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The NeuroD6 subtype of VTA neurons contributes to psychostimulant sensitization and behavioral reinforcement

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27 ZB planned experiments, performed histological and behavioral experiments, analyzed data,

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29 experiments, analyzed data, prepared figures; SS planned, performed, analyzed FSCV

30 experiments, prepared figures; VZ, planned, performed, analyzed patch clamp experiments,

31 prepared figures; BV performed histological experiments, analyzed data; SD planned and

performed histological experiments, prepared figures; BG produced and contributed *Vmat2$^{lox/lox}$* mice; CB planned FSCV experiments and analyzed data; TSH planned patch clamp experiments and analyzed data; ÅWM, conceived and planned all experiments, analyzed data, wrote the manuscript.

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96 **The NeuroD6 subtype of VTA neurons contributes to psychostimulant sensitization and**

97 **behavioral reinforcement**

98 *Short title*: NeuroD6 VTA neurons in reward-related behavior

99 **Abstract**

100 Reward-related behavior is complex and its dysfunction correlated with neuropsychiatric

101 illness. Dopamine neurons of the ventral tegmental area (VTA) have long been associated

102 with different aspects of reward function, but it remains to be disentangled how distinct VTA

103 dopamine neurons contribute to the full range of behaviors ascribed to the VTA. Here, a

104 recently identified subtype of VTA neurons molecularly defined by NeuroD6 (NEX1M) was

105 addressed. Among all VTA dopamine neurons, less than 15% were identified as positive for

106 NeuroD6. In addition to dopaminergic markers, sparse NeuroD6 neurons expressed the

107 *Vesicular glutamate transporter 2* gene. To achieve manipulation of NeuroD6 VTA neurons,

108 NeuroD6(NEX)-Cre-driven mouse genetics and optogenetics were implemented. First,

109 expression of *Vesicular monoamine transporter 2* was ablated to disrupt dopaminergic

110 function in NeuroD6 VTA neurons. Comparing $Vmat2^{lox/lox;NEX-Cre}$ conditional knockout

111 (cKO) mice with littermate controls, it was evident that baseline locomotion, preference for

112 sugar and ethanol, and place preference upon amphetamine- and cocaine-induced

113 conditioning were similar between genotypes. However, locomotion upon repeated

114 psychostimulant administration was significantly elevated above control level in cKO mice.

115 Second, optogenetic activation of NEX-Cre VTA neurons was shown to induce dopamine

116 release and glutamatergic post-synaptic currents within the nucleus accumbens. Third,

117 optogenetic stimulation of NEX-Cre VTA neurons *in vivo* induced significant place

118 preference behavior, while stimulation of VTA neurons defined by Calretinin failed to cause a

119 similar response. The results show that NeuroD6 VTA neurons exert distinct regulation over

120   specific aspects of reward-related behavior, findings that contribute to the current

121   understanding of VTA neurocircuitry.

122   **Significance statement**

123   Reward-related behavior is complex and its dysfunction is implicated in many

124   neuropsychiatric disorders, including drug addiction. Midbrain dopamine neurons of the

125   ventral tegmental area (VTA) are crucial for reward behavior, but due to recently uncovered

126   heterogeneity, it remains to be fully resolved how they regulate reward responsiveness and

127   how their dysfunction might contribute to disease. Here we show that the recently described

128   NeuroD6 (NEX) subtype of VTA dopamine neurons is involved in psychostimulant

129   sensitization and that optogenetic stimulation of NEX-Cre VTA neurons induces dopamine

130   release, glutamatergic post-synaptic currents and real time place preference behavior.

131   NeuroD6 VTA neurons thus exert distinct regulation over specific aspects of reward-related

132   behavior, findings that contribute to the current understanding of VTA neurocircuitry.

133

134   **Introduction**

135   The midbrain dopamine (mDA) system mediates a diverse spectrum of behaviors and their

136   dysfunction is correlated with a range of severe behavioral disorders including substance use

137   disorder, schizophrenia, ADHD and Parkinson´s disease (PD). Consequently, therapies based

138   on modulating the activity of the mDA system are commonly prescribed, however, due to

139   their unselective nature, current treatments often fail to alleviate symptoms and instead cause

140   adverse effects (Divac et al., 2014; Weintraub, 2008). One reason for the lack of successful

141   treatment is incomplete understanding of the underlying neurobiology. Indeed, it is

142   increasingly understood that the mDA system is highly heterogeneous (reviewed in Morales

143   and Margolis, 2017; Pupe and Wallén-Mackenzie, 2015). Beyond the classical separation into

5

144   the ventral tegmental area (VTA) and substantia nigra *pars compacta* (SNc), with VTA

145   projections to cortical and limbic target areas and SNc projections to the dorsal striatum

146   subserving cognitive/affective and motor functions, respectively (Björklund and Dunnett,

147   2007), a higher level of complexity is now being unfolded: Afferent and efferent projections,

148   electrophysiological patterns, capacity for glutamate or GABA co-release and responsiveness

149   to appetitive or aversive stimuli are some of the properties that distinguish mDA neurons from

150   each other (Beier et al., 2015; Faget et al., 2016; Lammel et al., 2011; Menegas et al., 2015).

151

152   Likely coupled to this functional diversity is a complex diversity in molecular identity.

153   Microarray-based analyses have identified gene expression patterns enriched in VTA over

154   SNc DA neurons (Chung et al., 2005; Greene et al., 2005; Viereckel et al., 2016) while single

155   cell profiling has begun to identify combinatorial gene expression patterns that molecularly

156   define subtypes of mDA neurons (Hook et al., 2018; La Manno et al., 2016; Poulin et al.,

157   2014). Based on this new knowledge, intersectional genetic approaches were recently

158   described in which the distinct projection pathways of several newly defined subtypes of

159   mDA neurons were identified (Poulin et al., 2018). By forwarding the current knowledge

160   towards molecularly defined, and thus targetable, subtypes of mDA neurons with distinct

161   projection patterns, these recent advances enhance the possibility of improving selectivity in

162   treatment of dopaminergic disorders. However, a key issue that remains to be resolved is how

163   each molecularly defined subtype of DA neuron contributes to the complex range of

164   behaviors ascribed to the mDA system.

165   The gene encoding the transcription factor NeuroD6 (aka NEX1M) has recently gained

166   attention due to its selective expression within subsets of VTA DA neurons while being

167   excluded from the SNc (Khan et al., 2017; Kramer et al., 2018; Viereckel et al., 2016). VTA

168   DA neurons are of particular interest for several reasons. First, the importance of VTA DA

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169   neurons in several aspects of behavioral reinforcement and conditioning has been established

170   through classical studies (reviewed in Di Chiara and Bassareo, 2007; Ikemoto, 2007), and

171   more recently, by the use of optogenetics (Ilango et al., 2014; Kim et al., 2012; Pascoli et al.,

172   2015; Tsai et al., 2009). However, detailed knowledge of the exact nature of those particular

173   DA neurons that contribute to each of these complex behaviors remains elusive. Second,

174   medial DA neurons mediate the most potent responsiveness to addictive drugs via their

175   projection to the nucleus accumbens shell (NAcSh) (Ikemoto and Bonci, 2014). The

176   possibility to ascribe specific aspects of drug responses to a distinct subtype of VTA DA

177   neurons would therefore enhance the understanding of addictive behavior. Third, certain VTA

178   neurons show resistance to degeneration in PD (Brichta and Greengard, 2014), however,

179   depending on their role in behavioral regulation, surviving VTA neurons might contribute to

180   non-motor symptoms including behavioral addictions (Cenci et al., 2015).

181   While *NeuroD6*-expressing DA neurons were recently identified as neuroprotected in

182   experimental PD (Kramer et al., 2018), the potential role of this newly described subtype of

183   VTA neurons in behavioral regulation has remained unexplored. Here, we implemented

184   NeuroD6-Cre mice (*aka* NEX-Cre) to create opportunities for targeting and manipulation of

185   the NeuroD6 subtype VTA neurons. We show that gene targeting of Vesicular monoamine

186   transporter 2 (VMAT2) within this particular DA neuron subtype elevated the locomotor

187   response to psychostimulants while activation of NeuroD6-Cre neurons by optogenetic

188   stimulation in the medial VTA induced DA release and glutamatergic post-synaptic responses

189   in the NAcSh. *In vivo* optogenetic activation of the NeuroD6-Cre VTA subpopulation in a

190   real-time place preference failed to trigger a conditioned response but induced place

191   preference upon direct stimulation. These results advance the current understanding of the

192   VTA circuitry by identifying discrete aspects of reward-related behavior correlated with the

193   NeuroD6 subtype VTA neurons.

194 **Materials and Methods**

195 **Mice**

196 Mice were provided with food and water *ad libitum* and housed according to Swedish

197 legislation (Animal Welfare Act SFS 1998:56) and European Union legislation (Convention

198 ETS 123 and Directive 2010/63/EU). Mice of either sex were used. Experiments were

199 conducted with permission from the local Animal Ethical Committees. DAT-Cre (Ekstrand et

200 al., 2007), Vglut2-Cre (Borgius et al., 2010), Calb2-Cre (Jackson laboratory,

201 RRID:MGI_4365741) and NeuroD6-Cre/NEX-Cre (Goebbels et al., 2006) transgenic mice

202 were bred with C57BL/6N Tac wildtype mice (Taconic) for optogenetics-based experiments.

203 NEX-Cre mice were also bred with *Vmat2$^{lox/lox}$* mice, in which exon 2 of the *Vmat2* gene is

204 flanked by LoxP sites (Narboux-Nême et al., 2011) to generate conditional knockout

205 (*Vmat2$^{lox/lox;NEX-Cre-tg}$*: cKO) mice in which *Vmat2* exon 2 is ablated upon NEX-Cre-mediated

206 recombination of LoxP sites. Littermate mice negative for the NEX-Cre-transgene served as

207 control mice (*Vmat2$^{lox/lox;NEX-Cre-wt}$*: Ctrl) (illustrated in Fig. 2*A)*. Mice were genotyped by PCR

208 using the following primer sequences: Cre (applies to DAT-Cre, NEX-Cre, and Calb2-Cre):

209 5'-ACG AGT GAT GAG GTT CGC AAG A-3'; 5'-ACC GAC GAT GAA GCA TGT TTA

210 G-3'; Vglut2-Cre: 5'-TTG CAT CGC ATT GTC TGA GTA G-3'; 5'-TTC CCA CAC AAG

211 ATA CAG ACT CC-3'; Vmat2Lox: 5'-GAC TCA GGG CAG CAC AAA TCT CC-3'; 5'-

212 GAA ACA TGA AGG ACA ACT GGG ACC C-3'.

213

214 *In situ* **hybridization**

215 <u>*In situ* hybridization (ISH) using radioactive oligoprobes</u>

216 The following probes sequences were used:

217 NeuroD6: NM_009717.2; bases 99-132, 933-966, 1256-1288

218 Th: NM_009377.1; bases 774-807, 272-305, 1621-1655

219   Vmat2exon1: NM_172523.3; bases18-51 and 83-116

220   Vmat2exon2: NM_172523.3; bases 201-237 and 240-276

221   Oligoprobes were 3' end-labeled with [alpha-^{35}S]dATP using terminal deoxynucleotidyl

222   transferase at a specific activity of $5x10^8$ d.p.m./µg. Sections were fixed in 3.7 %

223   formaldehyde in phosphate-buffered saline (PBS) for 1 h, washed in PBS, rinsed in water,

224   dehydrated in 70 % ethanol and air-dried. Hybridization was carried out at 42 °C for 16 h in

225   hybridization medium (Oramacell, France) containing the labeled antisense oligonucleotides

226   (3.10^5 cpm / 100 µl). Sections were washed to a final stringency of 0.5 SSC at 53 °C,

227   dehydrated in ethanol, air-dried and exposed to Fujifilm BioImaging Analyzer BAS-5000 for

228   15 days.

229

230   <u>Double and triple *in situ* hybridization using riboprobes (fluorescent *in situ* hybridization</u>

231   <u>(FISH) or combined FISH /brightfield *in situ* hybridization (FISH/ISH))</u>

232   The following probes sequences were used:

233   Calb2: NM_007586.1; bases 80-793

234   Dat (Slc6a3): NM_012694.2; bases 1015-1938

235   NeuroD6: NM_009717.2; bases 635-1419

236   Th: NM_009377.1; bases 456-1453

237   Vglut2 (Slc17a6): NM_080853.3; bases 2315-3244

238   Viaat (Slc32a1): NM_009508.2; bases 649-1488

239   Vmat 2 Probe 1: Vmat2: NM_0130331.1 (rat); bases 701-1439 (corresponds to exon 6-15 of

240   mouse sequence NM_172523.3)

241   Vmat2 Probe 2: NM_172523.3; bases142-274 i.e. the whole exon 2

242   Detection of Th, Dat, Vglut2, Viaat, Calb2, NeuroD6 mRNA and Vmat2 Probe 1 and Probe 2

243   mRNA in brain tissue using *in situ* hybridization was performed following a previously

244 published protocol (Viereckel et al., 2016). Briefly, mice were sacrificed and brains dissected.

245 Coronal cryosections were prepared, air-dried, fixed in 4% paraformaldehyde and acetylated

246 in 0.25% acetic anhydride/100 mM triethanolamine (pH=8) followed by hybridization for 18h

247 at 65 °C in 100 μl of formamide-buffer containing 1 μg/ml digoxigenin (DIG)-labeled probe

248 for colorimetric detection or 1 μg/ml DIG- or 1 μg/ml fluorescein-labeled probes for

249 fluorescent detection. Sections were washed at 65 °C with SSC buffers of decreasing strength,

250 and blocked with 20% FBS and 1% blocking solution. For colorimetric detection, DIG

251 epitopes were detected with alkaline phosphatase-coupled anti-DIG fab fragments at 1/500

252 and signal developed with NBT/BCIP. For fluorescent detection, sections were incubated

253 with HRP-conjugated anti-fluorescein antibody at 1/1000 concentration (Roche

254 Cat#11426346910, RRID:AB_840257). Signals were revealed with the TSA™ Kit (Perkin

255 Elmer Cat# NEL749A001KT) using biotin-tyramide at 1:75 concentration followed by

256 incubation with neutravidin Oregon Green conjugate at 1:750 (Molecular Probes Cat#A-6374,

257 RRID:AB_2315961). HRP-activity was stopped by incubation of sections in 0,1 M glycine

258 and 3% H_2O_2. DIG epitopes were detected with HRP conjugated anti-DIG antibody at 1:1000

259 (Roche Cat#11207733910, RRID:AB_514500) and revealed with TSA™ Kit (Perkin Elmer

260 Cat# NEL744A001KT) using Cy3 tyramide at 1:200. For triple FISH, TH mRNA was

261 detected with Dinitrophenyl (DNP)-labeled probe; NeuroD6 mRNA with DIG-labeled

262 probe and Vglut2 mRNA with fluorescein-labeled probe. The protocol was the same as

263 described above until revelation: DIG epitopes were detected with HRP anti-DIG fab

264 fragments at 1:3000 and revealed using Cy3 tyramide at 1:50 followed by glycine and H_2O_2

265 treatment. Fluorescein epitopes were detected with HRP anti-fluorescein fab fragments at

266 1:5000 and revealed using Cy2-tyramide at1:250 by glycine and H_2O_2 treatment. DNP

267 epitopes were detected with HRP anti-DNP fab fragments at 1:1000 and revealed using Cy5-

268 tyramide at 1:50, followed by incubation with DAPI. Fluorophore-tyramides were synthetized

269   as previously described (Hopman et al., 1998). All slides were scanned and analyzed on

270   NanoZoomer 2.0-HT Ndp2.view (Hamamatsu). Stereotaxic reference atlases (Franklin and

271   Paxinos, 2008; Fu et al., 2012) were used to outline anatomical borders.

272

273   *Validation of NEX-Cre-mediated recombination of floxed Vmat2 exon 2*

274   Upon genotyping, PCR-validated *Vmat2$^{lox/lox;NEX-Cre-tg}$* (cKO) and *Vmat2$^{lox/lox;NEX-Cre-wt}$* (Ctrl)

275   mice were sacrificed and brains analyzed by *in situ* hybridization to verify NEX-Cre-driven

276   recombination of the floxed exon 2 of the *Vmat2* gene in cKO mice. Littermate Ctrl mice

277   were used to validate wildtype Vmat2 mRNA. A Vmat2 mRNA 2-probe approach was

278   implemented to visualize cells positive for wildtype Vmat2 mRNA and cells positive for a

279   truncated Vmat2 mRNA generated upon NEX-Cre-driven recombination of the floxed *Vmat2*

280   exon 2. Probe 1 (green) was designed for detection of Vmat2 mRNA derived from exon 6-15

281   and Probe 2 (blue) for detection of mRNA from exon 2 (illustrated in Fig. 2*B*). In control

282   mice, both Probe 1 and Probe 2 can bind their target mRNA (wiltype Vmat2 mRNA).

283   Combination of Probe 1 and Probe 2 gives rise to combined blue and green labeling in

284   wildtype DA neurons. In cKO mice, *Vmat2* exon 2 will be deleted specifically in cells

285   expressing the NEX-Cre transgene, leading to production of Vmat2 mRNA missing exon 2

286   but maintaining exons exons 6-15. In Vmat2-expressing cells that do not express the NEX-

287   Cre transgene in cKO mice, wildtype Vmat2 mRNA will be produced. *Vmat2*-targeted cells

288   can thus be identified based on lack of blue color (Probe 2) and presence of green color only

289   (Probe 1). Thus, using the Vmat2 mRNA 2-probe-approach, the color shift from complete

290   overlap of blue and green color in Ctrl mice to the presence of green-only cells in cKO mice

291   is used to verify Cre-LoxP-mediated conditional knockout of the *Vmat2* gene.

292

293   **Immunohistochemistry**

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294   Detection of TH and eYFP proteins took place according to standard immunohistochemical

295   protocols using primary antibodies [mouse anti-TH (1:1000, Millipore Cat# MAB318,

296   RRID:AB_2201528), chicken anti-GFP (1:1000, Abcam Cat# ab13970, RRID:AB_300798)].

297   After overnight incubation, primary antibodies were removed and sections were incubated in

298   specific fluorophore-conjugated secondary antibodies (donkey anti-mouse Cy3, Millipore

299   Cat#AP192C, RRID:AB_11214096, donkey anti-chicken A488, Jackson ImmunoResearch

300   Labs Cat# 703-545-155, RRID:AB_2340375, both 1:500). Upon rinses, slides were

301   coverslipped using Fluoromount Aqueous mounting medium (Sigma-Aldrich Cat# F4680).

302   For bright-field detection of TH, the peroxidase-based method (ABC-kit; Vector Laboratories

303   Cat# PK-4001, RRID:AB_2336810) with DAB chromogen was used. Quantifications were

304   done manually on 3 mice per group. A stereotaxic atlas (Franklin and Paxinos, 2008) was

305   used to outline anatomical borders.

306

307   **Behavioral analysis**

308    $Vmat2^{lox/lox;NEX-Cre-tg}$ cKO and $Vmat2^{lox/lox;NEX-Cre-wt}$ Ctrl mice were analyzed in the following

309   behavioral tests:

310   <u>Baseline locomotion</u>

311   Spontaneous locomotion and habituation in a novel environment were monitored for 30 min

312   upon placing the mice in Makrolon® polycarbonate boxes containing 1.5 cm bedding and a

313   transparent plexiglas lid. Locomotor behavior of the mice was recorded by the EthovisionXT

314   software (Noldus, RRID:SCR_000441).

