $GABA_B$ receptors
49 inhibit DA release without requiring cholinergic interneurons. Furthermore, ambient levels of
50 GABA inhibited DA release, predominantly through $GABA_B$ receptors. These findings provide
51 further support for direct inhibition of DA release by GABA receptors, and furthermore, reveal that
52 striatal GABA operates a tonic inhibition on DA output that could critically influence striatal
53 output.

54 ## Introduction

55 The striatum plays key roles in promoting motivated behaviours and learned actions. Nigrostriatal
56 dopamine (DA) neurons release DA from immensely arborized structures with each neuron forming
57 ~10^5 en passant varicosities and reaching ~2.7% of striatum in rat (Matsuda et al., 2009). DA output
58 is gated by numerous striatal neuromodulators (Sulzer et al., 2016). Cholinergic interneurons (ChIs)
59 play a particularly powerful role in gating and driving DA release through nicotinic acetylcholine
60 receptors (nAChRs) on DA axons (Jones et al., 2001; Zhou et al., 2001; Rice and Cragg, 2004;
61 Zhang and Sulzer, 2004; Threlfell et al., 2012). ChIs make up 1-2% of striatal neurons (Oorschot,
62 1996), with the 98% remainder of striatal neurons being GABAergic. Striatal GABA neurons
63 include the principal spiny project neurons (SPNs) (~95%) as well as interneurons (~2-3%)
64 including ‘fast-spiking interneurons’ (FSIs) and ‘low threshold spiking interneurons’ (LTS)
65 amongst others (Gittis and Kreitzer, 2012), and furthermore, DA axons might provide a source of
66 co-released GABA (Tritsch et al., 2012, 2014; Kim et al., 2015). Striatal $GABA_A$ and $GABA_B$

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67 receptors have been shown to modulate DA release, but information about their localization to DA
68 axons is lacking. A role for ChIs as potential mediators of GABA regulation of DA has not been
69 excluded.

70 Striatal administration of pregnanolone, a positive allosteric modulator of the $GABA_A$
71 receptor, or bicuculline, an antagonist, respectively decreased and increased extracellular dopamine
72 in intact rats measured by microdialysis (Smolders et al., 1995), and muscimol, a $GABA_A$ agonist,
73 inhibited DA release from striatal synaptosomes (Ronken et al., 1993). A $GABA_A$-mediated
74 enhancement of DA release has been reported in guinea-pig striatal slices during prolonged
75 electrical pulse trains, but this is thought to arise indirectly via inhibition of H_2O_2 release from
76 striatal neurons during prolonged stimuli (Avshalumov et al., 2003). Striatal perfusion of $GABA_B$
77 agonist baclofen, or antagonist phaclofen, respectively decreased and increased extracellular
78 dopamine in intact rats (Smolders et al., 1995), and baclofen decreased electrically evoked DA
79 release in acute slices of mouse caudate-putamen (Schmitz et al., 2002) and nucleus accumbens
80 (Pitman et al., 2014). Findings in slice preparations are less confounded by potential effects on long
81 loop circuits *in vivo* that could regulate DA via changes in DA neuron firing, and therefore more
82 directly support a local mechanism of action. However, while both $GABA_A$ and $GABA_B$ receptors
83 are densely expressed throughout striatum (Ng and Yung, 2000; Waldvogel et al., 2004), only the
84 $GABA_B$ receptor has been indicated on structures that resemble DA axons: Ultrastructural studies
85 report $GABA_B$ receptors in striatal neuropil in monkey and rat (Charara et al., 1999; Yung et al.,
86 1999), consistent with, but not definitive evidence for, $GABA_B$ receptors on DA axons.

87 Both $GABA_A$ and $GABA_B$ receptors are present on identified ChIs (Waldvogel et al., 1998;
88 Yung et al., 1999). Since ChIs operate strong control over DA release, and can mediate effects of
89 multiple neuromodulators on DA including opioids, nitric oxide, glutamate, and insulin (Britt and
90 McGehee, 2008; Hartung et al., 2011; Stouffer et al., 2015; Kosillo et al., 2016), ChIs emerge as
91 key potential mediators of GABAergic inhibition of DA release. ChIs need to be excluded as

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92 potential mediators before direct regulation of DA by $GABA_A$ and/or $GABA_B$ receptors seems
93 more plausible.

94 Here, we explored the control of DA release by GABA, $GABA_A$ and $GABA_B$ receptors, in
95 the absence of ACh input to nAChRs. We assessed GABA receptor regulation of DA release when
96 evoked electrically in the presence of nAChR antagonist DHβE, and also when DA release was
97 evoked by targeted activation of DA axons using optogenetics when DA release is not under
98 nAChR control (Threlfell et al., 2012; Melchior et al., 2015). We find that $GABA_A$ and $GABA_B$
99 receptors can inhibit DA release independently from nAChR activation, providing further support
100 for a direct mechanism of action. Furthermore, we find that endogenous striatal GABA provides a
101 tonic inhibition of DA release.