315   <u>Sucrose preference test</u>

316   Preference to sucrose was assessed in the home cage of the mice. The mice were housed

317   individually in cages containing two drinking bottles. After 48h of habituation to the

318   experimental set up, they were presented to one bottle of tap water and one of sucrose

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319　solution (1, 3 and 10%) that were replaced and weighted every 24h. Each concentration was

320　tested twice and the position of the bottles was alternated to avoid side bias.

321　Ethanol preference test

322　Individually housed mice had access to one bottle of tap water and one of alcohol solution (3,

323　6 and 10%) that were replaced and weighted every 24h. Each concentration of ethanol was

324　tested four times.

325　Cocaine-induced locomotion

326　Mice were placed in Makrolon® polycarbonate boxes containing 1.5cm bedding and a

327　transparent plexiglass lid and their locomotor behavior was recorded 30 min pre- and 60 min

328　post-injection of saline or cocaine injections (5, 10 & 20 mg/kg, i.p.) on four consecutive

329　days. Locomotor behavior of the mice was recorded by the EthovisionXT software (Noldus,

330　RRID:SCR_000441).

331　Amphetamine sensitization

332　Upon habituation, mice received a saline injection (Day 1) followed by 4 days of

333　amphetamine injections (Days 2-5, 3 mg/kg, i.p.) followed by a last injection on Day 17.

334　Locomotion was recorded 30 min pre- and 1.5 h post-injection. Locomotor behavior of the

335　mice was recorded by the EthovisionXT software (Noldus, RRID:SCR_000441).

336　Conditioned-placed preference (CPP)

337　An apparatus (Panlab, Harvard Apparatus) consisting of two-main compartments [20cm (W)

338　x 18cm (L) x 25cm (H)] with distinct wall and floor texture patterns and one connecting,

339　transparent compartment [20cm (W) x 7cm (L) x 25cm (H)]. The CPP procedure was

340　conducted throughout six days. Firstly, during the Pre-test, the mice were placed in the

341　apparatus and left to freely explore. This session was used to assess initial preferences and to

342　calculate the preference score (see below). During the next four consecutive conditioning

343　days, the mice were constrained in one of the two main compartments and received drug

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344   injections (cocaine, 20 mg/kg or amphetamine, 3 mg/kg, i.p.) in the least preferred

345   compartment or saline injections in the opposite one. The conditioning sessions were repeated

346   twice a day [morning (a.m); afternoon (p.m)] and the treatment was alternated between days.

347   Thus, the mice received in total 4 injections of saline and 4 injections of the drug,

348   counterbalanced between sessions and genotypes. On the Test day, the mice were placed

349   again in the apparatus and were let to freely explore. The preference score was calculated by

350   subtracting the time in seconds the animal spent in the drug-paired compartment during pre-

351   test from the time spent in the same compartment during the test (ΔSec). All sessions lasted

352   30 minutes and the locomotor behavior of the mice was recorded by the EthovisionXT

353   software (Noldus, RRID:SCR_000441).

354

355   **Stereotaxic injections**

356   Optogenetic viruses were purchased from University of North Carolina, Vector Core, US.

357   DAT-Cre, Vglut2-Cre, Calb2-Cre and NEX-Cre mice (>8 weeks; >20g) were deeply

358   anesthetized with isofluorane and received infusions of 300nl of *AAV5-EF1a-DIO-*

359   *ChR2(H134)-eYFP* or *AAV5-EF1a-DIO-eYFP-WPREpA* in the right VTA (AP: -3.45 mm, L:

360   -0.2 mm, V: -4.4 mm according to (Franklin and Paxinos, 2008) at 100 nl min^{-1} flow rate. For

361   behavioral analysis, an optic fiber was implanted and stabilized above the right VTA (AP: -

362   3.4 mm, ML: -0.3 mm, DV: -4.0mm) using anchor screws and dental cement. A subset of

363   NEX-Cre mice was injected bilaterally with *AAV5-EF1a-DIO-ChR2(H134)-eYFP* before

364   fiber implantation. After post-mortem histological analysis, mice with limited transfection in

365   the VTA and/or misplaced optic fiber were excluded from statistical analysis.

366

367   **Imaging, cell counting and analysis of projection target areas**

368   Quantification of *in situ* hybridization. Manual counting of cells expressing mRNAs of

369   interest was performed in 2-3 mice per probe pair with Th mRNA as reference for outline of

370   the VTA and Th, Dat, Viaat or Vglut2 mRNA as reference for distinct cell soma. A signal for

371   a particular mRNA was considered as specific for a particular cell when five contiguous

372   fluorescent dots were present within the outline of the cell soma.

373   Quantification of immunohistochemistry. Sections of Calb2-Cre and NEX-Cre mice injected

374   with *AAV5-EF1a-DIO-ChR2(H134)-eYFP* containing the VTA (-3.28 mm to -3.80 mm from

375   bregma according to Franklin and Paxinos, 2008) were immunostained for eYFP and TH as

376   described above. Z-stacks in 4 different positions within the VTA, representative of subareas

377   (VTA1-VTA4 of which VTA1 and VTA3 represented medial VTA and VTA2 and VTA4

378   lateral VTA on two different bregma levels, Fig. 5*G*), were acquired using a Zeiss Confocal

379   microscope (LSM 700, 20x magnification). Co-labeling of YFP and TH was identified for

380   each   fluorescent   channel   and   counted   manually   using   the   ImageJ   software

381   (RRID:SCR_003070). A minimum of 3 mice of each genotype was processed and analyzed.

382   Analysis of projection areas. Fluorescent microscopy (Zeiss Confocal microscope) was used

383   to detect eYFP-positive fibers in sections derived from the whole brain of NEX-Cre, Calb2-

384   Cre, DAT-Cre and NEX-Cre mice injected into the VTA with *AAV5-EF1a-DIO-*

385   *ChR2(H134)-eYFP*. A minimum of 2 mice of each genotype was analyzed by 2 persons blind

386   to the genotype of the mice.

387

388   **Fast-scan cyclic voltammetry in slices**

389   For DA recordings in terminal areas upon photostimulation, DAT-Cre, Calb2-Cre and NEX-

390   Cre mice were injected with *AAV5-EF1a-DIO-ChR2(H134)-eYFP* or *AAV5-EF1a-DIO-*

391   *eYFP-WPREpA* as described above.

392 <u>Carbon Fiber Microelectrodes.</u> Carbon fiber working electrodes were fabricated by aspirating

393 7 μm diameter carbon fibers (Cytec engineered materials, Tempe, AZ) into borosilicate glass

394 capillaries (1.2 mm O.D., 0.69 mm I.D., Sutter Instrument Co, CA). Capillaries were adjusted

395 (Sutter Instrument, P-97) and sealed with epoxy (EpoTek 301, Epoxy Technology, MA).

396 Electrodes were tested on bath applications of known concentrations of DA. Only electrodes

397 showing good reaction kinetics (current *vs* time plots, and current *vs* voltage plots) were used.

398 <u>Fast-scan cyclic voltammetry (FSCV).</u> A Dagan Chem-Clamp potentiostat (Dagan

399 Corporation, MN) and two data acquisition boards (PCI-6221, National Instruments, TX) run

400 by the TH 1.0 CV program (ESA, MA) were used to collect all electrochemical data. Cyclic

401 voltammograms were obtained by applying a triangular waveform potential (-0.4 to $+1.3$ V

402 vs Ag/AgCl) repeated every 100 ms at a scan rate of 200 V/s (low pass Bessel filter at 3 kHz).

403 Each cyclic voltammogram was a background-subtracted average of 10 successive cyclic

404 voltammograms taken at the maximum oxidation peak current. All electrodes were allowed to

405 cycle for at least 15 min before recording to stabilize the background current. The recorded

406 current response was converted to DA concentration via *in vitro* electrode calibration with

407 standard DA solution after each experiment. For optically evoked DA release,

408 photostimulation during FSCV recordings was generated through a 3.4 Watt 447 nm LED

409 mounted on the microscope oculars and delivered through the objective lens.

410 Photostimulation was controlled via a DigiData 1440A, enabling control over duration and

411 intensity. Illumination intensity typically scaled to 3 mW/mm^2. Acquired data were analysed

412 and plotted using Matlab (RRID:SCR_001622) routines and statistical analysis was

413 performed using Prism 6.0 (GraphPad Software, La Jolla, CA, RRID:SCR_002798)

414

415 **Patch-clamp electrophysiology in slices**

16

416 For recordings of excitatory post synaptic currents (EPSCs) and inhibitory post synaptic

417 currents (IPSCs) upon optogenetic stimulation, Calb2-Cre and NEX-Cre mice (>8 weeks, >20

418 g) were injected with *AAV5-EF1a-DIO-ChR2(H134R)-eYFP* as described above. Mice were

419 deeply anaesthetized with pentobarbital (200 mg.kg^{-1} i.p.; Virbac) and perfused intracardially

420 with 10 ml ice-cold sucrose-artificial cerebrospinal fluid (ACSF) containing (in mM): 75

421 sucrose, 87 NaCl, 2.5 KCl, 7 MgCl$_2$, 0.5 CaCl$_2$, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$ and continuously

422 bubbled with carbogen (95% O2–5% CO2). 200 µm coronal brain slices were cut in sucrose-

423 ACSF. Slices were transferred to a perfusion chamber containing ACSF at 31 °C (in mM):

424 126 NaCl, 2.5 KCl, 1.2 MgCl$_2$, 2.4 CaCl$_2$, 1.4 NaH$_2$PO$_4$, 25 NaHCO$_3$, 11 glucose,

425 continuously bubbled with carbogen. After at least 45 min recovery, slices were transferred to

426 a recording chamber continuously perfused with ACSF (2–3 ml min^{-1}) maintained at 29–31

427 °C. Patch pipettes (3.5–5.5 MΩ) were pulled from borosilicate glass and filled with internal

428 recording solution containing (in mM): 120 CsCH$_3$SO$_3$, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5

429 TEA, 2.5 Mg-ATP, 0.25 Na-GTP, at pH 7.25 and 285 ± 5 mOsm. VTA neurons and terminals

430 were visualized by epifluorescence and visually guided patch recordings were achieved using

431 infrared differential interference contrast (IR-DIC) illumination (Axiocam MRm, Zeiss).

432 ChR2 was activated by flashing blue light (5-ms pulse width) through the light path of the

433 microscope using a light-emitting diode (UHP-LED460, Prizmatix) under computer control.

434 EPSCs and IPSCs were recorded in whole-cell voltage clamp (-60 mV and 0mV holding

435 potential respectively, Multiclamp 700B amplifier, Axon Instruments), filtered at 2 KHz,

436 digitized at 10 KHz (Axon Digidata 1550, Axon Instruments), and collected online using

437 pClamp 10 software (Molecular Device). Series resistance and capacitance were

438 electronically compensated before recordings. Estimated liquid-junction potential was 12mV

439 and left uncorrected. Series resistance and/or leak current were monitored during recordings

440 and cells that showed >25% change during recordings were considered unstable and

17

441   discarded. Single-pulse (5-ms) photostimuli were applied every 55 s and 10 photo-evoked

442   currents were averaged per neuron per condition. DMSO stock solution of 6,7-

443   dinitroquinoxaline-2,3-dione (DNQX, 10 mM, Sigma) was diluted 1,000-fold in ACSF and

444   bath applied. Current sizes were calculated by using peak amplitude from baseline. Decay

445   time constants (τ) were calculated by fitting an exponential function to each averaged current

446   trace using the following formula: $f(t) = e^{-t/\tau} + C$.

447

448   **Place preference upon optogenetic stimulation**

449   The three-compartment apparatus (Panlab, Harvard Apparatus) used in the CPP experiments

450   (above) was also implemented in the optogenetics-driven place preference experiments to

451   address real time place preference upon photostimulation (RT-PP) and conditioned response

452   (CR), the association to compartment previously paired with photostimulation. Similar to

453   protocols previously described by others (Qi et al., 2016; Root et al., 2014), the entry of the

454   mouse into one of the two main compartments was paired with intracranial VTA

455   photostimulation (10 ms pulse width, 20 Hz, 10 mW) while the interconnecting compartment

456   was not coupled to light stimulation (neutral) at all. The EthovisionXT tracking software

457   (Noldus, RRID:SCR_000441) was used to monitor behavior and trigger laser stimulation.

458   Behavior was assessed over the course of 8 experimental days (Fig. 8*A*) subdivided into two

459   recording phases with a minimum 3-day rest period in between ("Phase 1"; Days 3-5 and

460   "Reversal Phase"; Days 6-8). On Day 1 ("habituation"), the mouse was connected to the optic

461   fiber cord and allowed to acclimatize. On Day 2 ("pre-test"), the mouse was placed in the 3-

462   compartment apparatus for 15 min to freely explore, while attached to the optic fiber cord but

463   without receiving any photostimulation; the preference for each compartment was evaluated.

464   On Days 3, 4 ("RT-PP"), entry into the assigned light-paired compartment (non-preferred in

465   pre-test) resulted in blue laser photostimulation delivered as continuous train of pulses (10ms

466   pulse width, 20Hz, 10mW). On Day 5 ("CR"), the time spent in each compartment was

467   measured for 15 min with no delivery of photostimulation. In the Reversal phase, the protocol

468   was repeated but with stimulation in the opposite compartment compared to Phase 1. "High-

469   power" experiments followed the same structure except that the mice received a stimulation

470   of higher power (5ms pulse width, 20Hz, 20mW).

471   For the Neutral Compartment Preference (NCP) test, a modified version of the test described

472   above was used with the following changes: Entry into either one of the two main

473   compartments was coupled to light-stimulation, while only entry the interconnecting

474   compartment had no consequence. The experiment took place on three consecutive days:

475   During the first two days (Stim1 & Stim2), the mice received stimulation upon entry in any of

476   the main compartments while the third day was stimulation-free and used to study the

477   presence of any conditioned responses (CR) induced by the experience with the stimulation.

478

479   **Experimental design and statistical analysis**

480   Regular and repeated measures (RM) two-way ANOVA and unpaired t-tests were used to

481   compare mean scores of Ctrl and cKO mice in behavioral tests. To analyze cocaine-induced

482   locomotion during CPP, a mixed-effects model was used. Post hoc comparisons were

483   performed by Sidak's multiple comparison test. Unpaired t-test was used to compare mean

484   DA release between ChR2- and eYFP (control)-injected DAT-Cre, Calb2-Cre and NEX-Cre

485   mice for each region where the measurements were performed. Paired t-tests were used to

486   compare pre- and post- DNQX EPSP recordings. Two-way RM ANOVA with Day and

487   Chamber were used as factors throughout the optogenetic experiments followed by Tukey's

488   post hoc test. When the days of stimulation were averaged, one-way ANOVA was used to

489   unravel the effect of compartment (paired, unpaired, neutral) on time spent and Tukey's

490   multiple comparison test for post hoc analysis. Data are presented as mean ± SEM unless

491   stated otherwise. Data analysis was performed with Prism8 (RRID: SCR_002798). Detailed

492   statistical information is shown in Table 1.

493

494   **Results**

495   *NeuroD6 mRNA is found in a modest population of the medial VTA where it co-localizes*

496   *extensively with dopaminergic markers and with a glutamatergic marker to minor degree*

497   To address the distribution pattern and neurotransmitter identity of *NeuroD6*-expressing

498   neurons, double-labeling fluorescent *in situ* hybridization was first performed in which

499   NeuroD6 mRNA (Fig. 1*A, C*) was compared to Tyrosine hydroxylase (Th) mRNA encoding

500   the rate-limiting enzyme (TH) of DA synthesis (Fig. 1*B, C*). Using the distribution pattern of

501   Th mRNA as reference, DA neurons of the SNc and VTA were identified, including the

502   paranigral (PN), parainterfascicular (PIF), parabrachial pigmented nucleus (PBP),

503   interfascicular nucleus (IF) and rostral linear nucleus (RLi) subareas of the VTA (Fig. 1*A- C*).

504   NeuroD6 mRNA was excluded from the SNc, but was detected in scattered VTA neurons.

505   Most NeuroD6 neurons were found within the PN, PIF and PBP subareas of the VTA,

506   followed by fewer NeuroD6 neurons in the IF and RLi (Fig. 1*A, C).* Co-detection analysis

507   showed that all neurons detected as positive for NeuroD6 mRNA within the PN, PIF, PBP, IF

508   and RLi were positive for Th mRNA (Fig 1*C*). Quantification verified that 100% of NeuroD6

509   mRNA-positive cells in the PN/PIF, PBP, IF and RLi were positive for Th mRNA, while 12%

510   of all Th-expressing neurons within these VTA subareas contained NeuroD6 mRNA (Fig.

511   1*D*). To further address the dopaminergic identity of NeuroD6 neurons, co-detection of

512   NeuroD6 mRNA with Dat mRNA, encoding the Dopamine transporter (DAT), was

513   performed. Similar to the overlap between NeuroD6 and Th, all neurons detected as positive

514   for NeuroD6 mRNA in the VTA were positive for Dat mRNA (Fig. 1*E).* To address the

515   neurotransmitter identity of the NeuroD6-mRNA-positive VTA neurons, co-detection

516   analyses of NeuroD6 mRNA with Vesicular glutamate transporter 2 (Vglut2) and Vesicular

517   inhibitory amino acid transporter (Viaat) mRNAs were performed for identification of

518   glutamatergic and GABAergic neurons, respectively. NeuroD6 mRNA showed some co-

519   localization with Vglut2 mRNA (Fig. 1*F*), while no or very few NeuroD6-positive cells in the

520   VTA were detected as positive for Viaat mRNA (Fig. 1*G*). To address the overlap of

521   NeuroD6 mRNA with Vglut2 and Th mRNA in detail, triple-labeling *in situ* hybridization of

522   NeuroD6, Th and Vglut2 mRNAs was performed (Fig. 1*H-P*). This experiment confirmed

523   that all NeuroD6 VTA neurons within the PN, PIF, PBP, IF and RLi were detected as positive

524   for Th (Fig. 1*H, K, N)* and that some NeuroD6 neurons co-localized with Vglut2 (Fig. 1*I,L,N*).

525   Further, the experiment identified that these NeuroD6/Vglut2 double positive cells in the

526   VTA were positive for Th mRNA (Fig. *1J,M,N*). Quantification verified that 100% of

527   NeuroD6 VTA neurons were positive for Th (NeuroD6+/Th+), and showed that 12% of these

528   NeuroD6+/Th+ VTA neurons were also positive for Vglut2 mRNA. 12% thus displayed a

529   NeuroD6+/Th+/Vglut2+ triple-positive molecular phenotype, while remaining 88% of

530   NeuroD6/Th neurons were negative for Vglut2 (NeuroD6+/Th+/Vglut2-) (Fig. *1O*).

531   NeuroD6+/Th+/Vglut2+ and NeuroD6+/Th+/Vglut2- VTA neurons were distributed

532   throughout the VTA with highest density in PN, PIF and PBP subareas (Fig. *1M, P*).