102 Materials and methods

103 Animal preparation and surgery

104 Animals were either wild-type C57BL6 male mice (P35-70) (RRID: IMSR_JAX:000664) or DAT-
105 Cre mice injected with an adeno-associated virus encoding Cre-dependent ChR2. For experiments
106 with light activation, DAT-Cre mice were bred from homozygotes for DAT-internal ribosome entry
107 site (IRES)-Cre, obtained from Jackson Laboratories (B6.SJL-*Slc6a3*$^{tm1.1(cre)Bkmn}$ /J, stock 006660)
108 (RRID: IMSR_JAX:006660). Postnatal day 25-35 DAT-Cre mice were anesthetized with
109 isoflurane, placed in a small animal stereotaxic frame (David Kopf Instruments, Tujunga, CA) and
110 injected with an adeno-associated virus (~10^{12} genome copies per ml; UNC Vector Core Facility,
111 Chapel Hill, NC) encoding Cre-dependent ChR2 (AAV5-EF1α-DIO-hChR2(H134R)-eYFP)
112 (RRID: SCR_002448). A total volume of 1 μl virus solution was injected bilaterally (500 nl per
113 hemisphere/injection) into substantia nigra pars compacta (SNc, AP -3.1 mm, ML ±1.2 mm from
114 bregma, DV -4.25 mm from exposed dura mater). Virus solution was injected at an infusion rate of

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115 50 nl/min with a 32 gauge Hamilton syringe and withdrawn 5-10 min after the end of injection.
116 Virus-injected mice were used for experiments > 4 weeks after viral injection.

117 **Slice preparation**

118 Wild type C57BL6 animals (P35-70) or DAT-Cre mice (P53-63) were decapitated after cervical
119 dislocation, and the brains were extracted. Coronal slices, 300 µm thick, were prepared in ice-cold
120 HEPES-buffered artificial cerebrospinal fluid (aCSF) containing (in mM): 120 NaCl, 20 $NaHCO_3$,
121 6.7 HEPES acid, 5 KCl, 3.3 HEPES salt, 2 $CaCl_3$, 2 $MgSO_3$, 1.2 KH_2PO_4, and 10 glucose. Slices
122 were then maintained in HEPES-buffered artificial cerebrospinal fluid at room temperature for at
123 least 1h prior to recording. All procedures were carried out according to institutional guidelines and
124 conformed to the UK Animals (Scientific Procedures) Act 1986.

125 **Fast-scan cyclic voltammetry**

126 Evoked DA release was detected in acute slices using fast-scan cyclic voltammetry (FCV). Slices
127 were superfused with a bicarbonate-buffered aCSF saturated with 95% O_2/ 5% CO_2 at 31-32°C,
128 containing (in mM): 124 NaCl, 26 $NaHCO_3$, 3.8 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 1.3 KH_2PO_4, and 10
129 glucose. All experiments with electrical stimulation were conducted in the presence of 1 µM
130 dihydro-β-erythroidine (DHβE) to prevent the effects of nAChR activation on DA release (Zhou et
131 al., 2001; Rice and Cragg, 2004). Extracellular DA concentration ($[DA]_o$) was monitored using fast-
132 scan cyclic voltammetry (FCV) with 7 µm-diameter (tip length 50–100 µm) carbon fiber
133 microelectrodes (CFMs; tip length 50–100 µm) and a Millar voltammeter (Julian Millar, Barts and
134 the London School of Medicine and Dentistry) as described previously (Threlfell et al., 2010). In
135 brief, the scanning voltage was a triangular waveform (−0.7 V to +1.3 V range versus Ag/AgCl) at
136 a scan rate of 800 V/s and sampling frequency of 8 Hz. Signals were attributable to DA by the
137 potentials for peak oxidation and reduction currents (oxidation peak: +500–600 mV, reduction
138 peak:~−200 mV). Electrodes were calibrated *post hoc* with 2 µM DA in experimental media. None

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139 of the drugs altered electrode sensitivity to DA. Data were acquired and analysed using Axoscope
140 10.6 (Molecular Devices) and locally written Excel macros.

141 **Electrical and light stimulation**

142 Recordings were obtained from the dorsal striatum. Electrical stimulation was delivered by a local
143 bipolar concentric electrode (25 μm diameter, Pt/Ir; FHC). All experiments with electrical
144 stimulation were conducted in the presence of 1 μM DHβE which prevents nAChR activation.
145 Stimulation intensity was set to 80% maximal $[DA]_o$: ~0.6 mA. Applied stimuli were single 200 μs
146 pulses (1p) or five pulses (5p) at 5, 25, and 100 Hz. Mean peak $[DA]_o$ evoked by 1p was equivalent
147 to that of a 1 Hz train; 1p is used in frequency comparison to indicate maximum 1 Hz data.
148 Electrical stimulations were repeated at 2.5 min intervals, which allow stable release to be sustained
149 over several hours. Mean $[DA]_o$ evoked by a single electrical pulse in control conditions (in the
150 presence of DHβE) across release sites was 1.13 ± 0.06 μM.

151 Light stimulation was delivered by an LED system (OptoLED; Cairn Research). DA release
152 was evoked with full-field 470 nm blue light, pulse duration 2 ms. Experimental stimulation
153 intensity was determined by delivering a light pulse sufficient to drive approximately 50% maximal
154 $[DA]_o$. During recordings, slices were visualised on an upright microscope (Olympus BX50WI),
155 with fluorescence optics for visualizing eYFP. Mean $[DA]_o$ evoked by a single light pulse in control
156 conditions was 0.90 ± 0.09 μM.

157 **Experimental Design and Statistical Analysis**

158 Data are represented as means ± SEM, and “n” refers to the number of experiments. Each
159 experiment was performed at a single recording site in one brain slice. For each experiment at a
160 given recording site, data for each variable were obtained in at least triplicate before averaging to
161 obtain the value for that individual parameter. The number of animals in each data set is ≥ 3. Data
162 are expressed as extracellular concentration of dopamine ($[DA]_o$), or as $[DA]_o$ normalized to mean
163 peak $[DA]_o$ evoked by single pulses in control conditions. In all cases, $[DA]_o$ displayed typical

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164 kinetics to peak and to decay indicative of good slice quality, and were obtained from recording
165 sites which maintained sufficiently stable levels of release over time. No data were excluded after
166 acquisition.

167 Data acquired immediately prior to drug application were used as pre-drug control data and
168 were compared with data acquired after drug effects had equilibrated, after approximately 10–20
169 min of application. Ratios for $[DA]_o$ evoked by 5p/1p were obtained by dividing each 5p-evoked
170 $[DA]_o$ value with an average 1p-evoked $[DA]_o$ value in the same condition at that recording site. In
171 graphs representing peak $[DA]_o$ evoked by 1p over time, missing data points are timepoints when
172 data were acquired using different stimulation parameters interspersed among 1p stimulations.
173 Comparisons for statistical significance were assessed by One- or Two-way ANOVA, paired t-tests,
174 and Mann Whitney U tests where data were not normally distributed, using GraphPad Prism
175 (RRID: SCR_002798).

176 **Drugs**

177 Dihydro-β-erythroidine (DHβE), saclofen, and bicuculline were obtained from Tocris Bioscience.
178 Baclofen, picrotoxin, and GABA were obtained from Sigma Aldrich. Muscimol and CGP 55845
179 hydrochloride were obtained from Abcam. Stock aliquots of drugs were prepared at 1,000–10,000×
180 final concentrations in de-ionized water, aqueous acid (baclofen), or DMSO (picrotoxin) and stored
181 at −20°C. DHβE was present throughout all experiments with electrical stimulation.

182 Results

183 GABA$_A$ and GABA$_B$ receptors inhibit striatal DA release

184 We assessed whether GABA can modulate striatal DA release in the absence of nAChR activity
185 using antagonist DHβE (1 μM) to inhibit nAChRs as previously (Rice and Cragg, 2004; Threlfell et
186 al., 2012). We first confirmed in our hands that DHβE concentration was supramaximal for
187 inhibition of DA release, by confirming the concentration-response relationship for DHβE (1 nM –

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188 1 μM) on $[DA]_o$ evoked by a single electrical pulse. DHβE concentration-dependently inhibited
189 evoked $[DA]_o$ (Figure 1A; Sigmoidal concentration-response curves, R^2 = 0.93. Hill slope, -1.92,
190 IC_{50}, 11 nM, *n* = 9), and 1 μM DHβE was supramaximal for inhibition of $[DA]_o$.

191 GABA was applied to striatal slices in the presence of the nAChR antagonist DHβE (1 μM).
192 Bath application of GABA (100 μM, 5 mM) concentration-dependently reduced DA release evoked
193 by single electrical pulses by ~15-50% (Figure 1B,C; Two-way ANOVA: F (1, 10) = 48.32, $P <$
194 0.0001, 100 μM GABA, $U = 0$, $P = 0.016$, $n = 7$; $U = 6$, 5 mM, $P = 0.0079$, $n = 5$, Mann-Whitney
195 tests) indicating that GABA is capable of inhibiting DA release independently from any effects on
196 ChI input to nAChRs.

197 To identify which GABA receptors can inhibit DA release, we tested the effect of $GABA_A$
198 or $GABA_B$ agonists. Activation of $GABA_A$ receptors with muscimol (20 μM) suppressed DA
199 release evoked by single electrical pulses by ~30% (Figure 1D,E: $U = 0$, $P = 0.0079$, $n = 5$, Mann-
200 Whitney test) and this effect was prevented by prior application of $GABA_A$ channel blocker
201 picrotoxin, confirming an effect via $GABA_A$ receptors (Figure 1F,G: $U = 11$, $P = 0.85$, $n = 5$,
202 Mann-Whitney test). Activation of $GABA_B$ receptors with baclofen (10 μM) also suppressed DA
203 release evoked by single electrical pulses by ~25% (Figure 1H,I: $U = 0$, $P = 0.0006$, $n = 7$, Mann-
204 Whitney test) and this effect was confirmed to be due to $GABA_B$ receptors, by prior application of
205 the $GABA_B$ antagonist saclofen which prevented the effect of baclofen (Figure 1J,K: $U = 5$, $P =$
206 0.11, $n = 5$, Mann-Whitney test).

207 **GABA receptors on frequency-sensitivity of DA release**

208 We explored whether GABA receptor activation inhibited DA release during trains of stimuli and
209 across a range of frequencies. Muscimol significantly decreased $[DA]_o$ evoked by 5-pulse trains at
210 all frequencies tested (Figure 2A,B. Two-way ANOVA: Drug effect: F(1, 48) = 67.55; $P = 0.0025$,
211 Frequency effect: F(3, 48) = 259.6, $P < 0.0001$, $n = 7$) and furthermore, there was a significant
212 interaction between stimulation frequency and drug effect (Figure 2B; Two way ANOVA: F(3, 48)

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213 = 5.485, P = 0.0025), which was borne out by an increase in the 5p/1p ratio examined at 100 Hz
214 (Figure 3C; paired t test: t(6) = 3.46, P = 0.014). In other words, activation of $GABA_A$ receptors can
215 marginally promote the contrast in DA signals released by different firing patterns or rate.