533   *Conditional ablation of the Vmat2 gene in NeuroD6-Cre VTA neurons – a model for spatially*

534   *restricted DA deficiency*

535   To analyze the consequences of lost ability for vesicular packaging of DA in NeuroD6 VTA

536   DA neurons, the *Slc18a2/Vmat2* gene encoding the Vesicular monoamine transporter 2

537   (VMAT2) was targeted using a NeuroD6-Cre (NEX-Cre) transgenic mouse line. By breeding

538   NEX-Cre mice with *Vmat2^{lox/lox}* mice, *Vmat2^{lox/lox;NEX-Cre-tg}* (cKO) and littermate control (Ctrl)

539   mice were produced (Fig. 2*A*). Upon PCR-based analysis of genotype, brains from Ctrl and

540   cKO mice were analyzed by *in situ* hybridization to verify loss of full-length Vmat2 mRNA

21

541 in cKO mice. Due to the scarcity of NeuroD6-positive neurons in the VTA, a Vmat2 mRNA

542 2-probe approach was utilized to allow detection of gene-targeted neurons. Vmat2 Probe 1

543 was designed to detect all cells positive for Vmat2 mRNA, while Vmat2 Probe 2 was

544 designed to bind mRNA derived from exon 2, the exon targeted for recombination by Cre

545 recombinase (Fig. 2*B*). In the ventral midbrain of control mice, Probe 1 (green) and Probe 2

546 (blue) were detected throughout the VTA and SNc areas with complete overlap (Fig. 2*C*, left

547 panel). In the corresponding area of cKO mice, the majority of cells were positive for both

548 Probe 1 and Probe 2 with complete overlap (Fig. 2*C*, right panel). However, throughout the

549 PN, PIF, PBP, IF VTA subareas, sparse cells showing green color only (Probe 1) were

550 detected, thus visualizing *Vmat2*-gene targeted cells among the mass of VTA DA neurons

551 positive for both Vmat2 Probe 1 and 2 (Fig. 2*C*, right panel). Having confirmed NEX-Cre-

552 mediated recombination of the floxed *Vmat2* gene within scattered neurons of the VTA, other

553 brain areas in which monoaminergic neurons reside were addressed by oligo *in situ*

554 hybridization. Apart from the modest VTA population positive for NeuroD6 mRNA,

555 NeuroD6 mRNA was not detected within any other monoaminergic cell group, identified by

556 Th and Vmat2 mRNA (Fig. 2-1). However, as previously reported (Goebbels et al., 2006),

557 NeuroD6 was abundant in several non-dopaminergic brain structures, primarily the cerebral

558 cortex and hippocampus (Fig. 2-1). In accordance with the lack of NeuroD6 in all

559 monoaminergic cell groups apart from the VTA, Vmat2 Probe 1 and Probe 2 showed

560 complete overlap in these areas, including locus coeruleus, ventromedial hypothalamus and

561 nucleus raphe obscurus, while none displayed labeling from Probe 1 only (Fig. 2*D*). These

562 experiments showed that in cKO mice, Vmat2 mRNA was selectively ablated within the

563 VTA. To address if the targeted deletion of *Vmat2* in NeuroD6 neurons of the VTA affected

564 the morphology of the midbrain DA system, distribution patterns of Th mRNA and TH

22

565 protein were addressed, none of which revealed any gross anatomical difference in the

566 dopaminergic system between Ctrl and cKO mice (Fig. 2*E*, Fig. 2-1).

567 *Heightened locomotor response to psychostimulants upon gene-targeting of Vmat2 in NEX-*

568 *Cre VTA neurons*

569 To address if it is possible to dissociate an explicit behavioral role of DA neurotransmission

570 exerted by NeuroD6 VTA DA neurons from the range of behaviors ascribed to the mDA

571 system, *Vmat2$^{lox/lox;NEX-Cre-tg}$* cKO mice were tested in a battery of tests relevant to the mDA

572 system and compared to *Vmat2$^{lox/lox;NEX-Cre-wt}$* Ctrl mice. To assess body weight, mice were

573 weighed every week from weaning to adulthood. cKO mice were similar to their Ctrl

574 littermates weight-wise (effect of age: $F_{(4,158)} = 79.8$, $p < 0.001$; genotype: $F_{(1,158)} = 4.67$ $p =$

575 0.032; no age x genotype interaction, no post hoc differences between genotypes) (Fig. 3*A*).

576 <u>Baseline locomotion</u>

577 The habituation response to a novel environment, a gross measure of stress and exploratory

578 behavior, was addressed. Both Ctrl and cKO mice showed the same rate of reaching a stable

579 plateau in baseline locomotion (effect of time: $F_{(5,160)} = 69.5$, $p < 0.001$; effect of genotype:

580 $F_{(1,32)} = 0.00912$, $p = 0.535$) (Fig. 3*B*).

581 <u>Sucrose and ethanol preference</u>

582 A sucrose bottle preference test was next performed. Both Ctrl and cKO mice preferred the

583 ascending concentrations of sucrose solutions over water (effect of concentration: $F_{(2,66)} =$

584 151, $p < 0.001$), but no differences between the genotypes were observed (effect of genotype:

585 $F_{(1,33)} = 1.12$, $p = 0.297$) (Fig. 3*C*). The rewarding effect of alcohol was subsequently

586 measured by using increasing concentrations of ethanol (3, 6, 10%) presented in a bottle

587 preference test. Again, both Ctrl and cKO mice preferred the presented reward over water

588 (effect of concentration: $F_{(2,52)} = 14.2$, $p < 0.001$), but there was no difference between the

589 genotypes (effect of genotype: $F_{(1,26)} = 0.969$, $p = 0.334$). However, post hoc analysis showed

23

590   that Ctrl mice significantly preferred the 6% and 10% concentrations over the 3% solution

591   (§§§ $p < 0.001$ 3% vs 6% and 10% ethanol in ctrl mice), while a trend towards significant

592   differences in cKO mice was observed only between the 3% and 10% ethanol solutions (3%

593   vs 10%: $p < 0.072$) (Fig. 3*D*).

594   Cocaine- and amphetamine-induced locomotion

595   To address locomotor responses upon psychostimulant-injections, cocaine and amphetamine

596   administration protocols were applied and locomotion was measured. Upon administration of

597   acute ascending doses of cocaine (5, 10 and 20 mg/kg), both Ctrl and cKO mice displayed

598   increased locomotion in a dose-dependent manner, however, no significant differences were

599   observed between genotypes (effect of session: $F_{(3,99)} = 108$, $p < 0.001$; genotype, $F_{(1,33)} =$

600   1.65, $p = 0.208$; session x genotype interaction: $F_{(3,99)} = 1$, $p = 0.396$) (Fig. 3*E)*. Next, an

601   amphetamine sensitization protocol was applied. All mice responded to amphetamine with

602   hyperlocomotion, but the effect was significantly higher in cKO mice than control mice in

603   days 4, 5 and 17 of the experiment (effect of day: $F_{(5,160)} = 40.9$, $p < 0.001$; genotype, $F_{(1,32)} =$

604   9.09, $p = 0.005$; day x genotype interaction: $F_{(5,160)} = 4.79$; $p < 0.001$; ctrl vs cKO Day 4 $p =$

605   0.011, Day 5 $p < 0.001$, Day 17 $p = 0.029$) (Fig. 3*F)*.

606

607   CPP

608   In order to study the reinforcing effects of psychostimulants, a CPP procedure was applied

609   (Fig. 3*G*). Both Ctrl and cKO mice showed preference to the cocaine- or amphetamine-paired

610   compartment over the saline-paired compartment with no significant difference between

611   genotypes (ctrl vs cKO cocaine: $p = 0.860$, amphetamine $p = 0.744$) (Fig. 3*H,J*). In addition to

612   preference, locomotion was monitored during the conditioning sessions. cKO mice displayed

613   increased locomotor responses after repeated administration of cocaine compared to Ctrl mice

614   (Fig. 3*I*, effect of session; $F_{(3,75)} = 4.4$, $p = 0.006$; effect of genotype $F_{(1,25)} = 5.2$, $p = 0.031$, no

24

615   differences in post hoc analysis). In contrast, in the CPP paradigm, repeated administration of

616   amphetamine did not induce elevated locomotion in cKO over Ctrl mice (Fig. 3*K*, effect of

617   session; $F_{(3,85)}$ = 24.0, *p* < 0.001; effect of genotype $F_{(1,30)}$ = 0.0631, *p* = 0.803).

618

619   *NeuroD6 mRNA co-localizes partly with Calb2 mRNA but Calb2 mRNA is abundant*

620   *throughout VTA and SNc*

621   To further characterize the molecular identity of NeuroD6 VTA neurons, *in situ* hybridization

622   was next used to address the putative overlap between NeuroD6 and Calb2 mRNAs.

623   Distribution patterns of NeuroD6 and Calb2 mRNAs within midbrain DA neurons were

624   recently described without addressing their putative overlap (Viereckel et al., 2016). In

625   contrast to the selective localization of NeuroD6 mRNA within the VTA and its exclusion

626   from the SNc, Calb2 mRNA was abundant in both VTA and SNc (Fig. 4*A*). The restricted

627   number of NeuroD6 neurons in the VTA showed partial overlap with Calb2 mRNA: 54% of

628   all NeuroD6 VTA neurons were positive for Calb2 mRNA while 20% of Calb2 neurons

629   expressed NeuroD6 mRNA (Fig. 4*A*). Further quantification within the VTA showed that

630   Calb2 mRNA was detected in 51% of all Th-neurons, with a similar match of Calb2/Dat co-

631   localization at 50% (Fig. 4*B, C)*. Some Calb2 neurons in the VTA were positive for Vglut2

632   mRNA (7%)(Fig. 4*D*) while 20% of all Calb2 neurons in the VTA were positive for Viaat

633   mRNA (Fig 4*E*).

634

635   *Spatially restricted striatal innervation by NeuroD6-Cre and Calb2-Cre VTA neurons*

636   Next, to allow analysis of projections, signaling properties and behavioral regulation of NEX-

637   Cre and Calb2-Cre VTA neurons, optogenetics was implemented. Upon infusion of viral

638   particles carrying a double-floxed *DIO-ChR2-eYFP* genetic construct encoding both

639   *Channelrhodopsin* (ChR2) and the *enhanced yellow fluorescent protein* (eYFP) into the VTA,

640   mice were analyzed in different parameters. DAT-Cre and Vglut2-Cre transgenic mice were

641   used as controls based on their representation of VTA and SNc dopaminergic and

642   glutamatergic neurons, respectively (Hnasko et al., 2012; Pascoli et al., 2015; Qi et al., 2016;

643   Stuber et al., 2010; Yoo et al., 2016). First, Cre-driven expression of the *DIO-ChR2-eYFP*

644   construct in DAT-Cre, Vglut2-Cre, Calb2-Cre and NEX-Cre mice was analyzed histologically

645   by comparing YFP with TH immunolabeling (Fig. 5*A*). In DAT-Cre, Vglut2-Cre, Calb2-Cre

646   and NEX-Cre mice, YFP fluorescent labeling was identified in the VTA, verifying the

647   activity of each Cre-driver to recombine the floxed optogenetic construct (Fig 5*B, C, D, E, F*).

648   YFP co-localized extensively with TH in the VTA. YFP was strongest and most abundant in

649   the VTA of DAT-Cre mice, while Vglut2-Cre, Calb2-Cre and NEX-Cre mice all showed

650   lower amount of cells positive for YFP (Fig 5*B, C, D, E, F*). Next, to reveal target areas,

651   sections throughout the entire brain of all four Cre-driver mouse lines were analyzed and

652   compared. Some target areas were the same for all four Cre-drivers, including the NAcSh and

653   ventral pallidum, while others differed, such as the distribution within the medial and lateral

654   habenula (Table 2). Overall, the density of YFP-positive fibers was substantially lower in

655   NEX-Cre and Calb2-Cre mice than in DAT-Cre and Vglut2-Cre mice. Following analysis of

656   sections throughout the brain, the VTA and striatum were analyzed in more detail. DAT-Cre

657   mice showed strong cellular YFP labeling within all VTA subareas (sparse in RLi) and within

658   the SNc, primarily on the injected side (Fig. 5*C-C''*). YFP-positive fibers were distributed

659   across the striatal complex including primarily the dorsomedial striatum, NAcSh, NAc core

660   and the olfactory tubercle (OT) (Fig. 5*C-C''*). Vglut2-Cre mice showed YFP-labeled cell

661   bodies primarily in the medial VTA with fibers innervating the NAc and OT (Fig 5*D-D''*).

662   Next, Calb2-Cre and NEX-Cre mice were addressed. Calb2-Cre mice showed similar

663   distribution of YFP-labeling as DAT-Cre within VTA, but the density was more sparse than

664   in DAT-Cre mice (Fig. 5*E-E´*). YFP-positive fibers in the striatal complex were detected in

26

665   the OT (Fig. 5*E-E´´*). NEX-Cre mice showed a low number of YFP cells in the VTA (Fig. 5*F-*

666   *F''*), in accordance with the modest distribution of endogenous NeuroD6 mRNA described

667   above. Weak YFP fluorescence was detected in fibers throughout the NAcSh and OT (Fig.

668   5*F-F''*). The distribution pattern of YFP-positive cells in the VTA of NEX-Cre mice was

669   similar as distribution of endogenous NeuroD6 mRNA. However, the YFP appeared more

670   abundant than the above analyzed NeuroD6 mRNA. Quantification was performed to address

671   the overlap between YFP and TH. The majority of NEX-Cre/YFP and Calb2-Cre/YFP

672   neurons showed TH immunoreactivity, however, for both Cre-lines, a number of YFP cells

673   were negative for TH (NEX-Cre/ChR2: TH+: 4013 ± 21.72, eYFP+ 965 ± 4.17, double: 715

674   ± 3.24; Calb2-Cre/ChR2: TH+: 4187 ± 18.9, eYFP+: 1396 ± 6.04, double: 939 ± 4.69). In

675   total, 74% of NEX-Cre and 67% of Calb2-Cre neurons showed overlap between YFP and TH

676   (Fig. 5*G,H*).

677   *Optogenetic stimulation in striatal target areas of NeuroD6 and Calb2 VTA neurons verifies*

678   *DA release*

679   To address neurotransmitter release, extracellular DA concentration upon optogenetic

680   stimulation was recorded using fast-scan cyclic voltammetry in slice preparations. DAT-Cre,

681   NEX-Cre and Calb2-Cre mice injected with the same *DIO-ChR2-eYFP* construct as above

682   (Fig. 6*A*) were analyzed upon photostimulation and subsequent recording within the NAcSh

683   and OT (Fig. 6*B*). Cre-mice injected with *DIO-eYFP* were used as controls. DA levels (~1

684   μM) were readily recorded upon photostimulation in both the NAcSh of DIO-ChR2 injected

685   DAT-Cre (0.9699 ± 0.1471 μM) and NEX-Cre mice (0.4701 ± 0.08043 μM), while a lower

686   signal was obtained in the NAcSh of Calb2-Cre/ChR2 mice (0.01509 ± 0.002845 μM) (Fig.

687   6*C,D*). Upon photostimulation and recording in the OT, lower DA levels (~200 nM) than

688   those measured in the NAcSh were obtained in DAT-Cre/ChR2 mice (0.2129 ± 0.01291 μM)

689   while even smaller levels were detected in both Calb2-Cre/ChR2 (0.02097 ± 0.002712 μM)

690 and NEX-Cre/ChR2 mice (0.01362 ± 0.002304 μM) (Fig. 6*C,D*). Despite comparably low in

691 size, all DA levels recorded in mice expressing the ChR2-YFP were significantly larger than

692 in mice injected with the control virus (DAT-Cre, NAcSh ChR2: 0.9699 ± 0.1471 μM, eYFP:

693 0.006802 ± 0.0008813 μM, $t_{(9)}$=6.55 *p* < 0.0001, OT ChR2 0.2129 ± 0.01291 μM vs eYFP

694 0.004649 ± 0.0009871 μM, $t_{(9)}$=16.08 *p* < 0.0001; NEX-Cre, NAcSh ChR2: 0.4701 ± 0.08043

695 μM, eYFP: 0.0102 ± 0.001682 μM, $t_{(9)}$=5.716 *p* < 0.0001, OT ChR2: 0.01362 ± 0.002304

696 μM, eYFP: 0.005791 ± 0.0008003 μM, $t_{(9)}$=3.209 *p* = 0.0049; Calb2-Cre, NacSh ChR2:

697 0.01509 ± 0.002845 μM, eYFP: 0.006087 ± 0.001746 μM, $t_{(9)}$=2.696 *p* = 0.0148, OT ChR2:

698 0.02097 ± 0.002712 μM, eYFP 0.007081 ± 0.001315 μM, $t_{(9)}$=4.607 *p* = 0.0002) (Fig. 6*C,D*).

699

700 *Optogenetic stimulation in striatal target areas of NeuroD6 and Calb2 VTA neurons reveals a*

701 *glutamatergic post-synaptic response*

702 To address the presence of post-synaptic currents in NAcSh and OT neurons upon

703 optogenetic activation, patch clamp electrophysiology was implemented in NEX-Cre and

704 Calb2-Cre injected with *DIO-ChR2-eYFP* (Fig. 7). Upon optogenetic stimulation, 82% of

705 neurons in the NAcSh NEX-Cre mice (18 out of 22 cells) and 87% of OT neurons in Calb2-

706 Cre mice (13 out of 15 cells) showed excitatory post-synaptic currents (EPSCs, NEX-Cre

707 NAcSh, mean amplitude 28 ± 6.8 pA; Calb2-Cre OT, mean amplitude 39 ± 7.7 pA;) (Fig.

708 7*B,C*). In both cases, EPSCs were almost completely abolished after bath application of 10

709 μM of the AMPA receptor antagonist DNQX, demonstrating that the recorded currents are

710 AMPA receptor-mediated (NEX-Cre NAcSh mean amplitude: control: 33 ± 13 pA, DNQX

711 1.5 ± 0.96 pA $t_{(5)}$=2.602 *p* = 0.0481; Calb2-Cre OT*,* mean amplitude: control: 46 ± 16 pA,

712 DNQX: 0.74 ± 0.74 pA $t_{(4)}$=2.867 *p* = 0.0456) (Fig. 7*D*). The synaptic delay of the EPSCs

713 was short (NEX-Cre NAcSh 3.3 ± 0.25 ms; Calb2-Cre OT 3.6 ± 0.21 ms). In contrast, the

714 mean decay time was longer in the OT than in NAcSh (NEX-Cre NAcSh 5.3 ± 0.5 ms; Calb2-

715 Cre OT 7.8 ± 0.62 ms). No inhibitory/GABA-receptor mediated currents were observed

716 during recordings in either NEX-Cre or Calb2-Cre mice (Fig. 7*B*).

717

718 *Optogenetic activation of NeuroD6 VTA neurons, but not Calb2 VTA neurons, induces place*

719 *preference*

720 Finally, *in vivo* optogenetic stimulation in the VTA of NEX-Cre and Calb2-Cre mice was

721 applied to assess if this would induce place preference behavior. Again, DAT-Cre and

722 Vglut2-Cre mice were used as references for comparison to Calb2-Cre and NEX-Cre mice.

723 Mice received *DIO-ChR2-eYFP* or *DIO-eYFP* (control) injection and implantation of optic

724 fibers above the VTA (Fig. 8*A*,*G*), and were analyzed for real-time place preference (RT-PP)

725 and conditioned response (CR) (Fig. 8*A*).