216 Baclofen significantly decreased evoked $[DA]_o$ (Figure 2E. Two-way ANOVA: F(1, 48) =
217 6.271, Drug effect: P = 0.016; Frequency effect: F(3, 48) = 144.5, P < 0.0001, n = 7). We did not
218 detect a significant interaction between stimulation frequency and drug effect (Figure 2E: Two way
219 ANOVA: F(3, 48) = 0.040, P = 0.99), however, the 5p/1p ratio for $[DA]_o$ evoked at 100 Hz was
220 slightly increased with baclofen (Figure 2F: paired t test: t(6) = 3.272, P = 0.017), suggesting that
221 $GABA_B$ receptors only marginally change the contrast in DA signals released by different activity.

222 **GABA receptors inhibit DA release evoked by targeted optogenetic stimulation**

223 To explore whether the inhibition of DA release by GABA receptors depended on the co-activation
224 during electrical stimulation of some other local neuron type or input, we used an optogenetic
225 approach to selectively activate DA axons only. We expressed ChR2-eYFP in DA neurons and
226 axons in DAT-Cre mice using an established viral approach as previously (Threlfell et al., 2012;
227 Brimblecombe and Cragg, 2015). Either $GABA_A$ agonist muscimol, or $GABA_B$ agonist baclofen
228 suppressed DA release evoked by single blue light pulses (muscimol, Figure 3A,B; U = 0, P =
229 0.0079, n = 5; Mann-Whitney test; baclofen, Figure 3C,D; U = 0, P = 0.029, n = 4, Mann-Whitney
230 test), indicating that GABA receptor agonists do not require coincident activation of another striatal
231 input to suppress DA release. We also confirmed that as with electrical stimulation, the effects of
232 muscimol and baclofen were prevented by prior application of antagonists for respectively $GABA_A$
233 (picrotoxin, Figure 3C,D; effect of agonist, P = 0.36, n = 6, Mann-Whitney test, U = 12) or $GABA_B$
234 receptors (saclofen, Figure 3G,H; effects of agonist, P = 0.127, n = 5, Mann-Whitney test, U = 5).

235 **GABA operates a tonic inhibition on DA release**

236 Some striatal GABA neurons are tonically active, and it has been reported that there is an ambient
237 GABA tone in striatum (Ade et al., 2008; Kirmse et al., 2008, 2009; Santhakumar et al., 2010;

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238 Cepeda et al., 2013). We therefore, we tested whether there was tonic inhibition of DA release, by
239 exploring the effects of GABA receptor antagonists on DA release, evoked either optogenetically or
240 electrically.

241 We found that co-application of $GABA_A$ and $GABA_B$ antagonists bicuculline (10 μM) and
242 CGP 55845 (2 μM) respectively, significantly enhanced $[DA]_o$ evoked by single light pulses in
243 DAT-Cre ChR2-expressing mice by ~20% (Figure 4A,B; $P = 0.0079$, $n = 5$, Mann-Whitney test, U
244 $= 0$) indicating a tonic inhibition of DA release by endogenous striatal GABA in the absence of
245 other stimuli. We probed which receptors could mediate inhibition of DA release by endogenous
246 GABA, using electrical stimulation in wild-type mice (in the presence of DHβE). $GABA_A$
247 antagonist bicuculline appeared to slightly elevate but did not significantly increase evoked $[DA]_o$
248 (Figure 4C,D,G. Two-way ANOVA: Effect of drug, $F(1,44) = 1.58$, $P = 0.215$; Effects of
249 frequency: $F(3,44) = 128.4$, $P < 0.0001$, $n = 5$), or interact with stimulation frequency (Figure 4C:
250 Two way ANOVA: $F(3, 44) = 0.115$, $P = 0.95$) or 5p:1p ratio (Figure 4H $t(7) = 1.217$, $P = 0.263$,
251 paired t-test). $GABA_B$ antagonist saclofen significantly increased evoked $[DA]_o$ (Figure 4E,F,I.
252 Two-way ANOVA: Effect of drug, $F(1,24) = 4.87$, $P = 0.0371$; Effects of frequency, $F(3,24) =$
253 57.8, $P < 0.0001$). There was no significant interaction with stimulation frequency (Figure 4C: Two
254 way ANOVA: $F(3,24) = 0.205$, $P = 0.89$), or 5p:1p ratio (Figure 4J, $t(3) = 1.49$, $P = 0.233$, paired t-
255 test).

256 **Discussion**

257 Here we show that GABA, $GABA_A$ and $GABA_B$ receptors in dorsal striatum inhibit DA release,
258 and that these actions are not mediated via regulation of striatal ACh acting at nAChRs. By
259 eliminating ChIs as a necessary site of action of GABA, a direct action on DA axons becomes
260 indicated as a site for GABA action on DA transmission. Furthermore, we show that GABA
261 receptor antagonists increase DA release evoked by single optogenetic stimuli, revealing that there

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262 is tonic inhibition of DA release by endogenous GABA arising from a tonically active source or
263 ambient level.

264 **GABA receptor influence on DA output does not require striatal ACh**

265 We found that both $GABA_A$ and $GABA_B$ receptor ligands can inhibit DA release whether evoked
266 electrically in the presence of nAChR antagonist or optogenetically by targeted activation of ChR2-
267 expressing DA axons in DAT-Cre mice. Light-activated DA release is not under tonic control by
268 striatal nAChRs in these stimulation conditions (Threlfell et al., 2012; Melchior et al., 2015). Since
269 GABA receptor effects were seen in the absence of nAChR activation, they are unlikely to require
270 GABA receptors on ChIs. These findings are consistent with a study in NAc indicating that $GABA_B$
271 mediated control of DA is independent of ACh input (Pitman et al., 2014). We note that muscarinic
272 M5 receptors on DA axons have been suggested in some studies to regulate DA release (Bendor et
273 al., 2010; Shin et al., 2015), but in our hands, electrically evoked DA release in the presence of
274 DHβE, and optogenetically evoked DA release, were not attenuated by muscarinic receptor
275 antagonists (Threlfell et al., 2010, 2012) and so these effects of GABA and GABA receptors cannot
276 be mediated by alternative actions of ACh at M5 mAChRs on DA axons.

277 Activation of GABA receptors had limited effects on the frequency sensitivity of evoked
278 DA release. Activation of $GABA_A$ but not $GABA_B$ receptors interacted in a statistically significant
279 manner with frequency and number of stimulus pulses to slightly promote the ratio of $[DA]_o$ evoked
280 by high frequency trains over single pulse release, but the effect size was modest. Thus, GABA
281 receptor activation primarily limits the overall amplitude of DA output with only a minor additional
282 enhancement in the frequency filtering, unlike ACh which profoundly changes the relationship of
283 DA output to presynaptic activity (Rice and Cragg, 2004; Zhang and Sulzer, 2004).

284 **Tonic Inhibition**

285 We found that GABA receptor antagonists can enhance DA release evoked by a short single
286 optogenetic stimulus pulse to ChR2-expressing DA axons in DAT-Cre mice, suggesting that DA

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287 release is under tonic inhibition by GABA. Light activation is targeted to DA axons, and should not
288 activate GABA release from other striatal neurons. Furthermore, the stimulus is sufficiently short
289 that evoked DA release should not be under the control of any GABA that might be co-released by
290 this stimulus. Mesostriatal DA neurons can apparently synthesise, store, and co-release GABA
291 (Tritsch et al., 2014; Kim et al., 2015) that can evoke inhibitory currents in post-synaptic medium
292 spiny neurons, but it is unlikely that any GABA co-released with DA could simultaneously gate the
293 concurrent release of DA evoked by the same single 2 ms stimulus. Rather, it is more likely that DA
294 release is under tonic inhibition by a pre-existing striatal GABA tone. A tonic inhibition is
295 consistent with previous reports that there is an ambient GABA tone in striatum, detected as a
296 $GABA_A$ receptor-mediated current in postsynaptic neurons even in *ex vivo* preparations (Ade et al.,
297 2008; Kirmse et al., 2008, 2009; Santhakumar et al., 2010; Cepeda et al., 2013). Our findings
298 indicate that tonic inhibition of DA release can readily be detected, at least for $GABA_B$ receptors.

299 An ambient GABA tone that can act on DA axons to limit DA output should not be entirely
300 surprising. At least one type of GABAergic interneuron, the low threshold spiking (LTS)
301 interneuron, is capable of autonomous firing in striatal slices (Beatty et al., 2012). Furthermore,
302 striatal neurons are ~95% GABAergic, and thus even low levels of GABA release per neuron could
303 potentially summate for significant impact. No GABAergic axoaxonic synapses have been
304 identified on DA axons, but this need not preclude an interaction. GABA can spillover for
305 extrasynaptic actions in many neurons (Farrant and Nusser, 2005) and might act on DA axons
306 through extrasynaptic effects. The volume of striatum reached by the extensive axonal arbour of a
307 single DA neuron (Matsuda et al., 2009) can be calculated from striatal neuron counts (Oorschot,
308 1996) to encompass ~70,000 GABAergic neurons (plus additional non-neuronal cells that might
309 provide a source of GABA). This large number of potential GABA sources might readily spillover
310 and/or otherwise summate to provide an ambient GABA tone that can act at GABA receptors to
311 limit DA output. Striatal GABA might therefore be in a position to influence striatal output not only

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312 though direct actions on output neurons and local interneuron and input networks, but also by
313 governing DA transmission.

314 **Direct vs indirect actions of GABA receptors**

315 The site of GABA receptor localization remains undefined. DA neurons in substantia nigra express
316 $GABA_A$ and $GABA_B$ receptors and these are certainly functional in somatodendritic compartments
317 (Bowery et al., 1987; Nicholson et al., 1992; Boyes and Bolam, 2003; Brazhnik et al., 2008), but
318 their trafficking and localization to DA axons has not yet been demonstrated. $GABA_B$ receptors
319 have been immunocytochemically detected in striatal neuropil, consistent with a localization of
320 these receptors on DA axons (Charara et al., 1999), but $GABA_A$ receptors have not yet been shown
321 on DA axons. It can be difficult to verify the ultrastructural location of membrane bound receptors
322 using classic immunocytochemical methods, which depend on appropriate fixation methods and
323 conditions for antibody penetration, and absence of proof is not proof of absence.

324 Key candidates for alternative sites of action can however be eliminated. Here, we exclude
325 actions of GABA-receptors on ChIs acting through downstream nAChRs. Furthermore, GABAergic
326 interneurons are unlikely as intermediaries: for example, if $GABA_A$ receptors are acting indirectly
327 through an intermediary GABA circuit that modulates DA via downstream $GABA_B$ receptors, then
328 $GABA_A$ receptor agonists would be expected to inhibit the intermediary GABA circuit, and
329 therefore increase DA release, which is opposite to that seen here. The remaining candidate
330 locations for the GABA receptors that regulate DA are therefore either an undisclosed neuron or
331 other cell type with currently unknown actions on DA, or more parsimoniously, DA axons
332 themselves. Direct responses to GABA have been described for axons of several other neurons
333 throughout the nervous system (Kullmann et al., 2005; Trigo et al., 2008; Bucher and Goaillard,
334 2011). In CNS neurons, $GABA_A$ receptors can reduce axonal spike amplitude and propagation and
335 promote spike failures (Zhang and Jackson, 1995; Ruiz et al., 2003; Verdier et al., 2003), whereas

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336 $GABA_B$ receptors inhibit voltage-gated Ca^{2+} channels (Sun and Chiu, 1999). Similar mechanisms
337 could account for GABAergic inhibition of DA release from DA axonal arbours.