726

727 <u>Analysis of RT-PP and CR in DAT-Cre and Vglut2-Cre mice</u>

728 DAT-Cre mice displayed a significant preference to the light-paired compartment on every

729 day of stimulation (Fig. 8*B* left, effect of compartment $F_{(2,18)} = 51.8$, $p < 0.001$; day x

730 compartment interaction $F_{(12,108)} = 33$, $p < 0.001$, *** $p < 0.001$ paired vs unpaired

731 compartment). This place preference was also evident when the effect of stimulation was

732 averaged for the four experimental days (Fig. 8*B*, right, effect of compartment $F_{(2,6)} = 166$, $p <$

733 0.001 ***$p < 0.001$ vs paired compartment). In the absence of stimulation, on days 5 and 8,

734 DAT-Cre mice demonstrated a conditioned response for the previous light-paired

735 compartment (Fig. 8*B*, ***$p < 0.001$ paired vs unpaired). Control mice (DAT-Cre negative or

736 DAT-Cre injected with *DIO-eYFP*) did not display any preference towards the stimulation

737 (Fig. 8-1*A*, left effect of compartment $F_{(2,4)} = 4.26$, $p = 0.102$; day x compartment interaction,

738 $F_{(12,24)} = 0.898$ $p = 0.562$, *$p < 0.05$ paired vs unpaired compartment; right: effect of

739   compartment $F_{(2,6)} = 48.7$ $p < 0.001$, ***$p < 0.001$ ###$p < 0.001$ neutral vs paired and

740   unpaired respectively; *Fig. 8-1B,* left effect of compartment $F_{(2,4)} = 27.9$, $p = 0.004$, day x

741   compartment interaction $F_{(12,24)} = 0.767$ $p = 0.677$, ***$p < 0.001$ paired vs unpaired

742   compartment; right: effect of compartment $F_{(2,6)} = 2.97$, $p = 0.127$; *Fig. 8-1C,* left: effect of

743   compartment $F_{(2,10)} = 18.6$, $p < 0.001$, day x compartment interaction $F_{(12,60)} = 0.963$, $p = $

744   0.494, right: effect of compartment $F_{(2,6)} = 9.27$, $p = 0.015$, *$p < 0.05$ #$p < 0.05$ neutral vs

745   paired and unpaired respectively). These results were in accordance with the literature (Yoo et

746   al., 2016) and thereby validated the experimental setup. In contrast to the strong place

747   preference induced by stimulation in DAT-Cre mice, Vglut2-Cre mice analyzed in the same

748   setup displayed a preference for the unpaired compartment (Fig. 8*C,* left: effect of

749   compartment $F_{(2,12)} = 40.9$, $p < 0.001$ and day x compartment interaction $F_{(12,72)} = 16.1$, $p < $

750   0.001, ***$p < 0.001$ paired vs unpaired; right: effect of compartment $F_{(2,6)} = 162$, $p < 0.001$,

751   ***$p < 0.001$ vs paired, ###$p < 0.001$ vs unpaired). To further verify this observation, the

752   protocol was modified so that the mice would receive photostimulation upon entry to either

753   one of the main compartments but not upon entry into the interconnecting neutral

754   compartment ("Neutral Compartment Preference", Fig. 8-1*Ii*). Once again, Vglut2-Cre mice

755   preferred to spend time in the area lacking stimulation (Fig. 8-1*Iii*, effect of compartment $F_{(2,8)}$

756    $= 70.9$, $p < 0.001$ and day x compartment interaction $F_{(4,16)} = 6.90$ $p = 0.002$, **$p < 0.01$, ***p

757   < 0.001 neutral vs paired compartments, Fig. 8-1*Iiii* effect of compartment $F_{(2,2)} = 54.2$, $p = $

758   0.018, *$p < 0.05$ neutral vs paired compartments). In the current setups, optogenetic VTA-

759   stimulation of DAT-Cre mice thus leads to place preference while same stimulation of

760   Vglut2-Cre mice causes an avoidance to any compartment that activates photostimulation.

761

762   Analysis of RT-PP and CR in Calb2-Cre and NEX-Cre mice

763 Using these behaviors as references and for comparison in the place preference setup, Calb2-

764 Cre mice showed a strikingly different behavior: Neither preference nor avoidance was

765 detected but instead, mice spent equal amount of time in both main compartments (Fig. 8*D*,

766 left, effect of compartment $F_{(2,12)} = 27$, $p < 0.001$, day x compartment interaction, $F_{(12,72)} =$

767 1.45, $p = 0.163$ and no differences between paired vs unpaired across days; right: effect of

768 compartment $F_{(2,6)} = 90.1$, $p < 0.001$, no differences between paired vs unpaired, ***$p <$

769 0.001, ###$p < 0.001$ neutral vs paired and unpaired, respectively). When analyzing if

770 optogenetic activation of NEX-Cre VTA neurons would cause place preference, a significant

771 behavioral response towards the photostimulation was observed (Fig. 8*E* left: effect of

772 compartment $F_{(2,8)} = 76.8$, $p < 0.001$, day x compartment interaction, $F_{(12,48)} = 4.63$, $p <$

773 0.001). NEX-Cre mice responded weakly to VTA-photostimulation on Days 3 and 4, but on

774 Days 6 and 7, NEX-Cre mice preferred the light-paired compartment (*$p = 0.02$, ***$p <$

775 0.001 paired vs unpaired). However, no CR was observed on either Day 5 or 8 (Fig.8*E* left).

776 By averaging the results of all four RT-PP days, NEX-Cre mice showed a significant

777 preference for paired over unpaired and neutral compartments (*Fig.* 8*E* right, effect of

778 compartment $F_{(2,6)} = 39.7$, $p < 0.001$ *$p = 0.013$ ***$p < 0.001$ vs paired, ##$p = 0.008$ neutral

779 vs unpaired).

780

781 Analysis of RT-PP and CR in DAT-Cre, Calb2-Cre and NEX-Cre mice using higher power

782 stimulation

783 While the result above demonstrated that activation of NEX-Cre VTA neurons induced place

784 preference behavior, higher power stimulation (5 ms pulse width, 20 Hz, 20 mW) was

785 subsequently used to test if these laser parameters would boost the observed behavioral

786 response. Again, DAT-Cre mice showed a strong preference for the light-paired chamber

787 (*Fig.* 8-1*E* left, effect of compartment $F_{(2,6)} = 105$, $p < 0.001$, day x compartment interaction

788   $F_{(12,36)}$ = 22.6, $p < 0.001$, ***$p < 0.001$ paired vs unpaired; right, effect of compartment $F_{(2,6)}$

789   = 404, $p < 0.001$, ***$p < 0.001$ unpaired and neutral vs paired) while Calb2-Cre mice

790   continued not to respond to the VTA photostimulation (*Fig.* 8-1*F,* left: effect of compartment

791   $F_{(2,12)}$ = 12.5, $p = 0.001$, day x compartment interaction $F_{(12,72)}$ = 0.469, $p = 0.927$; right:

792   effect of compartment $F_{(2,6)}$ = 47.3, $p < 0.001$, ***$p < 0.001$ & ###$p < 0.001$ neutral vs

793   unpaired and paired). In contrast, NEX-Cre mice showed a significant preference also with

794   this higher power stimulation (*Fig.* 8-1*G* left, effect of compartment $F_{(2,6)}$ = 48.3, $p < 0.001$,

795   day x compartment interaction $F_{(12,36)}$ = 8.58, $p < 0.001$, **$p = 0.003$, ***$p < 0.001$ paired vs

796   unpaired; right, effect of compartment $F_{(2,6)}$ = 178, $p < 0.001$, ***$p < 0.001$ paired vs unpaired

797   and neutral. ###$p < 0.001$ unpaired vs neutral). Finally, to further validate the role of NEX-

798   Cre VTA neurons in place preference, a subset of NEX-Cre mice was bilaterally injected with

799   *DIO-ChR2-eYFP* and tested in the same protocol under normal and high-power light

800   stimulation (*Fig.* 8-1*H* and *Fig.* 8*F*). Mice preferred the light-paired side over the unpaired

801   under both conditions, and high-power stimulation accentuated the preference towards the

802   light paired compartment which reached a 3-fold increase compared to the unpaired (standard

803   power: *Fig.* 8-1*H*, left, effect of compartment $F_{(2,6)}$ = 43.3, $p < 0.001$, day x compartment

804   interaction $F_{(12,36)}$ = 2.13, $p = 0.04$; right, effect of compartment $F_{(2,6)}$ = 331, $p < 0.001$, ***$p <$

805   0.001 vs paired ###$p < 0.001$ vs unpaired. High power: *Fig.* 8*F*, left, effect of compartment

806   $F_{(2,6)}$ = 36.5, $p < 0.001$, day x compartment interaction $F_{(12,36)}$ = 9.03, $p < 0.001$, ***$p < 0.001$

807   paired vs unpaired; right, effect of compartment $F_{(2,6)}$ = 106 $p < 0.001$, ***$p < 0.001$ paired vs

808   unpaired and neutral, #$p = 0.011$ unpaired vs neutral). However, unlike DAT-Cre mice, NEX-

809   Cre mice did not show any CR in any RT-PP experiment (*Fig.* 8*E* day 5 paired vs unpaired

810   p>0.999, day 8 paired vs unpaired $p = 0.937$; *Fig.* 8*F* day 5 paired vs unpaired $p > 0.999$, day

811   8 paired vs unpaired $p = 0.989$).

812

813 **Discussion**

814 It is well established that the VTA is involved in a range of functions, including behavioral

815 reinforcement, reward, aversion, motivation and incentive salience (Morales and Margolis,

816 2017). However, an area of active investigation is how the VTA can possess the ability to

817 contribute to all of these diverse functions, some even contrasting. It is now becoming

818 increasingly clear that functional diversity within the mDA system might be matched by

819 molecular and anatomical heterogeneity (Lammel et al., 2011, 2012; Morales and Margolis,

820 2017; Poulin et al., 2018; Roeper, 2013). Why is this important? The possibility to determine

821 the exact identity of neurons that contribute to a particular behavior opens up entirely new

822 perspectives in the opportunity to selectively target only those neurons that contribute to

823 clinical symptoms without side-effects caused by affecting adjacent neuronal population. In

824 this study, we used Cre-driven mouse genetics and optogenetics to begin to disentangle the

825 contribution of the newly described NeuroD6 VTA subtype (Khan et al., 2017; Kramer et al.,

826 2018; Viereckel et al., 2016) in reward-related behaviors commonly ascribed to the VTA DA

827 system. The main finding of our studies is that despite their restricted number, NeuroD6 VTA

828 neurons contribute to psychostimulant-induced hyperlocomotion and that their activation

829 induces place preference behavior.

830 *NeuroD6 VTA neurons represent a modest neuronal population within the VTA with*

831 *molecular capacity for dopaminergic and glutamatergic neurotransmission*

832 In the current study, we showed that NeuroD6 VTA neurons constitute a modest proportion

833 (circa 12%) of all VTA neurons expressing the gene encoding TH within the PN, PIF, PBP,

834 IF and RLi subareas. Within these VTA subareas, all NeuroD6-positive neurons were positive

835 for both Th and Dat mRNAs, markers of dopaminergic neurons. In addition, while no or very

836 few NeuroD6 neurons were positive for Viaat mRNA, a marker of GABAergic neurons, 12%

33

837 of the NeuroD6/Th double-positive neurons within the VTA were positive for Vglut2 mRNA,

838 suggesting a capacity for dual dopaminergic/glutamatergic neurotransmission. Indeed,

839 DA/glutamate co-release has in several studies been shown as a property of certain mDA

840 neurons where it has been proposed to play a role in reward-related behavior reinforced by

841 DA (recently reviewed in Trudeau and El Mestikawy, 2018). The identification of co-labeling

842 of NeuroD6 mRNA with Th, Dat and Vglut2 mRNAs within distinct neurons was partly in

843 accordance with our analysis of a NEX-Cre transgenic mouse line, implemented here to

844 achieve manipulation of the NeuroD6 VTA neurons, which identified substantial co-

845 localization between NEX-Cre-driven reporter gene expression (YFP) and TH

846 immunofluorescence. However, lack of TH/YFP co-localization was also identified. The

847 findings showing that the majority of NeuroD6 VTA neurons expressed DA markers fitted

848 with our electrophysiological data in which optogenetic VTA stimulation of NEX-Cre

849 neurons enabled the identification of DA release, as further discussed below. Further,

850 optogenetic stimulation also gave rise to EPSCs of glutamatergic nature, while no

851 GABAergic currents were detected, in agreement with the co-localization of NeuroD6 mRNA

852 with Vglut2 mRNA but lack of significant co-localization with Viaat mRNA.

853

854 In the context of transgenic mice, it is noteworthy that our result showing non-complete

855 overlap between NEX-Cre-driven reporter gene expression and TH, which contrasts the

856 parallel finding that all VTA neurons positive for endogenous NeuroD6 mRNA also label for

857 Th mRNA, are in accordance with a recent study in which a substantial number of non-

858 dopaminergic NEX-Cre VTA neurons were identified (Kramer et al., 2018). Collectively,

859 these findings propose that interpretation of VTA-data originating from the current NEX-Cre

860 mouse line should be considered with awareness of complex downstream neurocircuitry.

861 Further, as extensively discussed in the literature, regulatory promoters implemented

862 experimentally to drive Cre expression may give rise to transient and/or ectopic Cre activity

863 that fails to mimic endogenous gene expression due to gene regulatory events, not least during

864 developmental phases. Indeed, patterns of ectopic Cre activity have been described for other

865 transgenic mouse lines, including DAT-Cre and TH-Cre transgenic mouse lines commonly

866 implemented for the study of DA neurons (Lammel et al., 2015; Lindeberg et al., 2004;

867 Morales and Margolis, 2017; Nordenankar et al., 2015; Pupe and Wallén-Mackenzie, 2015;

868 Stamatakis et al., 2013; Stuber et al., 2015). While the current NEX-Cre transgenic line has

869 been thoroughly validated recently for the study of VTA neurons (Khan et al., 2017; Kramer

870 et al., 2018), to direct selectivity to VTA DA neurons, we here implemented a conditional

871 genetic approach to specifically abrogate vesicular packaging of DA in NEX-Cre neurons,

872 while we used optogenetically-driven neuronal activation to study effects upon direct

873 stimulation of NEX-Cre VTA neurons.

874

875 *Targeting of the Vmat2 gene in NEX-Cre VTA DA neurons allowed identification of a role in*

876 *psychostimulant-mediated response*

877 To enable the study of how reward-related behaviors classically associated with the mDA

878 system would be affected if the NEX-Cre DA neuron subtype lost its ability for dopaminergic

879 function, a conditional gene-targeting approach was implemented in which VMAT2 was

880 ablated specifically from NEX-Cre neurons. Since we could show that NeuroD6 and Vmat2

881 mRNAs only co-localized within the VTA, no other monoaminergic population should suffer

882 from loss of VMAT2 by this approach. Indeed, the results confirmed that Vmat2 mRNA was

883 selectively knocked out within the VTA, while all other monoaminergic neurons maintained

884 normal Vmat2 mRNA. Thus, the $Vmat2^{lox/lox;NEX-Cre}$ mouse line forms a new mouse model of

885 DA-release deficiency from a restricted group of VTA DA neurons characterized by NeuroD6

886 promoter activity. Based on the importance of mDA system in processing natural and drug

887   rewards (Baik, 2013; Di Chiara and Bassareo, 2007; Ikemoto, 2007; Kalivas et al., 1992;

888   Robinson and Berridge, 1993), we addressed the behavioral responses of *Vmat2$^{lox/lox;NEX-Cre-tg}$*

889   cKO mice and *Vmat2$^{lox/lox;NEX-Cre-wt}$* control mice to sugar, ethanol and the psychostimulants

890   amphetamine and cocaine. cKO mice displayed higher locomotor activation upon repeated

891   administration of psychostimulants than control mice. In contrast, sugar preference and

892   conditioned place preference to cocaine and amphetamine were similar between cKO and

893   control mice, and both genotype groups showed a preference for increasing dose of ethanol,

894   albeit in different patterns.

895   While acute administration of cocaine failed to cause differences in locomotor responses

896   between cKO and control mice, repeated administration caused exaggerated locomotor

897   behavior in cKO mice when measured in the CPP paradigm. In contrast, with repeated

898   amphetamine injections, the locomotor response was elevated above control levels in the open

899   field, but not in the CPP. The tests implemented were designed to study different behavioral

900   parameters, and results obtained in different setups and by different drugs are therefore not

901   directly comparable. What may seem as apparent discrepancies might be related to several

902   different properties. Firstly, the size and properties of the test environment were substantially

903   different between setups. The open field test took place in an environment that resembled the

904   home-cage. Locomotion was recorded during the conditioning phase when the mice were

905   confined to a much smaller compartment with specific patterns and no bedding. Secondly, the

906   injection regime differed between tests. In the open field, mice received acute injections of

907   cocaine or were sensitized to amphetamine by receiving daily injections after a 30-min

908   habituation period. In contrast, in the CPP experiment, the mice received in total four

909   injections of the drug in two non-consecutive days without any previous habituation period.

910   Finally, the recording period was shorter in the CPP compared to the open field (30 min vs

911   1.5 h), a parameter that could mask the long-lasting effects of amphetamine on locomotion.

36

912   Further, the observation of heightened, rather than reduced, psychostimulant-induced

913   locomotion might seem counter-intuitive: Loss of VMAT2 should lead to decreased

914   packaging and release of DA which might be expected to cause reduced locomotion

915   compared to control levels. However, the results obtained from our spatially selective cKO

916   mice are similar to the heightened amphetamine-induced hyperlocomotion observed in a

917   study of mice heterozygous for *Vmat2* in all DAT-Cre neurons (Isingrini et al., 2016). Thus,

918   lowering the level of VMAT2 throughout all DAT-Cre neurons or ablating it within the NEX-

919   Cre VTA DA population give rise to similar behavioral consequences. Further analyses

920   focused around VMAT2 in psychomotor behavior will be necessary to pin-point this matter,

921   however, developmental adaptations, a common feature of KO strategies induced during

922   embryonal development, may underlie the heightened locomotor response.

923   *Optogenetic stimulation of the VTA in NEX-Cre mice induces DA release and glutamatergic*

924   *EPSCs*

925   Complementary to the cKO approach, we used optogenetics-based experiments in which the

926   NEX-Cre VTA population could be directly stimulated. This type of manipulation provides

927   high spatial and temporal resolution (Deisseroth, 2015) and thus has the advantage of

928   enabling selective stimulation of Cre-driven neurons in real time with the benefit of directly

929   pin-pointing the role of molecularly defined neurons in measurable behavior. By analysis of

930   optogenetic reporter gene (eYFP) expression upon injection into the VTA of NEX-Cre mice,

931   we showed that NeuroD6 VTA neurons projected mainly to the NAcSh of the striatal

932   complex, with substantially lower density than observed upon similar injection in DAT-Cre

933   and Vglut2-Cre mice used here as controls (Hnasko et al., 2012; Pascoli et al., 2015; Qi et al.,

934   2016; Stuber et al., 2010; Yoo et al., 2016). NEX-Cre VTA projections also reached several

935   additional areas, but with even lower density than seen in the NAcSh, including the OT,

936   medial habenula and ventral pallidum. In accordance with the co-localization of eYFP with

937   TH immunoreactivity, we could verify that NEX-Cre VTA neurons released DA in both the

938   NAcSh and OT upon optogenetic stimulation in the VTA. Although the levels were lower

939   than those observed upon similar stimulation of DAT-Cre-positive VTA neurons, they were

940   significantly higher than those observed in control experiments, demonstrating that the NEX-

941   Cre VTA population indeed releases measurable amounts of DA in their target areas. To

942   investigate whether the TH-negative cellular population, present most profoundly in the

943   medial VTA, was of glutamatergic or GABAergic nature, patch-clamp electrophysiology was

944   performed which showed that optogenetic stimulation of NEX-Cre terminals induced EPSCs,

945   but not IPSCs, in NAcSh, thus verifying glutamatergic neurotransmission. While

946   glutamatergic post-synaptic currents were evidently a result of the optogenetic stimulation of

947   NEX-Cre VTA neurons, it remains to be established if the rare endogenous NeuroD6/Vglut2

948   double-positive neurons observed in our histological analysis are sufficiently potent to drive a

949   similar post-synaptic response in the natural situation, that is, upon excitation of the NeuroD6

950   VTA neurons in a non-transgenic context. Finally, the current setup did not allow us to

951   conclude if the EPSCs were of mono-or polysynaptic nature. The short onset of EPSCs was

952   suggestive of monosynaptic transmission, however, electrophysiological approaches

953   combined with pharmacological agents will be necessary to fully define the signaling

954   properties.

955   *Optogenetic stimulation of NEX-Cre VTA neurons reveals a role in place preference behavior*

956   Optogenetic stimulation of the mDA system of TH-Cre and DAT-Cre mice has been

957   demonstrated to potently induce DA release and real time place preference (Stuber et al.,

958   2010; Tsai et al., 2009; Yoo et al., 2016). The same type of activation of VTA in Vglut2-Cre

959   mice has been described to cause post-synaptic glutamatergic currents and to induce either

960   place preference or place avoidance, depending on stimulation parameters (Hnasko et al.,

961   2012; Qi et al., 2016; Wang et al., 2015; Yoo et al., 2016). Using DAT-Cre and Vglut2-Cre

962   mice as references, we could show here that optogenetic stimulation within the VTA of NEX-

963   Cre mice induced a significant preference for the light-paired compartment. The magnitude of

964   the preference observed was, however, smaller in NEX-Cre than in DAT-Cre mice. This

965   difference is likely related to the substantially smaller population of VTA neurons activated

966   upon photostimulation in the NEX-Cre compared to DAT-Cre VTA and the different

967   projection patterns of these neuronal populations: VTA-injection of ChR2-YFP in DAT-Cre

968   mice results in strong YFP-fluorescence in all innervation areas ascribed to the mDA system.

969   In contrast, the same injection into the VTA of NEX-Cre mice results in substantially lower

970   YFP-derived fluorescence in the VTA and sparse fluorescence in target areas.

971   Despite smaller magnitude, the ability of NEX-Cre VTA neurons to induce real time place

972   preference is an important finding as it demonstrates the possibility of identifying spatially

973   restricted groups of VTA neurons that are sufficient to induce a measurable behavior. Further

974   arguing for the importance of this result, the optogenetically induced preference behavior

975   displayed by NEX-Cre mice was strengthened by viral injections in bilateral, rather than

976   unilateral, manner as well as by increased laser power. The results of these experimental

977   manipulations suggest that the enhanced recruitment of NEX-Cre neurons strengthened the

978   behavioral output. While additional studies will be required to completely disentangle the

979   behavioral role of NeuroD6 VTA neurons, the current optogenetics-based setup already

980   enabled us to demonstrate that VTA activation in NEX-Cre mice could induce place

981   preference in real-time, but that it failed to result in conditioned response, defined as

982   significant place preference even in absence of actual optogenetic stimulation. This contrasts

983   the strong conditioned response observed in the DAT-Cre mice, and hence, activation of VTA

984   populations in NEX-Cre mice and DAT-Cre mice differ in more than one parameter:

985 Magnitude in real time place preference and presence of a detectable conditioned response. In

986 contrast to the preference behavior displayed by NEX-Cre and DAT-Cre mice, optogenetic

987 stimulation of VTA Vglut2-Cre neurons led to real time place avoidance defined here as

988 reduced time spent in the stimulation-paired compartment. This result is consistent with a

989 recent study which found that real time avoidance coincided with a frequency-dependent

990 increase in entries to the light-paired compartment and robust self-stimulation in an operant

991 task (Yoo et al., 2016). In contrast, another study found that photostimulation of Vglut2-Cre

992 neurons in VTA induced modest real time place preference and self-stimulation (Wang et al.,

993 2015). These data show that the behavioral effects of VTA glutamate neuron stimulation are

994 sensitive to the task, including the design of the apparatus and stimulus parameters. In this

995 context, it is noteworthy that VTA neurons of the NEX-Cre transgenic mouse line, with their

996 mixture of dopaminergic and glutamatergic signaling properties, might have shown lower

997 level of place preference than DAT-Cre mice not only due to the smaller number of neurons

998 and more sparse projections, but also as their activation might have caused a glutamate-

999 mediated avoidance behavior that counterbalanced the behavioral preference for light-

1000 stimulation.

1001

1002 *NeuroD6 and Calb2 mRNAs show partial overlap but NEX-Cre and Calb2-Cre VTA neurons*

1003 *have distinct projections and role in behavior*

1004 Parallel to the focus on NeuroD6 VTA neurons in neurocircuitry and behavioral regulation,

1005 our histological analysis enabled us to identify a degree of co-localization between NeuroD6

1006 and Calb2 mRNAs. While NeuroD6 mRNA was uniquely found in the VTA and excluded

1007 from the SNc, Calb2 mRNA was found distributed throughout these dopaminergic areas.

1008 However, histological analysis showed a degree of co-localization between NeuroD6 and

1009 Calb2 mRNAs, a finding which adds to the recent molecular description of NeuroD6 as co-

40

1010   localized with gastrin-releasing peptide (GRP) and additional markers (Khan et al., 2017;

1011   Kramer et al., 2018; Poulin et al., 2018). Beyond the partial co-localization of NeuroD6 and

1012   Calb2 mRNAs, the results show that Calb2 VTA neurons constitute a substantially larger

1013   proportion within the mDA population, show considerable expression of the gene encoding

1014   VIAAT, and are present in the SNc, an area devoid of NeuroD6 neurons. Our

1015   neurophysiological circuitry analyses of Calb2-Cre mice showed that Calb2-Cre VTA

1016   neurons belong to the category of VTA/SNc neurons that projects to the OT where their

1017   stimulation resulted in DA release and glutamatergic post-synaptic currents. While it was

1018   recently described that activation of dopaminergic fibers from VTA to the medial OT can

1019   induce place preference in DAT-Cre mice (Zhang et al., 2017), a similar response was not

1020   observed here upon Calb2-Cre VTA stimulation. These differences might be explained by the

1021   difference in density of the innervation patterns in the OT between the DAT-Cre and Calb2-

1022   Cre mice. The difference in preference behavior between NeuroD6-Cre and Calb2-Cre mice

1023   shows that distinct VTA neurocircuitry is crucial for the behavioral output.

1024   *Unraveling the behavioral roles of NeuroD6 VTA neurons stands to benefit current decoding*

1025   *of VTA-related disorders*

1026   The behavioral complexity mediated by the VTA is implicated in a range of neuropsychiatric

1027   conditions including substance use disorder, schizophrenia and ADHD for which clinical

1028   interventions based on increasing, decreasing, stabilizing or modulating the mDA system are

1029   commonly prescribed. In addition, since VTA DA neurons are less susceptible to

1030   degeneration in PD than SNc DA neurons, molecular differences are intensively searched for.

1031   GRP, in several studies identified as a marker for VTA DA neurons (Chung et al., 2005;

1032   Greene et al., 2005; La Manno et al., 2016; Viereckel et al., 2016) was recently shown to co-

1033   localize with NeuroD6 (Kramer et al., 2018). Several lines of evidence suggest that a discrete

1034   NeuroD6/GRP VTA subtype should be of specific interest: Overexpression of the gene

1035   encoding GRP increased the survival rate of cultured DA neurons in a parkinsonian

1036   experimental model (Chung et al., 2005) and GRP-positive mDA neurons remain in biopsies

1037   from deceased PD patients (Viereckel et al., 2016). Further, NeuroD6 increases neuronal

1038   survival in a toxin model of PD (Kramer et al., 2018). The NeuroD6/GRP VTA subtype might

1039   thereby possess resistance to PD. Our current results show that, despite their modest

1040   representation within the VTA, NeuroD6-expressing VTA neurons are implicated in distinct

1041   aspects of reward-related behavior. Their resistance to PD may thus contribute to the cause of

1042   behavioral dysfunction observed in the non-motor symptom domain of PD, including

1043   treatment-induced complications that resemble aspects of neuropsychiatric diseases, such as

1044   behavioral addictions (Cenci et al., 2015).

1045   Current molecular profiling of DA neuron subtypes should prove valuable for prospects of

1046   selective treatment in conditions related to VTA dysfunction. Of essence to achieve such

1047   selectivity is the systematic decoding of the explicit behavioral roles mediated by distinct

1048   VTA neurons. In this study, we initiated such analysis and now propose that NeuroD6 VTA

1049   neurons are of particular interest for further analysis of motivated and addictive behavior as

1050   they are here implicated in reward-related behavior measured as real time place preference

1051   and as their controlled dysregulation alters the responsiveness to psychostimulants. Our

1052   findings should prove useful for future investigations aimed at advancing the knowledge of

1053   VTA neurocircuitry in healthy conditions and in neuropsychiatric illness implicating the

1054   VTA.

1055   **Figure Legends**

1056   **Figure 1. NeuroD6 mRNA is found in a modest population of the VTA, co-localizes with**

1057   **dopaminergic markers and partially with a glutamatergic marker**

1058   (*A-G*) Double-fluorescent *in situ* hybridization (FISH) in the ventral midbrain of adult wild-

1059   type mice detecting the following mRNAs: *A, A'* NeuroD6 (red); *B, B'* Th (green); *C*, *C'*

1060   NeuroD6 (red) and Th (green). Th/NeuroD6 mRNA overlap shown in yellow. Low

1061   magnification to the left; close-ups to the right. Schematic outline shows borders for SNc and

1062   subregions of VTA: PN, PIF, PBP, IF, RLi. *D*, Quantification of percentage of NeuroD6-

1063   positive cells among all Th VTA cells; all NeuroD6 cells are positive for Th mRNA. *E*,

1064   NeuroD6 (red) and Dat (green), inset with high-magnification of Dat/NeuroD6 mRNA

1065   overlap (yellow); *F*, NeuroD6 (red) and Vglut2 (green); *G*, NeuroD6 (red) and Viaat (green),

1066   inset with high-magnification of Viaat-negative/NeuroD6-positive (red). (*H-P*) Triple-

1067   labeling FISH in the ventral midbrain of adult wild-type mice detecting: *H*, Th (blue); *I*,

1068   NeuroD6 (red); *J*, Vglut2 (green) mRNAs and their co-localization: *K*, NeuroD6/Th; *L*,

1069   NeuroD6/Vglut2; *M*, Th/NeuroD6/Vglut2. Cellular closeups: *N*, NeuroD6/Th (top),

1070   NeuroD6/Vglut2 (middle), Th/NeuroD6/Vglut2 (bottom). Arrows point to NeuroD6 mRNA-

1071   positive cells. *O*, Quantification of percentage of NeuroD6+/Th+/Vglut2+ and

1072   NeuroD6+/Th+/Vglut2+ neurons of the VTA; *P*, Schematic illustration of distribution pattern

1073   of NeuroD6+/Th+/Vglut2+ and NeuroD6+/Th+/Vglut2+ neurons within the VTA (same as

1074   shown with experimental data in *M*). NeuroD6+/Th+/Vglut2- cells in magenta;

1075   NeuroD6+/Th+/Vglut2+ cells in cyan.

1076   Abbreviations: VTA, ventral tegmental area; SNc, substantia nigra *pars compacta*; PBP,

1077   parabrachial pigmented nucleus; PN, paranigral nucleus; PIF, parainterfascicular nucleus;

1078   RLi, rostral linear nucleus; IF, interfascicular nucleus.

1079

1080   **Figure 2. Conditional ablation of the Vmat2 gene in NEX-Cre neurons – a model for**

1081   **spatially restricted DA deficiency**

1082   *A*, Breeding strategy for generation of mice gene-targeted for Vmat2 in VTA NEX-Cre

1083   neurons. NEX-Cre transgenic mice were mated to *Vmat2$^{lox/lox}$* mice to generate NEX-Cre-

1084   positive mice homozygous for *Vmat2$^{lox/lox}$* (*Vmat2$^{lox/lox;NEX-Cre-tg}$*: cKO mice) and littermate

1085   control mice homozygous for *Vmat2$^{lox/lox}$* and negative for the NEX-Cre transgene

1086   (*Vmat2$^{lox/lox;NEX-Cre-wt}$*: Ctrl mice). *B*, 2-probe approach for detection of Vmat2 mRNA by *in*

1087   *situ* hybridization. Probe 1 detects exons 6-15 and Probe 2 detects exon 2 of the Vmat2 gene.

1088   Exon 2 is floxed in *Vmat2$^{lox/lox}$* mice leading to failure of Probe 2-binding to Vmat2 mRNA in

1089   cKO neurons. *C*, Implementation of Vmat2 mRNA 2-probe approach in *Vmat2$^{lox/lox;NEX-Cre-wt}$*

1090   (Ctrl, left panel) and *Vmat2$^{lox/lox;NEX-Cre-tg}$* (cKO, right panel) brains. Wildtype neurons are

1091   positive for both Vmat2 probes, while cKO neurons are only positive for Probe 1 due to

1092   targeted deletion of exon 2 (detected by Probe 2). Probe 1 detected in green and Probe 2

1093   detected in blue results in green-blue double-labeling in wild-type cells and green-only

1094   labeling in cKO cells. Green arrows point to green-only cells, i.e. VMAT2 cKO cells. *D*,

1095   Vmat2 mRNA 2-probe *in-situ* hybridization in additional monoaminergic areas. *E*, TH

1096   immunohistochemistry in Ctrl and cKO midbrain and striatum.

1097   Abbreviations: LC, locus coeruleus; ROB, raphe nucleus obscurus; VMH, ventromedial

1098   hypothalamus; VTA, ventral tegmental area; SNc, substantia nigra *pars compacta;* DStr,

1099   dorsal striatum; NAc, nucleus accumbens; OT, olfactory tubercle.

1100   **Figure 3. Altered responsiveness to psychostimulants upon ablation of Vmat2 gene**

1101   **expression in NeuroD6 VTA neurons**

1102   Color coding: *Vmat2$^{lox/lox;NEX-Cre-wt}$* (Ctrl) in white; *Vmat2$^{lox/lox;NEX-Cre-tg}$* (cKO) in green. *A*,

1103   Weight curve for Ctrl (N=14) and cKO (N=23) mice. Data presented as mean weight in grams

1104   for each week ± SEM (+ $p<0.05$ effect of genotype; ### $p<0.001$, effect of age). *B*, Baseline

1105   locomotion in novel environment. Ctrl (N=17) and cKO (N=17). Data expressed as mean

1106   distance moved in 5 min bins ± SEM (###$p<0.001$, effect of time). *C*, Sucrose preference

1107   expressed as percentage of preference for sucrose over tap water ± SEM. Ctrl (N=14) and

1108   cKO (N=21). (###p<0.001 effect of sucrose concentration). *D*, Ethanol preference expressed

1109   as percentage of preference for ethanol solution over tap water ± SEM. Ctrl (N=14) and cKO

1110   (N=14) (###p<0.001 effect of ethanol concentration, §§§ p<0.001 3% vs 6% and 10% in ctrl

1111   mice). *E,* Cocaine-induced locomotion. Top: Administration schedule. Bottom: Average

1112   distance moved 1 hour post-injection of saline and 5, 10, 20 mg/kg of cocaine; Ctrl (N=14)

1113   and (N=21) mice. Data expressed as total distance moved during the 1h recording period ±

1114   SEM. (### p<0.001 effect of session). *F*, Amphetamine-induced locomotion. Top:

1115   Administration schedule. Bottom: Average distance moved 1.5 hours post-injection; Ctrl

1116   (N=17) and cKO mice (N=17). Data presented as mean of total distance moved in cm ± SEM

1117   for each session ++ p<0.01 effect of genotype, ### p<0.001 effect of session, * p<0.05 & ***

1118   p<0.001 cKO vs Ctrl). *G*, Conditioned place preference (CPP). Illustration of setup and

1119   administration schedule. *H & J*, Preference score displayed as Δsec, the difference between

1120   time spent in drug-paired compared during pretest and test ± SEM, positive value indicates

1121   preference (cocaine: Ctrl N=12, cKO N=15; amphetamine: Ctrl N=13, cKO N=16). *I & K*,

1122   Cocaine- and amphetamine-induced locomotion during conditioning in the CPP setup

1123   displayed as distance moved in 30 min ± SEM (cocaine: Ctrl N=12, cKO N=15;

1124   amphetamine: Ctrl N=15, cKO N=17, + p=0.031 effect of genotype, ## p=0.006, ### p<0.001

1125   effect of session).

1126

1127   **Figure 4 NeuroD6 mRNA co-localizes partly with Calb2 mRNA, but Calb2 mRNA is**

1128   **abundant throughout VTA and SNc**

1129   Double-labeling fluorescent *in situ* hybridization in the ventral midbrain of adult wild-type

1130   mice detecting the following mRNAs: *A*, NeuroD6 (red) and Calb2 (green), inset with high-

1131   magnification of overlap (yellow), pie charts illustrating quantification of overlap between

45

1132   NeuroD6 and Calb2; **B**, Calb (red) and Th (green), inset with high-magnification of overlap

1133   (yellow), pie charts illustrating quantification of overlap between Th and Calb2; **C**, Calb2

1134   (red) and Dat (green), inset with high-magnification of Dat/Calb2 mRNA overlap (yellow),

1135   pie chart illustrating quantification of overlap between Th and Calb2; **D**, Calb2 (red) and

1136   Vglut2 (green), inset with high-magnification of Vglut2/Calb2 mRNA overlap (yellow) in

1137   blue square and Vglut2-negative/Calb2-positive (red) in white square, pie chart illustrating

1138   quantification of overlap between Th and Calb2; **E**, Calb2 (red) and Viaat (green), inset with

1139   high-magnification of Viaat negative or positive (red, yellow, green) in white square.

1140   Abbreviations: VTA, ventral tegmental area; SNc, substantia nigra *pars compacta*; PBP,

1141   parabrachial pigmented nucleus; PN, paranigral nucleus; PIF, parainterfascicular nucleus;

1142   RLi, rostral linear nucleus; IF, interfascicular nucleus.

1143

1144   **Figure 5. Spatially restricted striatal innervation by NeuroD6 and Calb2 VTA neurons**

1145   **A**, Schematic illustration of stereotaxic injection into VTA of Cre-dependent *DIO-ChR2-*

1146   *eYFP* DNA construct packaged into AAV. **B**, Representative VTA neurons immunopositive

1147   for TH (red), YFP (green) or both (yellow) (*DIO-ChR2-eYFP*-injected NEX-Cre mice). **C-F**,

1148   Representative pictures of VTA (left panels) and striatal complex (right panels) in *DIO-ChR2-*

1149   *eYFP*-injected DAT-Cre (**C-C''**); Vglut2-Cre (**D-D''**); Calb2-Cre (**E-E''**); NEX-Cre (**F-F''**)

1150   mice. Panel far right: Schematic summary of striatal innervation pattern. Additional target

1151   areas listed in *Table 1*. **G-F**. Quantification of YFP and TH immunofluorescent overlap:

1152   Schematic illustration of 4 representative VTA areas selected for counting, shown as squares

1153   and labeled VTA 1-4 (**G**); Results of quantifications shown in histograms for each VTA area

1154   and the total sum (**H**).

1155   Abbreviations: PBP, parabrachial pigmented nucleus; PN, paranigral nucleus; PIF,

1156   parainterfascicular nucleus; RLi, rostral linear nucleus; IF, interfascicular nucleus; SNc,

1157   substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata*, IPR: interpeduncular

1158   nucleus, rostral subnucleus; IPC: interpeduncular nucleus, caudal subnucleus; DMStr,

1159   dorsomedial striatum; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; aca;

1160   anterior commissure, anterior part; OT, olfactory tubercle.