338 In conclusion, we show not only that $GABA_A$ and $GABA_B$ receptors can gate DA output,
339 but that there is a tonic inhibition by endogenous GABA through an apparent ambient GABA tone.
340 Besides directly regulating striatal output, striatal GABA tone might therefore also govern striatal
341 integration via dampening DA output. We exclude cholinergic interneurons acting through nAChRs
342 on DA axons as a candidate intermediary for GABA regulation of DA, thereby adding support to
343 the hypothesis that GABA is acting directly on DA axons. With the assistance of new genetic,
344 tagging and imaging tools, future studies should revisit the potential localization of $GABA_A$ and
345 $GABA_B$ receptors to DA axons.

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465 ## Figure Legends

466 **Figure 1. GABA, $GABA_A$ or $GABA_B$ agonists inhibit DA release. (A)** Mean peak $[DA]_o$ (±
467 SEM) evoked by 1 electrical pulse vs applied DHβE concentration. Sigmoidal concentration-
468 response curve fit, R^2 = 0.93, *n* = 9. **(B,D,F,H,J)** Mean peak $[DA]_o$ (± SEM) versus time and
469 (**C,E,G,I,K**) mean $[DA]_o$ (± SEM) vs time, evoked by 1 electrical pulse before and after application
470 of: (B,C) 100 μM, *n* = 7, or 5 mM GABA, *n* = 5, ****P*<0.001, Two-way ANOVA, (D,E) 20 μM
471 muscimol, *n* = 5; (F,G) 20 μM muscimol in the presence of 100 μM picrotoxin, *n* = 5; (H,I) 10 μM
472 baclofen, *n* = 7; (J,K) 10 μM baclofen in the presence of 100 μM saclofen, *n* = 5. Data are
473 normalised to peak $[DA]_o$ prior to drug application. Shaded areas are used to obtain illustrated data
474 and statistical comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001, Mann-Whitney tests. The nAChR
475 antagonist DHβE (1 μM) is present throughout.

476 **Figure 2. $GABA_A$ and $GABA_B$ receptor agonists modify DA release during pulse trains.**
477 **(A,D)** Mean $[DA]_o$ (± SEM) vs time and (**B,E**) mean peak $[DA]_o$ (± SEM) vs stimulation frequency
478 for 1- or 5-pulse trains in control conditions (*black*), 20 μM muscimol, *n* = 7 (A,B) (*red*) or 10 μM
479 baclofen (D,E) (*blue*). Two-Way ANOVA, effect of drug **P*<0.05, ****P*<0.001, drug x frequency
480 interaction ††*P*<0.01. Data are normalised to peak $[DA]_o$ prior to drug application. **(C,F)** Ratio of
481 peak $[DA]_o$ released by 5p vs 1p (100 Hz) in control conditions, muscimol (C), or baclofen (F).
482 **P*<0.05*,* paired t-tests vs control. The nAChR antagonist DHβE (1 μM) is present throughout.

483 **Figure 3. GABA receptors inhibit optogenetically stimulated DA release.** (**A,C,E,G**) Mean
484 peak $[DA]_o$ (± SEM) versus time and (**B,D,F,H**) mean $[DA]_o$ (± SEM) vs time, evoked by 1 light
485 pulse before and during application of (A,B) 20 μM muscimol, *n* = 5, (C,D) 20 μM muscimol in the
486 presence of 100 μM picrotoxin, *n* = 5, (E,F) 10 μM baclofen, *n* = 4, or (G,H) 10 μM baclofen in the
487 presence of 100 μM saclofen, *n* = 6. Data are normalised to mean peak $[DA]_o$ prior to drug
488 application. **P*<0.05, ***P*<0.01, Mann-Whitney tests.

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489 **Figure 4. GABA receptor antagonists increase DA release. (A)** Mean peak $[DA]_o$ (± SEM)
490 versus time and (**B**) mean $[DA]_o$ (± SEM) vs time, evoked by 1 light pulse before and in the
491 presence of 3.5 μM CGP 55845 and 10 μM bicuculline (GABAR antagonists). $^{**}P<0.01$, Mann-
492 Whitney test. **(C,E)** Mean peak $[DA]_o$ evoked by 1 electrical pulse with the application of 10 μM
493 bicuculline (C) or 100 μM saclofen (E), all in the presence of 1 μM DHβE. **(D,F)** Mean $[DA]_o$ (±
494 SEM) vs time in control conditions (*black*), 10 μM bicuculline (D) (*red*) or 100 μM saclofen (F)
495 (*blue*). **(G,I)** Mean peak $[DA]_o$ (± SEM) vs stimulation frequency in control (*black*), with
496 bicuculline (G), or saclofen (I). Data are normalised to peak 1p-evoked $[DA]_o$ prior to drug
497 application. Two-way ANOVA, effect of drug $^{*}P<0.05$. **(H,J)** Ratio of peak $[DA]_o$ released by 5p
498 vs 1p (100Hz) in control conditions, bicuculline (H), or with saclofen (J).