1161

1162   **Figure 6 Optogenetic stimulation in striatal target areas of NeuroD6 and Calb2 VTA**

1163   **neurons verifies DA release**

1164   *A*, Schematic representation of stereotaxic injection into VTA of Cre-dependent *DIO-ChR2-*

1165   *eYFP* and *DIO-eYFP* (Ctrl); FSCV recording sites within NAcSh and OT (red dots). *B*.

1166   Illustration of the experimental setup. *C*, Representative light-evoked dopamine recordings

1167   from injected DAT-Cre (left), NEX-Cre (middle) and Calb2-Cre (right) mice in the NAcSh

1168   (top) and the OT (bottom). *D*, Quantification of photostimulation-evoked dopamine release in

1169   the NAc shell (left) and OT (right). N=10 recording sites per group for each region. Mice used

1170   for the recordings: DAT-Cre/ChR2 N=2, DAT-Cre/eYFP N=2, NEX-Cre/ChR2 N=3, NEX-

1171   Cre/ eYFP N=2 Calb2-Cre/ChR2 N=3, Calb2-Cre/eYFP N=2. Box-and-whisker plots: Center

1172   lines indicate medians, box edges represent the interquartile range, whiskers extend to the

1173   minimal and maximal values (*p<0.05, **p<0.01, ***p<0.001 ChR2 vs ctrl).

1174   Abbreviations: DStr, dorsal striatum; NAcSh, nucleus accumbens shell; OT, olfactory

1175   tubercle.

1176

1177   **Figure 7. Optogenetic stimulation in striatal target areas of NeuroD6 and Calb2 VTA**

1178   **neurons reveals glutamatergic post-synaptic responses.**

1179   *A*, Representative picture from patch-clamp slice electrophysiology in NAcSh of NEX-Cre

1180   mice and OT of Calb2-Cre mice injected with *DIO-ChR2-eYFP*. *B*. Representative traces of

1181   photostimulation-evoked post synaptic currents recorded from NAcSh cells from NEX-Cre

47

1182 and OT cells from Calb2-Cre mice injected with *DIO-ChR2-eYFP*. **C**. Pie charts represent the

1183 percentage of cells showing excitatory post synaptic currents (white) vs negative (black) upon

1184 photostimulation of terminals in the NAcSh (N=18 cells from 4 mice) of NEX-Cre mice, and

1185 OT (N=14 cells from 4 mice) of Calb2-Cre mice. The y axis shows amplitude in pA; each

1186 circle represents one cell and bold lines the mean amplitude ± SEM. **D**. Patch-clamp

1187 recordings pre-(control) and post-bath application of DNQX upon photostimulation in NAcSh

1188 of NEX-Cre/ChR2 (left, N=6 cells from 3 mice) and OT of Calb2-Cre/ChR2 mice (right, N=5

1189 cells from 3 mice). Each circle represents one cell (* p<0.05 control vs DNQX).

1190 Abbreviations: NAcSh, Nucleus accumbens shell; OT, Olfactory tubercle

1191

1192

1193 **Figure 8. Optogenetic activation of NeuroD6 VTA neurons, but not Calb2 VTA neurons,**

1194 **induces place preference**

1195 *A*, Schematic drawing of stereotaxic injection into VTA of Cre-dependent *DIO-ChR2-eYFP*,

1196 and of experimental setup for real-time place preference (RT-PP) analysis. **B-F**. Time spent in

1197 light-paired (blue), unpaired (white during Phase 1, black during Reversal Phase) and neutral

1198 (gray) compartments shown as mean percentage of time spent in each compartment ± SEM

1199 (left) (* p<0.05, *** p<0.001 paired vs unpaired compartment); average percentage of time

1200 spent in each compartment during days 3,4,6 & 7 ± SEM (bar graphs; right) (* p<0.05, ***

1201 p<0.001 vs light-paired compartment; # p<0.05, ## p<0.01, ### p<0.001 vs unpaired

1202 compartment). DAT-Cre N=10; Vglut2-Cre N=7; Calb2-Cre N=7; NEX-Cre N=5. **F**. High-

1203 power stimulation of bilaterally injected NEX-Cre mice (N=4). **G**. Schematic illustration of

1204 optical fiber placement in mice analyzed in RT-PP analysis.

1205 Abbreviation: NS, non-significant.

1206

1207 **Table 1**. **Statistical analysis of results obtained in behavioral and electrophysiological**

1208 **experiments.**

1209

1210 **Table 2**. **Projection areas of VTA neurons represented in NEX-Cre and Calb2-Cre mice**

1211 **compared with DAT-Cre and Vglut2-Cre mice.** Summary of projection areas for VTA

1212 neurons virally injected with optogenetic constructs (*DIO-ChR2-eYFP*) in DAT-Cre, Vglut2-

1213 Cre, Calb2-Cre and NEX-Cre mice, respectively, and detected as YFP-positive fibers.

1214 + indicates presence of YFP-positive fibers; - indicates absence of YFP-positive fibers; (+)

1215 indicates low presence of fibers.

1216

1217 **Figure 2-1** ***In situ*** **hybridization for detection of Th, NeuroD6 and Vmat2 mRNA**.

1218 Analysis of Th, NeuroD6 and Vmat2 (two probes, covering mRNA derived from exon 1 and

1219 exon 2, respectively) mRNAs using radioactively labeled oligo-probes on sections throughout

1220 the whole brains of *Vmat2$^{lox/lox/NEX-Cre-wt}$* (Ctrl) and *Vmat2$^{lox/lox/NEX-Cre-tg}$* (cKO) mice.

1221

1222 **Figure 8-1. Complementary data from behavioral optogenetics.** DAT-Cre-negative mice

1223 injected with *DIO-ChR2-eYFP* (*A*) and DAT-Cre-positive mice injected with *DIO-eYFP* mice

1224 (*B*) did not show preference to the light paired compartment so their results were pooled

1225 together (*C*). *D*. Schematic illustration of optical fiber placement for mice analyzed in RT-PP

1226 analysis. *E-G*. RT-PP testing under high power (20mW, 5ms, 20Hz) stimulation for DAT-

1227 Cre/ChR2 (*E*), Calb2-Cre/ChR2 (*F*) and NEX-Cre/ChR2 (*G*). *H*. RT-PP results for bilaterally

1228 injected NEX-Cre/ChR2 mice. (*A-C, E-H*: graphs: *p<0.05, **p<0.01, ***p<0.001 paired vs

1229 unpaired; bar graphs, *p<0.05, ***p<0.001 vs paired, # p<0.05, ### p<0.001 vs unpaired). *I*.

1230 Vglut2-Cre/ChR2 tested in the Neutral Compartment Preference (NCP) test. *Ii*. Schematic of

1231 the experimental setup. *Iii-iii*. Mice spent significantly more time in the light-unpaired neutral

49

1232   compartment (*p<0.05, **p<0.01, ***p<0.001 neutral vs Paired1 and Paired2 compartments).

1233   (DAT-Cre negative N=3, DAT-Cre/eYFP=3, DAT-Cre/ChR2 high power N=4, Calb2-

1234   Cre/ChR2 high power N=7, NEX-Cre/ChR2 N=4, NEX-Cre/ChR2 bilateral N=4).

1235   Abbreviation: NS, non-significant.

1236

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Figure	Data structure	Type of test	Sample Size	Statistical data
Figure 3*A* Weight analysis of ctrl and cKO mice	Normally distributed	Two-way ANOVA followed by Sidak's multiple comparison test	Ctrl N=14 (M=8, F=6) cKO N=23 (M=15, F=8)	Interaction: p = 0.996, $F_{(4,158)}$ = 0.0447 Week: p <0.001, $F_{(4,158)}$ = 79.8 Genotype: p = 0.032, $F_{(1,158)}$ = 4.67 **Multiple comparisons** Ctrl vs cKO w4 p=0.908; 95% CI: -3.55 to 1.75 w5 p=0.966; 95% CI: -2.70 to 1.57 w6 p=0.876; 95% CI: -2.91 to 1.35 w7 p=0.720; 95% CI: -3.19 to 1.15 w8 p=0.783; 95%CI: -3.11 to 1.23
Figure 3*B* Baseline locomotion of ctrl and cKO mice for 30 min in 5-min bins	Normally distributed	Two-way RM ANOVA followed by Sidak's multiple comparison test	Ctrl N=17 (M=8, F=9) cKO N=17 (M=13, F=4)	Interaction: p = 0.256, $F_{(5,160)}$ = 1.33 Time: p < 0.001, $F_{(5,160)}$ = 69.5 Genotype: p = 0.535, $F_{(1,32)}$ = 0.00912 **Multiple comparisons** Ctrl vs cKO 5 p>0.999; 95% CI: -287 to 211 10 p>0.999; 95% CI: -217 to 282 15 p=0.952; 95% CI: -170 to 329 20 p>0.999; 95% CI: -236 to 263 25 p=0.886; 95% CI: -346 to 153 30 p=0.993; 95% CI: -195 to 304
Figure 3*C* Sucrose preference of ctrl and cKO mice for 1, 3 and 10% sucrose solutions	Normally distributed	Two-way RM ANOVA followed by Sidak's multiple comparison test	Ctrl N=14 (M=8, F=6) cKO N=21 (M=13, F=8)	Interaction: p = 0.475, $F_{(2,66)}$ = 0.752 Concentration: p < 0.001, $F_{(2,66)}$ = 151 Genotype: p = 0.297, $F_{(1,33)}$ = 1.12 **Multiple comparisons** Ctrl vs cKO 1% p>0.999; 95% CI: -5.21 to 5.69 3% p=0.294; 95% CI: -1.83 to 9.08 10% p=0.991; 95% CI: -4.85 to 6.05
Figure 3*D* Ethanol preference of ctrl and cKO mice for 3, 6 and 10% ethanol concentrations	Normally distributed	Two-way RM ANOVA followed by Sidak's multiple comparison test	Ctrl N=14 (M=7, F=7) cKO N=14 (M=6, F=8)	Interaction: p = 0.129, $F_{(2,52)}$ = 2.13 Concentration: p < 0.001, $F_{(2,52)}$ = 14.2 Genotype: p = 0.334, $F_{(1,26)}$ = 0.969 **Multiple comparisons** Ctrl vs cKO 3% p=0.983; 95% CI: -9.31 to 7.11 6% p=0.453; 95% CI: -3.68 to 12.7 10% p=0.396; 95% CI: -3.38 to 13.0 Ctrl 3% vs 6% p<0.001; 95% CI: -14.7 to -3.45 3% vs 10% p<0.001; 95% CI: -16.9 to -5.58 6% vs 10% p=0.733; 95% CI: -7.78 to 3.52 cKO 3% vs 6% p=0.354; 95% CI: -9.11 to 2.18 3% vs 10% p=0.072; 95% CI: -10.9 to 0.354 6% vs 10% p=0.814; 95% CI: -7.47 to 3.82
Figure 3*E* Injection-induced locomotion for ctrl and cKO mice after saline and 5,10 & 20 mg/kg injections of cocaine	Normally distributed	Two-way RM ANOVA followed by Sidak's multiple comparison test	Ctrl N=14 (M=8, F=6) cKO N=21 (M=13, F=8)	Interaction: p = 0.396, $F_{(3,99)}$ = 1 Session: p<0.001, $F_{(3,99)}$ = 108 Genotype: p = 0.208, $F_{(1,33)}$ = 1.65 **Multiple comparisons** Ctrl vs cKO Saline p=0.966; 95% CI: -3437 to 5436 5 mg/kg. p=0.962; 95% CI: -3410 to 5464 10 mg/kg. p=0.887; 95% CI: -3015 to 5858 20 mg/kg. p=0.152; 95% CI: -802 to 8071
Figure 3*F* Amphetamine- induced (3 mg/kg) locomotion under a sensitization protocol for ctrl and cKO mice	Normally distributed	Two-way RM ANOVA followed by Sidak's multiple comparison test	Ctrl N=17 (M=8, F=9) cKO N=17 (M=13, F=4)	Interaction: p<0.001, $F_{(5,160)}$ = 4.79 Session: p<0.001, $F_{(5,160)}$ = 40.9 Genotype: p = 0.005, $F_{(1,32)}$ = 9.09 **Multiple comparisons** Ctrl vs cKO Day1 p>0.999; 95% CI: -13977 to 12091 Day2 p=0.266; 95% CI: -3371 to 22696 Day3 p=0.063; 95% CI: -407 to 25661

				Day4 p=0.011; 95% CI: -2481 to 28549 Day5 p<0.001; 95% CI: -6873 to 32941 Day17 p=0.029; 95% CI: -928 to 26996
Figure 3H (top panel) Cocaine (20 mg/kg, i.p) CPP for ctrl and cKO mice	Normally distributed	Unpaired t-test	Ctrl N=12 (M=6, F=6) cKO N=15 (M=6, F=9)	t-test ctrl vs cKO p=0.860; 95% CI: -162.0 to 136.1
Figure 3H (bottom panel) Amphetamine (3 mg/kg, i.p.) CPP for ctrl and cKO mice	Assumed normality	Unpaired t-test	Ctrl N=13 (M=6, F=7) cKO N=16 (M=9, F=7)	t-test ctrl vs cKO p=0.744; 95% CI: -365.5 to 264.3
Figure 3I (top panel) Cocaine-induced locomotion during the CPP for ctrl and cKO mice	Assumed normality	Two-way RM ANOVA followed by Sidak's multiple comparison test	Ctrl N=12 (M=6, F=6) cKO N=15 (M=6, F=9)	Interaction: p=0.652, $F_{(3,75)}$ = 0.5 Session: p=0.006, $F_{(3,75)}$ = 4.4 Genotype: p = 0.031, $F_{(1,25)}$ = 5.2 **Multiple comparisons** Ctrl vs cKO Injection 1: p=0.373; 95% CI: -6850 to 1526 Injection 2: p=0.067; 95% CI: -8185 to 191 Injection 3: p=0.115; 95% CI: -7818 to 558 Injection 4: p=0.475; 95% CI: -6591 to 1785
Figure 3I (bottom panel) Amphetamine- induced locomotion during the CPP for ctrl and cKO mice	Normally distributed	Mixed-effects model (REML) followed by Sidak's multiple comparison test	Ctrl N=15 (M=7, F=8) cKO N=17 (M=10, F=7)	Interaction: p=0.567, $F_{(3,85)}$ = 0.680 Session: p<0.001, $F_{(3,85)}$ = 24.0 Genotype: p = 0.803, $F_{(1,30)}$ = 0.0631 **Multiple comparisons** Ctrl vs cKO Injection 1: p=0.941; 95% CI: -2522 to 1473 Injection 2: p=0.931; 95% CI: -1431 to 2517 Injection 3: p=0.995; 95% CI: -1783 to 2331 Injection 4: p=0.989; 95% CI: -1671 to 2327
Figure 6D (left) Optically evoked DA release in NAcSh of DAT-, NEX- and Calb2-Cre mice injected with ChR2 or eYFP	Normally distributed	Unpaired t-test	10 observations for each group and virus DAT-Cre/Chr2 N=2 (M=0, F=2) DAT-Cre/eYFP N=2 (M=1, F=1) NEX-Cre/Chr2 N=3 (M=2, F=1) NEX-Cre/eYFP N=2 (M=0, F=2) Calb2-Cre/Chr2 N=3 (M=1, F=2) Calb2-Cre/eYFP N=2 (M=0, F=2)	t-test DAT-Cre/ChR2 vs DAT-Cre/eYFP p<0.0001; 95% CI: -1.272 to -0.6540 NEX-Cre/ChR2 vs NEX-Cre/eYFP p<0.0001; 95% CI: -0.6289 to -0.2909 Calb2-Cre/ChR2 vs Calb2-Cre/eYFP p=0.0148; 95% CI: -0.01602 to -0.001988
Figure 6D (right) Optically evoked DA release in OT of DAT-, NEX- and Calb2-Cre mice injected with ChR2 or eYFP	Normally distributed	Unpaired t-test	As above	**t-test** DAT-Cre/ChR2 vs DAT-Cre/eYFP p<0.0001; 95% CI: -0.2354 to -0.1810 NEX-Cre/ChR2 vs NEX-Cre/eYFP p=0.0049; 95% CI: -0.01295 to -0.002704

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				Calb2-Cre/ChR2 vs Calb2-Cre/eYFP p=0.0002; 95% CI: -0.02022 to -0.007554
Figure 7D (left panel) Optically evoked EPSCs in NAcSh of NEX-Cre/ChR2 mice before (control) and after DNQX bath application	Assumed normality	Paired t-test	6 cells from 3 NEX-Cre/ChR2 mice (M=3, F=0)	p=0.0481; 95% CI: -61.86 to -0.3739
Figure 7D (right panel) Optically evoked EPSCs in OT of Calb2-Cre/ChR2 mice before (control) and after DNQX bath application	Assumed normality	Paired t-test	5 cells from 3 Calb2-Cre/ChR2 mice (M=2, F=1)	p=0.0456; 95% CI: -89.88 to -1.444
Figure 8B (left) Behavioral analysis of DAT-Cre/ChR2 mice throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=10 (M=2, F=8)	Interaction: p < 0.001, $F_{(12,108)}$ = 33 Day: p = 0.435, $F_{(6,54)}$ = 1 Compartment: p < 0.001, $F_{(2,18)}$ = 51.8 **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired p=0.513; 95% CI: -38.4 to -6.29 Day3 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: 21.7 to 66.4 Paired vs neutral p<0.001; 95% CI: 35.7 to 80.4 Day4 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: 37.6 to 82.3 Paired vs neutral p<0.001; 95% CI: 47.3 to 92.0 Day5 (CR) Paired vs Unpaired p<0.001; 95% CI: 11.5 to 56.1 Paired vs neutral p<0.001; 95% CI: 26.7 to 71.4 Day6 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -65.7 to -21.0 Paired vs neutral p<0.001; 95% CI: 34.4 to 79.1 Day7 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -85.0 to -40.4 Paired vs neutral p<0.001; 95% CI: 43.1 to 87.7 Day8 (CR) Paired vs Unpaired p<0.001; 95% CI: -67.2 to -22.5 Paired vs neutral p<0.001; 95% CI: 33.6 to 78.3 Reversal parameters: Day3 paired vs Day6 unpaired p<0.001; 95% CI: 22.8 to 67.4 Day3 paired vs Day7 unpaired p<0.001; 95% CI: 32.5 to 77.2 Day4 paired vs Day6 unpaired p<0.001; 95% CI: 32.3 to 76.9 Day4 paired vs Day7 unpaired p<0.001; 95% CI: 42.0 to 86.7 Day5 paired vs Day8 unpaired p<0.001; 95% CI: 17.5 to 62.2
Figure 8B (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for DAT-Cre/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=10 (M=2, F=8)	Compartment p < 0.001, $F_{(2,6)}$ = 166 **Multiple comparisons** Paired vs unpaired p<0.001; 95% CI: 41.1 to 63.9 Paired vs neutral p<0.001; 95% CI: 51.7 to 74.5 Unpaired vs neutral p=0.066; 95% CI: -0.808 to 22.0
Figure 8-1A (left) Behavioral analysis of DAT-Cre negative mice injected with AAV-ChR2 throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=3 (M=0, F=3)	Interaction: p = 0.562, $F_{(12,24)}$ = 0.898 Day: p = 0.569, $F_{(6,12)}$ = 0.830 Compartment: p = 0.102, $F_{(2,4)}$ = 4.26 **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired p=0.010; 95% CI: -49.6 to -4.05 Day3 (RT-PP) Paired vs Unpaired p=0.074; 95% CI: 44.5 to 1.05 Paired vs neutral p=0.292; 95% CI: -5.3 to 40.3 Day4 (RT-PP) Paired vs Unpaired p=0.236; 95% CI: -41.0 to 4.57 Paired vs neutral p=0.055; 95% CI: -0.241 to 45.3 Day5 (CR) Paired vs Unpaired p=0.074; 95% CI: -44.5 to 1.08

				Paired vs neutral p=0.204; 95% CI: -4.08 to 41. 5
				Day6 (RT-PP)
				Paired vs Unpaired p=0.998; 95% CI: -30.3 to 15.3
				Paired vs neutral p<0.001; 95% CI: 12.0 to 57.6
				Day7 (RT-PP)
				Paired vs Unpaired p=0.863; 95% CI: -34.5 to 11.0
				Paired vs neutral p=0.001; 95% CI: 9.18 to 54.8
				Day8 (CR)
				Paired vs Unpaired p=0.012; 95% CI: -49.2 to -3.6
				Paired vs neutral p<0.001; 95% CI: 22.5 to 68.1
				Reversal parameters:
				Day3 paired vs Day6 unpaired p=0.995; 95% CI: -30.8 to 14.8
				Day3 paired vs Day7 unpaired p>0.999; 95% CI: -27.0 to 18.5
				Day4 paired vs Day6 unpaired p>0.999; 95% CI: -28.0 to 17.6
				Day4 paired vs Day7 unpaired p>0.999; 95% CI: -24.2 to 21.4
				Day5 paired vs Day8 unpaired p>0.999; 95% CI: -21.3 to 24.3
Figure 8-1A (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for DAT-Cre negative/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=3 (M=0, F=3)	Compartment p < 0.001, $F_{(2,6)}$ = 48.7 **Multiple comparisons** Paired vs unpaired p=0.358; 95% CI: -15.8 to 5.46 Paired vs neutral p<0.001; 95% CI: 16.1 to 37.3 Unpaired vs neutral p<0.001; 95% CI: 21.2 to 42.5
Figure 8-1B (left) Behavioral analysis of DAT-Cre/eYFP throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=3 (M=0, F=3)	Interaction: p = 0.677, $F_{(12,24)}$ = 0.767 Day: p = 0.935, $F_{(6,12)}$ = 0.281 Compartment: p = 0.004, $F_{(2,4)}$ = 27.9 **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired p<0.001; 95% CI: -62.8 to -22.5 Day3 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -64.9 to -24.6 Paired vs neutral p=0.198; 95% CI: -3.52 to 36.7 Day4 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -63.5 to -23.2 Paired vs neutral p=0.222; 95% CI: -3.85 to 36.4 Day5 (CR) Paired vs Unpaired p<0.001; 95% CI: -65.9 to -25.6 Paired vs neutral p=0.251; 95% CI: -4.21 to 36.1 Day6 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -70.7 to -30.5 Paired vs neutral p<0.001; 95% CI: 45.8 to 86.1 Day7 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -55.5 to -15.2 Paired vs neutral p<0.001; 95% CI: 31.3 to 71.6 Day8 (CR) Paired vs Unpaired p<0.001; 95% CI: -66.1 to -25.8 Paired vs neutral p<0.001; 95% CI: -42.7 to 82.9 Reversal parameters: Day3 paired vs Day6 unpaired p>0.999; 95% CI: -17.8 to 22.5 Day3 paired vs Day7 unpaired p>0.999; 95% CI: -23.1 to 17.2 Day4 paired vs Day6 unpaired p>0.999; 95% CI: -17.4 to 22.9 Day4 paired vs Day7 unpaired p>0.999; 95% CI: -22.7 to 17.5 Day5 paired vs Day8 unpaired p>0.999; 95% CI: -20.4 to 19.9
Figure 8-1B (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for DAT-Cre /eYFP mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=3 (M=0, F=3)	Compartment p = 0.127, $F_{(2,6)}$ = 2.97 **Multiple comparisons** Paired vs unpaired p>0.999; 95% CI: -55.6 to 54.5 Paired vs neutral p=0.171; 95% CI: -17.5 to 92.6 Unpaired vs neutral p=0.165; 95% CI: -16.9 to 93.1
Figure 8-1C (left) Behavioral analysis of DAT-Cre controls (pooled) throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=6 (M=0, F=6)	Interaction: p = 0.494, $F_{(12,60)}$ = 0.963 Day: p = 0.929, $F_{(6,30)}$ = 0.306 Compartment: p < 0.001 $F_{(2,10)}$ = 18.6 **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired p<0.001; 95% CI: -48.6 to -20.8 Day3 (RT-PP)

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				Paired vs Unpaired $p<0.001$; 95% CI: -47.1 to -19.4
				Paired vs neutral $p=0.004$; 95% CI: 3.15 to 30.9
				Day4 (RT-PP)
				Paired vs Unpaired $p<0.001$; 95% CI: -44.7 to -16.9
				Paired vs neutral $p<0.001$; 95% CI: 5.52 to 33.3
				Day5 (CR)
				Paired vs Unpaired $p<0.001$; 95% CI: -47.6 to -19.8
				Paired vs neutral $p=0.003$; 95% CI: 3.42 to 31.2
				Day6 (RT-PP)
				Paired vs Unpaired $p<0.001$; 95% CI: -42.9 to -15.1
				Paired vs neutral $p<0.001$; 95% CI: 36.5 to 64.3
				Day7 (RT-PP)
				Paired vs Unpaired $p<0.001$; 95% CI: -37.4 to -9.64
				Paired vs neutral $p<0.001$; 95% CI: 27.8 to 55.6
				Day8 (CR)
				Paired vs Unpaired $p<0.001$; 95% CI: -50.1 to -22.3
				Paired vs neutral $p<0.001$; 95% CI: 40.2 to 67.9
				Reversal parameters:
				Day3 paired vs Day6 unpaired $p>0.999$; 95% CI: -16.7 to 11.1
				Day3 paired vs Day7 unpaired $p>0.999$; 95% CI: -17.5 to 10.3
				Day4 paired vs Day6 unpaired $p>0.999$; 95% CI: -15.1 to 12.7
				Day4 paired vs Day7 unpaired $p>0.999$; 95% CI: -15.9 to 11.9
				Day5 paired vs Day8 unpaired $p>0.999$; 95% CI: -13.3 to 14.5
Figure 8-1C (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for DAT-Cre control mice (pooled)	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=6 (M=0, F=6)	Compartment $p = 0.015$, $F_{(2,6)} = 9.27$ **Multiple comparisons** Paired vs unpaired $p=0.946$; 95% CI: -30.6 to 24.8 Paired vs neutral $p=0.028$; 95% CI: 4.44 to 59.8 Unpaired vs neutral $p=0.019$; 95% CI: 7.30 to 62.7
Figure 8-1E (left) Behavioral analysis of DAT-Cre/ChR2 mice tested on high power, throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=10 (M=2, F=8)	Interaction: $p < 0.001$, $F_{(12,36)} = 22.6$ Day: $p = 0.455$, $F_{(6,18)} = 1$ Compartment: $p < 0.001$, $F_{(2,6)} = 105$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired $p>0.999$; 95% CI: -45.7 to 26.7 Day3 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: 26.2 to 98.6 Paired vs neutral $p<0.001$; 95% CI: 38.5 to 111 Day4 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: 42.7 to 115 Paired vs neutral $p<0.001$; 95% CI: 47.1 to 119 Day5 (CR) Paired vs Unpaired $p<0.001$; 95% CI: 17.4 to 89.8 Paired vs neutral $p<0.001$; 95% CI: 16.5 to 88.8 Day6 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -98.0 to -25.6 Paired vs neutral $p<0.001$; 95% CI: 36.5 to 109 Day7 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -109 to -37.0 Paired vs neutral $p<0.001$; 95% CI: 42.3 to 115 Day8 (CR) Paired vs Unpaired $p=0.407$; 95% CI: -62.5 to 9.72 Paired vs neutral $p=0.030$; 95% CI: 1.98 to 74.3 Reversal parameters: Day3 paired vs Day6 unpaired $p<0.001$; 95% CI: 26.5 to 98.9 Day3 paired vs Day7 unpaired $p<0.001$; 95% CI: 32.1 to 104 Day4 paired vs Day6 unpaired $p<0.001$; 95% CI: 34.8 to 107 Day4 paired vs Day7 unpaired $p<0.001$; 95% CI: 40.5 to 113 Day5 paired vs Day8 unpaired $p<0.001$; 95% CI: 4.15 to 76.5
Figure 8-1E (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for DAT-Cre/ChR2 mice under high	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=4 (M=0, F=4)	Compartment $p < 0.001$, $F_{(2,6)} = 404$ **Multiple comparisons** Paired vs unpaired $p<0.001$; 95% CI: 59.9 to 78.2 Paired vs neutral $p<0.001$; 95% CI: 68.1 to 86.5 Unpaired vs neutral $p=0.074$; 95% CI: -0.934 to 17.4

power stimulation				
Figure 8C (left) Behavioral analysis of Vglut2-Cre/ChR2 mice throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=7 (M=2, F=5)	Interaction: $p < 0.001$, $F_{(12,72)} = 16.1$ Day: $p = 0.181$, $F_{(6,36)} = 1.58$ Compartment: $p < 0.001$, $F_{(2,12)} = 40.9$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired $p>0.999$; 95% CI: -28.1 to 26.6 Day3 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -71.9 to -17.2 Paired vs neutral $p>0.999$; 95% CI: -18.0 to 36.7 Day4 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -68.1 to -13.4 Paired vs neutral $p>0.997$; 95% CI: -16.9 to 37.8 Day5 (CR) Paired vs Unpaired $p=0.998$; 95% CI: -37.8 to 16.9 Paired vs neutral $p=0.019$; 95% CI: 2.42 to 57.1 Day6 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: 32.9 to 87.6 Paired vs neutral $p>0.999$; 95% CI: -28.8 to 25.9 Day7 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: 27.0 to 81.7 Paired vs neutral $p>0.999$; 95% CI: -28.9 to 25.8 Day8 (CR) Paired vs Unpaired $p=0.783$; 95% CI: -10.8 to 43.9 Paired vs neutral $p=0.055$; 95% CI: -0.268 to 54.4 Reversal parameters: Day3 paired vs Day6 unpaired $p<0.001$; 95% CI: -78.7 to -24.1 Day3 paired vs Day7 unpaired $p<0.001$; 95% CI: -74.8 to -20.1 Day4 paired vs Day6 unpaired $p<0.001$; 95% CI: -77.1 to -22.4 Day4 paired vs Day7 unpaired $p<0.001$; 95% CI: -73.2 to -18.5 Day5 paired vs Day8 unpaired $p=0.952$; 95% CI: -41.0 to 13.7
Figure 8C (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for Vglut2-Cre/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=7 (M=2, F=5)	Compartment $p < 0.001$, $F_{(2,6)} = 162$ **Multiple comparisons** Paired vs unpaired $p<0.001$; 95% CI: -60.2 to -39.7 Paired vs neutral $p=0.469$; 95% CI: -6.08 to 14.5 Unpaired vs neutral $p<0.001$; 95% CI: 43.9 to 64.4
Figure 8D (left) Behavioral analysis of Calb2-Cre/ChR2 mice throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=7 (M=0, F=7)	Interaction: $p = 0.163$, $F_{(12,72)} = 1.45$ Day: $p = 0.567$, $F_{(6,36)} = 0.813$ Compartment: $p < 0.001$, $F_{(2,12)} = 27$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired $p=0.096$; 95% CI: -33.3 to 1.13 Day3 (RT-PP) Paired vs Unpaired $p=0.343$; 95% CI: -30.6 to 3.82 Paired vs neutral $p=0.010$; 95% CI: 2.52 to 37.0 Day4 (RT-PP) Paired vs Unpaired $p>0.999$; 95% CI: -21.1 to 13.4 Paired vs neutral $p<0.001$; 95% CI: 8.22 to 42.7 Day5 (CR) Paired vs Unpaired $p>0.999$; 95% CI: 13.4 to 47.9 Paired vs neutral $p<0.001$; 95% CI: -15.7 to 18.8 Day6 (RT-PP) Paired vs Unpaired $p>0.999$; 95% CI: -18.2 to 16.2 Paired vs neutral $p<0.001$; 95% CI: 13.0 to 47.5 Day7 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -17.6 to 16.9 Paired vs neutral $p>0.999$; 95% CI: 10.8 to 45.2 Day8 (CR) Paired vs Unpaired $p<0.001$; 95% CI: -13.1 to 21.3

				Paired vs neutral $p>0.999$; 95% CI: 7.45 to 41.9 Reversal parameters: Day3 paired vs Day6 unpaired $p=0.991$; 95% CI: -24.6 to 9.91 Day3 paired vs Day7 unpaired $p=0.995$; 95% CI: -24.2 to 10.2 Day4 paired vs Day6 unpaired $p>0.999$; 95% CI: -19.5 to 15.0 Day4 paired vs Day7 unpaired $p>0.999$; 95% CI: -19.1 to 15.3 Day5 paired vs Day8 unpaired $p>0.999$; 95% CI: -17.2 to 17.3
Figure 8D (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for Calb2-Cre/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=7 (M=0, F=7)	Compartment $p < 0.001$, $F_{(2,6)} = 90.1$ **Multiple comparisons** Paired vs unpaired $p=0.297$; 95% CI: -11.4 to 3.42 Paired vs neutral $p<0.001$; 95% CI: 18.5 to 33.3 Unpaired vs neutral $p<0.001$; 95% CI: 22.4 to 37.3
Figure 8-1F Behavioral analysis of Calb2-Cre/ChR2 mice tested on high power, throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=7 (M=0, F=7)	Interaction: $p = 0.927$, $F_{(12,72)} = 0.469$ Day: $p = 0.661$, $F_{(6,36)} = 0.688$ Compartment: $p = 0.001$, $F_{(2,12)} = 12.5$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired $p=0.104$; 95% CI: -33.5 to 1.28 Day3 (RT-PP) Paired vs Unpaired $p=0.995$; 95% CI: -24.4 to 10.4 Paired vs neutral $p=0.019$; 95% CI: 1.56 to 36.3 Day4 (RT-PP) Paired vs Unpaired $p>0.999$; 95% CI: -22.6 to 12.2 Paired vs neutral $p<0.001$; 95% CI: 47.1 to 119 Day5 (CR) Paired vs Unpaired $p=0.742$; 95% CI: -28.2 to 6.54 Paired vs neutral $p=0.015$; 95% CI: 1.87 to 36.6 Day6 (RT-PP) Paired vs Unpaired $p=0.937$; 95% CI: -26.3 to 8.46 Paired vs neutral $p<0.001$; 95% CI: 11.1 to 45.9 Day7 (RT-PP) Paired vs Unpaired $p>0.999$; 95% CI: -22.3 to 12.5 Paired vs neutral $p<0.001$; 95% CI: 7.32 to 42.1 Day8 (CR) Paired vs Unpaired $p=0.976$; 95% CI: -25.5 to 9.30 Paired vs neutral $p<0.001$; 95% CI: 7.52 to 42.3 Reversal parameters: Day3 paired vs Day6 unpaired $p>0.999$; 95% CI: -17.0 to 17.8 Day3 paired vs Day7 unpaired $p>0.999$; 95% CI: -18.4 to 16.4 Day4 paired vs Day6 unpaired $p>0.999$; 95% CI: -15.1 to 19.6 Day4 paired vs Day7 unpaired $p>0.999$; 95% CI: -16.5 to 18.2 Day5 paired vs Day8 unpaired $p>0.999$; 95% CI: -17.5 to 17.3
Figure 8-1F (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for Calb2-Cre/ChR2 mice under high power stimulation	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=7 (M=0, F=7)	Compartment $p < 0.001$, $F_{(2,6)} = 47.3$ **Multiple comparisons** Paired vs unpaired $p=0.988$; 95% CI: -8.15 to 8.97 Paired vs neutral $p<0.001$; 95% CI: 15.1 to 32.3 Unpaired vs neutral $p<0.001$; 95% CI: 14.7 to 31.8
Figure 8E (left) Behavioral analysis of NEX-Cre/ChR2 mice throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=5 (M=1, F=4)	Interaction: $p < 0.001$, $F_{(12,48)} = 4.63$ Day: $p = 0.307$, $F_{(6,24)} = 1.27$ Compartment: $p < 0.001$, $F_{(2,8)} = 76.8$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired $p>0.999$; 95% CI: -18.7 to 24.9 Day3 (RT-PP) Paired vs Unpaired $p=0.414$; 95% CI: -5.70 to 37.9 Paired vs neutral $p<0.001$; 95% CI: 17.1 to 60.7 Day4 (RT-PP) Paired vs Unpaired $p>0.999$; 95% CI: -17.5 to 26.1 Paired vs neutral $p<0.001$; 95% CI: 16.6 to 60.3 Day5 (CR) Paired vs Unpaired $p>0.999$; 95% CI: 12.0 to 55.6