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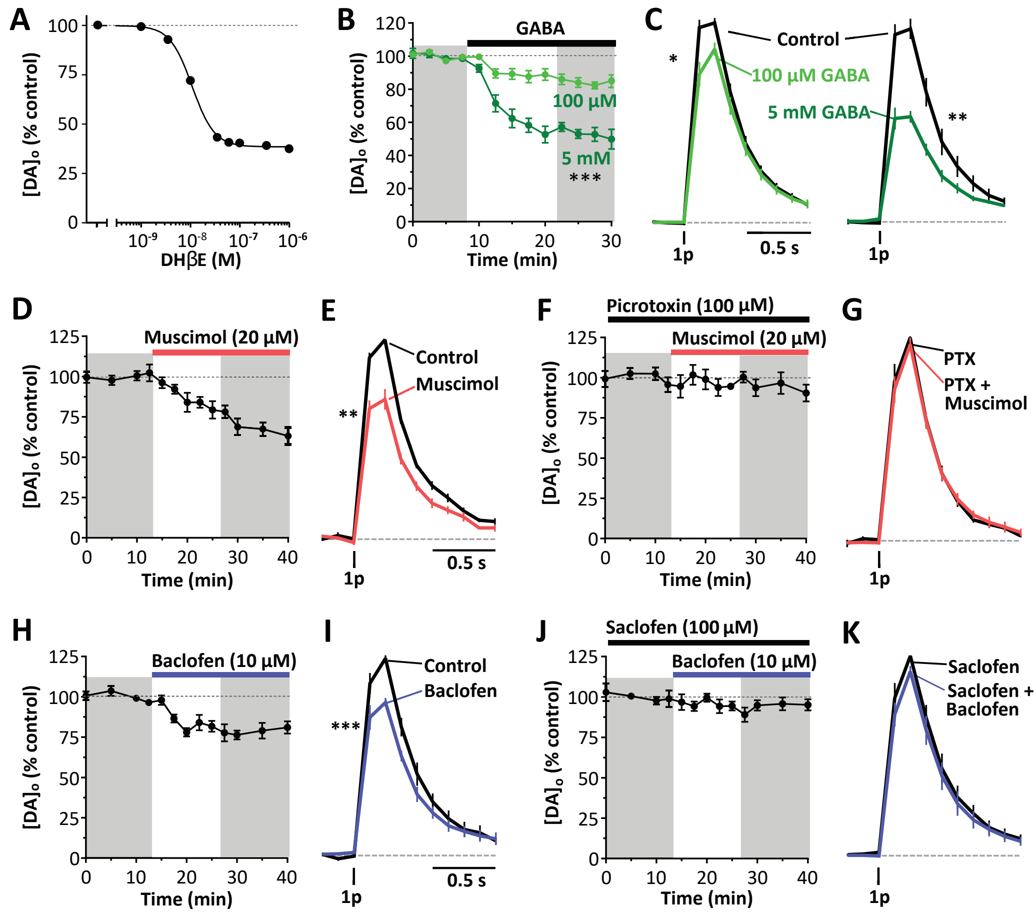

Figure 1
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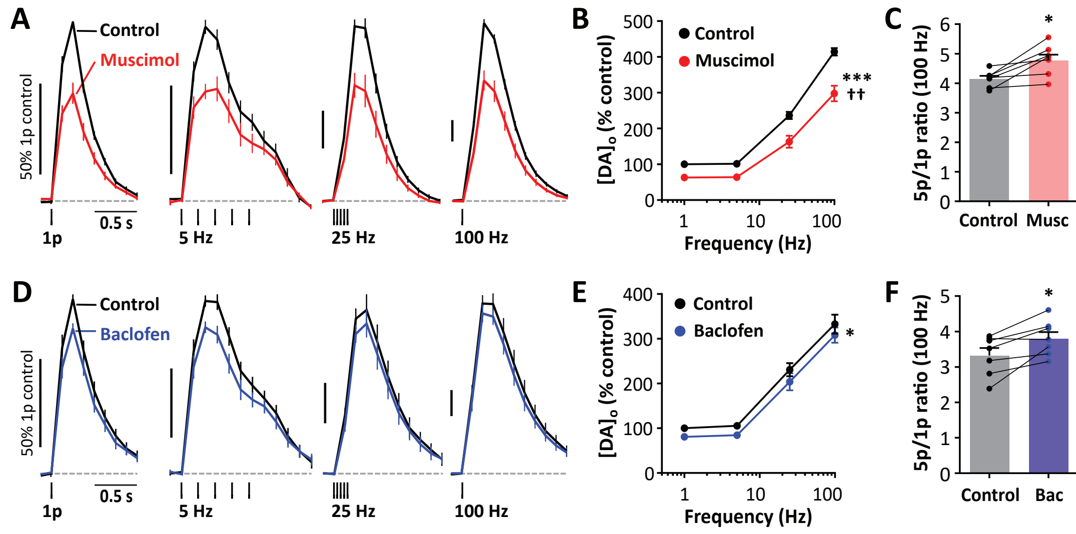