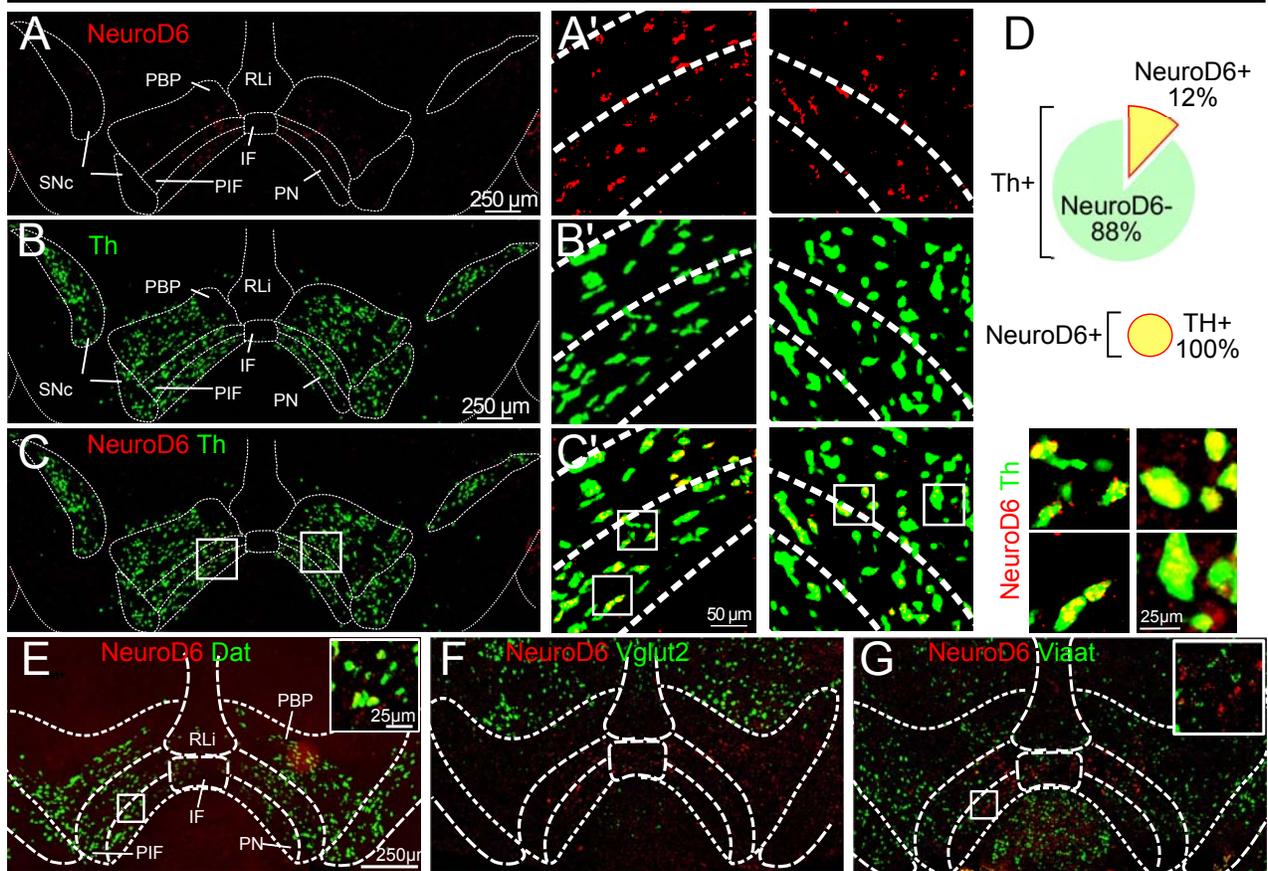|				Paired vs neutral $p<0.001$; 95% CI: -5.03 to 38.6 Day6 (RT-PP) Paired vs Unpaired $p=0.020$; 95% CI: -45.5 to -1.92 Paired vs neutral $p<0.001$; 95% CI: 15.1 to 58.7 Day7 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -51.8 to -8.16 Paired vs neutral $p<0.001$; 95% CI: 20.5 to 64.1 Day8 (CR) Paired vs Unpaired $p=0.937$; 95% CI: -32.7 to 10.9 Paired vs neutral $p<0.001$; 95% CI: 16.3 to 59.9 Reversal parameters: Day3 paired vs Day6 unpaired $p=0.049$; 95% CI: 0.0239 to 43.6 Day3 paired vs Day7 unpaired $p=0.016$; 95% CI: 2.38 to 46.0 Day4 paired vs Day6 unpaired $p=0.252$; 95% CI: -4.06 to 39.5 Day4 paired vs Day7 unpaired $p=0.105$; 95% CI: -1.71 to 41.9 Day5 paired vs Day8 unpaired $p=0.998$; 95% CI: -14.0 to 29.6
Figure 8E (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for NEX-Cre/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=5 (M=1, F=4)	Compartment $p < 0.001$, $F_{(2,6)} = 39.7$ Multiple comparisons Paired vs unpaired $p=0.013$; 95% CI: 5.03 to 32.0 Paired vs neutral $p<0.001$; 95% CI: 25.7 to 52.6 Unpaired vs neutral $p=0.008$; 95% CI: 7.16 to 34.1
Figure 8-1G (left) Behavioral analysis of NEX-Cre/ChR2 mice tested on high power, throughout the opto-behavioral experiments	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=4 (M=1, F=3)	Interaction: $p < 0.001$, $F_{(12,36)} = 8.58$ Day: $p = 0.252$, $F_{(6,18)} = 1.44$ Compartment: $p < 0.001$, $F_{(2,6)} = 48.3$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired $p=0.369$; 95% CI: -25.0 to 3.62 Day3 (RT-PP) Paired vs Unpaired $p=0.358$; 95% CI: -3.54 to 25.1 Paired vs neutral $p<0.001$; 95% CI: 16.0 to 44.6 Day4 (RT-PP) Paired vs Unpaired $p=0.003$; 95% CI: 3.97 to 32.6 Paired vs neutral $p<0.001$; 95% CI: 24.9 to 53.5 Day5 (CR) Paired vs Unpaired $p=0.084$; 95% CI: -0.819 to 27.8 Paired vs neutral $p<0.001$; 95% CI: 19.7 to 48.3 Day6 (RT-PP) Paired vs Unpaired $p=0.087$; 95% CI: -27.8 to 0.877 Paired vs neutral $p<0.001$; 95% CI: 17.7 to 46.4 Day7 (RT-PP) Paired vs Unpaired $p<0.001$; 95% CI: -34.7 to -6.03 Paired vs neutral $p<0.001$; 95% CI: 21.8 to 50.5 Day8 (CR) Paired vs Unpaired $p=0.798$; 95% CI: -22.5 to 6.11 Paired vs neutral $p<0.001$; 95% CI: 13.7 to 42.3 Reversal parameters: Day3 paired vs Day6 unpaired $p=0.203$; 95% CI: -2.36 to 26.3 Day3 paired vs Day7 unpaired $p=0.028$; 95% CI: 0.881 to 29.5 Day4 paired vs Day6 unpaired $p=0.005$; 95% CI: 3.12 to 31.8 Day4 paired vs Day7 unpaired $p<0.001$; 95% CI: 6.36 to 35.0 Day5 paired vs Day8 unpaired $p=0.202$; 95% CI: -2.35 to 26.3
Figure 8-1G (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for NEX-Cre/ChR2 mice under high power stimulation	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=4 (M=1, F=3)	Compartment $p < 0.001$, $F_{(2,6)} = 178$ **Multiple comparisons** Paired vs unpaired $p<0.001$; 95% CI: 10.1 to 21.3 Paired vs neutral $p<0.001$; 95% CI: 28.8 to 40.0 Unpaired vs neutral $p<0.001$; 95% CI: 13.1 to 24.3
Figure 8-1H (left) Behavioral analysis of bilaterally injected NEX-Cre/ChR2 mice throughout the opto-	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple	N=4 (M=0, F=4)	Interaction: $p = 0.040$, $F_{(12,36)} = 2.13$ Day: $p = 0.384$, $F_{(6,18)} = 1.13$ Compartment: $p < 0.001$, $F_{(2,6)} = 43.3$ **Multiple comparisons** (of interest)

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behavioral experiments		comparison test		Day2 (Pre-test) Paired vs Unpaired p=0.999; 95% CI: -50.7 to 25.1 Day3 (RT-PP) Paired vs Unpaired p=0.998; 95% CI: -24.6 to 51.2 Paired vs neutral p=0.017; 95% CI: 4.27 to 80.1 Day4 (RT-PP) Paired vs Unpaired p=0.768; 95% CI: -15.7 to 60.2 Paired vs neutral p=0.003; 95% CI: 10.3 to 86.1 Day5 (CR) Paired vs Unpaired p=0.974; 95% CI: -21.2 to 54.7 Paired vs neutral p=0.015; 95% CI: 4.63 to 80.5 Day6 (RT-PP) Paired vs Unpaired p>0.999; 95% CI: -49.6 to 26.3 Paired vs neutral p=0.029; 95% CI: 2.13 to 78.0 Day7 (RT-PP) Paired vs Unpaired p=0.999; 95% CI: -52.5 to 23.3 Paired vs neutral p=0.019; 95% CI: 3.69 to 79.5 Day8 (CR) Paired vs Unpaired p=0.185; 95% CI: -70.1 to 5.78 Paired vs neutral p<0.001; 95% CI: 14.2 to 90.0 Reversal parameters: Day3 paired vs Day6 unpaired p=0.999; 95% CI: -25.0 to 50.8 Day3 paired vs Day7 unpaired p=0.995; 95% CI: -23.6 to 52.3 Day4 paired vs Day6 unpaired p=0.952; 95% CI: -20.0 to 55.8 Day4 paired vs Day7 unpaired p=0.999; 95% CI: -18.6 to 57.3 Day5 paired vs Day8 unpaired p=0.668; 95% CI: -14.1 to 61.8
Figure 8-1*H* (right) time spent in paired, unpaired and neutral compartments during the 4 RT-PP days for bilaterally injected NEX-Cre/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=4 (M=0, F=4)	Compartment $p < 0.001$, $F_{(2,6)} = 331$ **Multiple comparisons** Paired vs unpaired p<0.001; 95% CI: 10.1 to 20.8 Paired vs neutral p<0.001; 95% CI: 37.7 to 48.4 Unpaired vs neutral p<0.001; 95% CI: 22.2 to 32.9
Figure 8*F* (left) Behavioral analysis of bilaterally injected NEX-Cre/ChR2 mice throughout the opto-behavioral experiments, tested on high power	Normally distributed	Two-way RM ANOVA followed by Tukey's multiple comparison test	N=4 (M=0, F=4)	Interaction: $p < 0.001$, $F_{(12,36)} = 9.03$ Day: $p = 0.310$, $F_{(6,18)} = 1.29$ Compartment: $p < 0.001$, $F_{(2,6)} = 36.5$ **Multiple comparisons** (of interest) Day2 (Pre-test) Paired vs Unpaired p=0.982; 95% CI: -42.9 to 17.4 Day3 (RT-PP) Paired vs Unpaired p=0.349; 95% CI: -7.34 to 53.0 Paired vs neutral p<0.001; 95% CI: 20.0 to 80.3 Day4 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: 14.1 to 74.4 Paired vs neutral p<0.001; 95% CI: 30.8 to 91.1 Day5 (CR) Paired vs Unpaired p>0.999; 95% CI: -29.7 to 30.6 Paired vs neutral p=0.002; 95% CI: 9.24 to 69.5 Day6 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -76.1 to -15.8 Paired vs neutral p<0.001; 95% CI: 28.5 to 88.8 Day7 (RT-PP) Paired vs Unpaired p<0.001; 95% CI: -74.4 to -14.1 Paired vs neutral p<0.001; 95% CI: 27.0 to 87.3 Day8 (CR) Paired vs Unpaired p=0.989; 95% CI: -42.4 to 17.9 Paired vs neutral p=0.006; 95% CI: 6.19 to 66.5 Reversal parameters: Day3 paired vs Day6 unpaired p=0.009; 95% CI: 5.24 to 65.5 Day3 paired vs Day7 unpaired p=0.011; 95% CI: 4.65 to 64.9 Day4 paired vs Day6 unpaired p<0.001; 95% CI: 16.0 to 76.3 Day4 paired vs Day7 unpaired p<0.001; 95% CI: 15.4 to 75.7 Day5 paired vs Day8 unpaired p>0.999; 95% CI: -20.8 to 39.5
Figure 8*F* (right) time spent in	Assumed	RM One-Way	N=4	Compartment $p < 0.001$, $F_{(2,6)} = 106$

paired, unpaired and neutral compartments during the 4 RT-PP days for NEX-Cre/ChR2 mice bilaterally injected and under high power stimulation	normality	ANOVA followed by Tukey's multiple comparison test	(M=0, F=4)	**Multiple comparisons** Paired vs unpaired $p<0.001$; 95% CI: 27.1 to 51.6 Paired vs neutral $p<0.001$; 95% CI: 44.5 to 69.0 Unpaired vs neutral $p=0.011$; 95% CI: 5.19 to 29.7
Figure 8-1*lii* Behavioral analysis of Vglut2-Cre throughout the NCP experiments	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=5 (M=0, F=5)	Interaction: $p = 0.002$, $F_{(4,16)} = 6.90$ Day: $p = 0.410$, $F_{(2,8)} = 1$ Compartment: $p < 0.001$, $F_{(2,8)} = 70.9$ **Multiple comparisons** (of interest) Stimulation 1 Neutral vs Paired1 $p=0.009$; 95% CI: -93.5 to -10.6 Neutral vs Paired2 $p=0.004$; 95% CI: -98.6 to -15.7 Paired 1 vs Paired2 $p>0.999$; 95% CI: -36.4 to 46.5 Stimulation 2 Neutral vs Paired1 $p<0.001$; 95% CI: -113 to -29.9 Neutral vs Paired2 $p<0.001$; 95% CI: -112 to -29. Paired 1 vs Paired2 $p>0.999$; 95% CI: -42.3 to 40.6 CR Neutral vs Paired1 $p=0.998$; 95% CI: -49.8 to 33.1 Neutral vs Paired2 $p>0.999$; 95% CI: -35.5 to 47.4 Paired 1 vs Paired2 $p=0.938$; 95% CI: -55.7 to 27.2
Figure 8-1*liii* time spent in paired1, paired2 and neutral compartments during the 2 NCP days for Vglut2-Cre/ChR2 mice	Normally distributed	RM One-Way ANOVA followed by Tukey's multiple comparison test	N=5 (M=0, F=5)	Compartment $p = 0.018$, $F_{(2,2)} = 54.2$ **Multiple comparisons** Paired1 vs Paired2 $p=0.951$; 95% CI: -38.9 to 43.2 Paired1 vs Neutral $p=0.023$; 95% CI: -103 to -20.7 Paired2 vs Neutral $p=0.021$; 95% CI: -105 to -22.8

Table 2

Area	Cre-driver			
	DAT	Vglut2	Calb2	NEX
Anterior olfactory area	+	+	+	+
Medial prefrontal cortex (infralimbic, prelimbic, and anterior cingulate cortices)	+	+	+	+
(Medial) orbital cortex	+	+	+	+
Nucleus accumbens shell	+	+	(+)	+
Nucleus accumbens core	+	-	-	+
Dorsomedial Striatum	+	-	-	-
Olfactory tubercle	+	+	+	+
Cingulate cortex	+	+	+	+
Septum/septal nuclei	+	+	-	+
Diagonal band of Broca	+	+	+	+
Ventral pallidum	+	+	+	+
Bed nuclei of the stria terminalis	+	+	+	+
Preoptic area	+	+	+	+
Lateral habenula	+	+	-	-
Medial habenula	-	-	+	+
Hippocampus	-	-	-	+
Dentate gyrus	-	-	-	+
Amygdala	+	+	+	+
Hypothalamic area	+	+	-	+

1

Double labeling *in situ* hybridization

A — NeuroD6
PBP, RLi, IF, SNc, PIF, PN — 250 μm

A'

B — Th
PBP, RLi, IF, SNc, PIF, PN — 250 μm

B'

C — NeuroD6 Th

C' 50 μm

NeuroD6 Th 25μm

D
Th+ [NeuroD6+ 12% / NeuroD6- 88%]
NeuroD6+ [TH+ 100%]

E — NeuroD6 Dat — PBP, RLi, IF, PIF, PN — 250μm — 25μm

F — NeuroD6 Vglut2

G — NeuroD6 Viaat — 25μm

Triple labeling *in situ* hybridization

H — RLi, PBP, IF, PIF, PN — Th — 200μm

I — NeuroD6

J — Vglut2

K — NeuroD6 Th

L — NeuroD6 Vglut2

M — Th NeuroD6 Vglut2

N — NeuroD6 Th — 35μm
NeuroD6 Vglut2
Th NeuroD6 Vglut2

O
NeuroD6+/Th+/Vglut2+ 12%
NeuroD6+/Th+/Vglut2- 88%

P
• NeuroD6+/Th+/Vglut2-   • NeuroD6+/Th+/Vglut2+
RLi, PBP, IF, PN, PIF, SNc

A NeuroD6-mediated targeting of Vmat2

$NEX\text{-}Cre^{tg/wt}$ $Vmat2^{lox/lox}$ $Vmat2^{lox/lox;NEX\text{-}Cre\text{-}wt}$(Ctrl)
$Vmat2^{lox/lox;NEX\text{-}Cre\text{-}tg}$(cKO)

B Vmat2 mRNA 2-probe approach

Detection of full-length Vmat2 mRNA

Probe2   Probe1   *Probe 1 and 2 bind to Vmat2 mRNA*

Detection of Vmat2 mRNA following Cre-mediated excision of exon2

Probe1   *Probe1 binds Vmat2 mRNA*
No binding of Probe2

C

$Vmat2^{lox/lox;NEX\text{-}Cre\text{-}wt}$(Ctrl) $Vmat2^{lox/lox;NEX\text{-}Cre\text{-}tg}$(cKO)

PBP   RLi   PBP   RLi
SNc   IF   SNc   IF
PIF   PN   PIF   PN
Probe1   250µm   50µm   Probe1

Probe2   Probe2

Probe1   Probe1
Probe2   Probe2

SNc   VTA   SNc   VTA
125µm

VTA: ○ Probe1+/Probe2+   ○ Probe1+/Probe2-

D

$Vmat2^{lox/lox;NEX\text{-}Cre\text{-}wt}$(Ctrl) $Vmat2^{lox/lox;NEX\text{-}Cre\text{-}tg}$(cKO)

LC   ROB   LC   ROB
Probe 1   125µm
Probe 2

VMH

E

$Vmat2^{lox/lox;NEX\text{-}Cre\text{-}wt}$(Ctrl) TH $Vmat2^{lox/lox;NEX\text{-}Cre\text{-}tg}$(cKO) TH

VTA   SNc   DStr   VTA   SNc
NAc
500µm   OT   1mm

Vmat2^{lox/lox;NEX-Cre-wt}(Ctrl)　　*Vmat2^{lox/lox;NEX-Cre-tg}*(cKO)

A AAV/EF1a-DIO-hChR2(H123R)-eYFP

DAT-Cre
Vglut2-Cre
Calb2-Cre
NEX-Cre

B NEX-Cre

TH | eYFP | eYFP TH

50µm

VTA

Striatal areas

C DAT-Cre — eYFP

RLi
PBP
SNc
IF
PIF
PN
1mm

C' eYFP TH

C'' DMStr
NAcC
aca
NAcSh
OT
500µm

NAcSh
OT
100µm

D Vglut2-Cre — eYFP

RLi
PBP
SNc
IF
PIF
PN
1mm

D' eYFP TH

D'' NAcSh
OT

E Calb2-Cre — eYFP

RLi
PBP
SNc
IF
PIF
PN
1mm

E' eYFP TH

E'' NAcSh
OT

F NEX-Cre — eYFP

RLi
PBP
SNc
IF
PIF
PN
1mm

F' eYFP TH

F'' NAcSh
OT

G

SNc
SNr
PBP
RLi
PN
IF
PIF IPR
-3.28mm – -3.52mm

SNc
SNr
RLi
PBP
IF
PIF
IPR
PN
IPC
-3.64mm – -3.80mm

-3.28mm – -3.52mm
VTA1 VTA2

-3.64mm – -3.80mm
VTA3 VTA4

H

■ NEX-Cre/ChR2-eYFP
■ Calb2-Cre/ChR2-eYFP
▨ TH+   ☐ TH-

VTA1 67% | 33%
       57% | 43%

VTA2 80% | 20%
       70% | 30%

VTA3 59% | 41%
       68% | 32%

VTA4 78% | 22%
       65% | 35%

Average 74% | 26%
VTA1-4   67% | 33%

A AAV/EF1a-DIO-hChR2(H123R)-eYFP (ChR2)
   or AAV/EF1a-DIO-eYFP-WPREpA (Ctrl)

● Recording site

DAT-Cre
Calb2-Cre
NEX-Cre

DStr
NAc
OT
VTA

B Volts (V) 1.3 /\ -0.4 8.5 ms

nA

-0.4   1.3   447 nm light

C

DAT-Cre   NEX-Cre   Calb2-Cre

NAcSh

OT

ChR2 eYFP   Current (nA) -15 -30   Photostimulation: 447 nm, 3s duration, 10 ms pulse width, 20 Hz

D

DAT-Cre | NEX-Cre | Calb2-Cre

NAcSh evoked DA release (µM)

***   ***   *

ChR2 Ctrl   ChR2 Ctrl   ChR2 Ctrl

DAT-Cre | NEX-Cre | Calb2-Cre

OT evoked DA release (µM)

***   **   ***

ChR2 Ctrl   ChR2 Ctrl   ChR2 Ctrl

A

NacC
recording pipette
NacSh

OT
recording pipette

B

NEX-Cre NacSh

$V_h = 0\ mV$

$V_h = -60\ mV$

$20\ \mu M\ DNQX$

$20\ ms$

Calb2-Cre OT

$V_h = 0\ mV$

$V_h = -60\ mV$

$20\ \mu M\ DNQX$

$20\ ms$

C

NEX-Cre NAcSh | Calb2-Cre OT

82% 18%

87% 13%

amplitude (pA)

☐ Connected ■ Unconnected

D

NEX-Cre NAcSh | Calb2-Cre OT

amplitude (pA)

*

*

control DNQX

control DNQX