Figure 2
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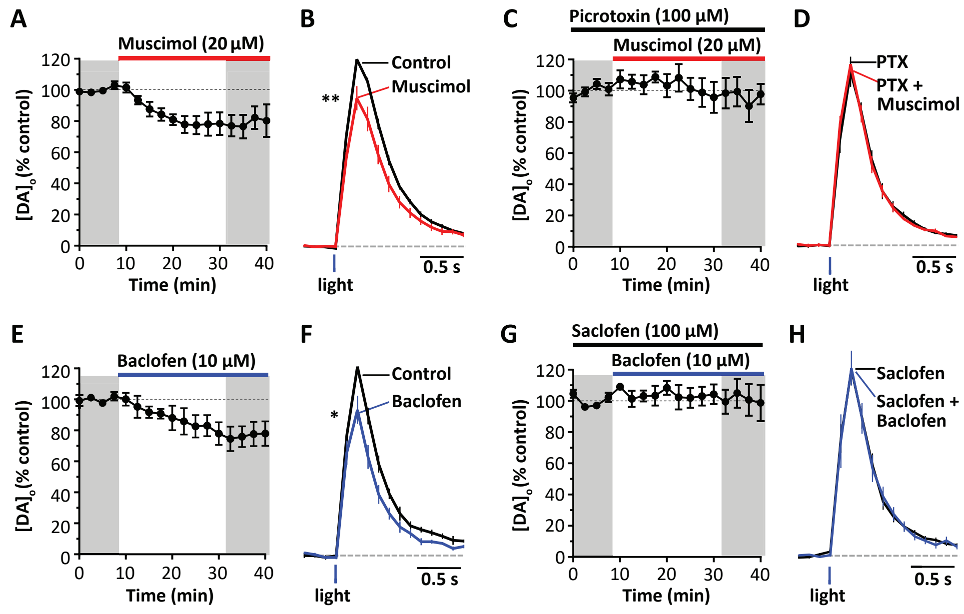

Figure 3
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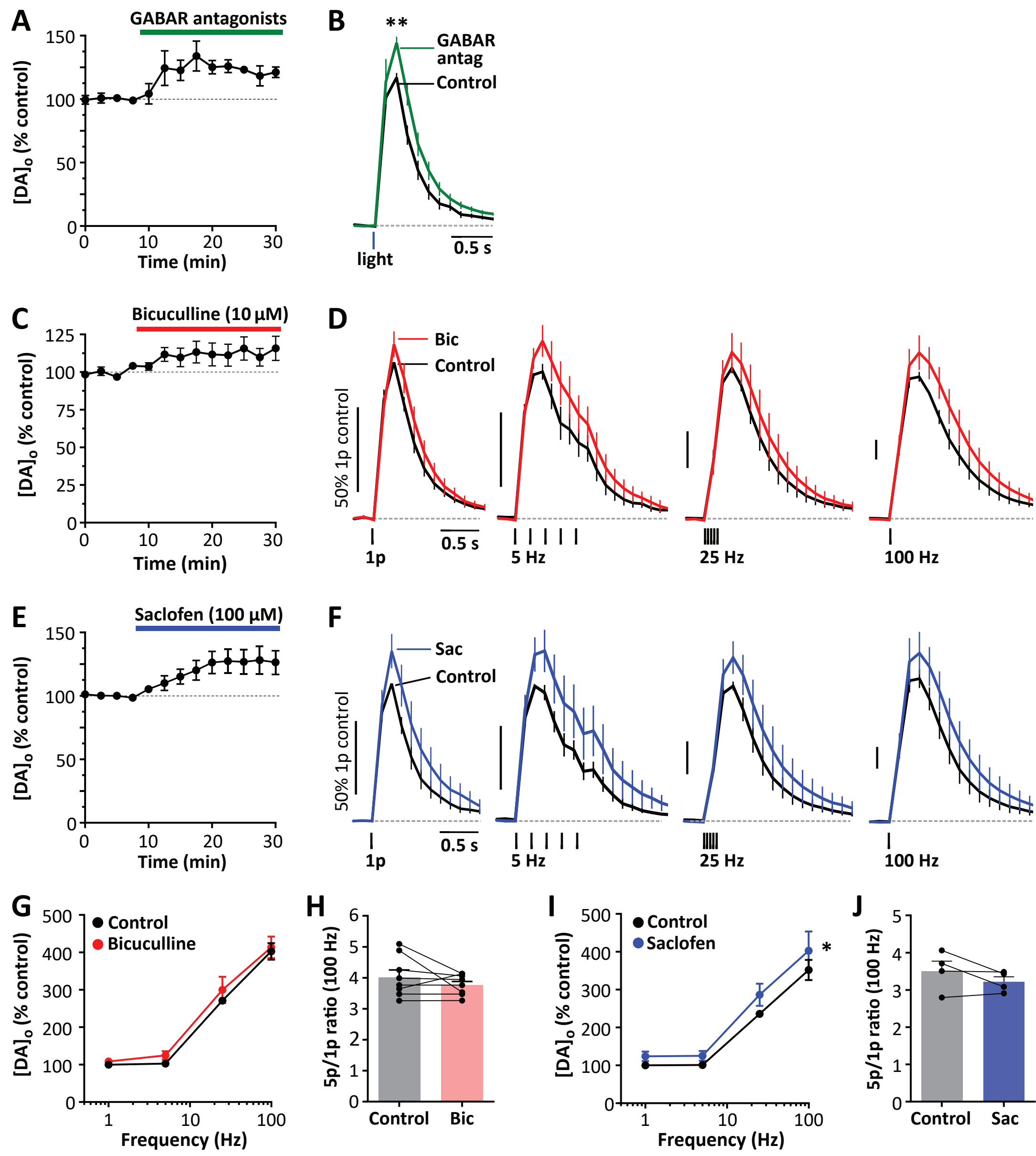

Figure 4
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