Reduced cue-induced reinstatement of cocaine-seeking behavior in Plcb1+/- mice

Running title: *Plcb1* is involved in cocaine-seeking behavior.

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ABSTRACT

Background and Purpose: Cocaine addiction causes serious health problems and no effective treatment is available yet. We previously identified a genetic risk variant for cocaine addiction in the *PLCB1* gene and found this gene upregulated in postmortem brains of cocaine abusers and in human dopaminergic neuron-like cells after an acute cocaine exposure. Here, we functionally tested the contribution of *PLCB1* gene to cocaine addictive properties in mice.

Experimental approach: We used heterozygous *Plcb1* knockout mice (*Plcb1+/-*) and characterized their behavioral phenotype. Subsequently, mice were trained for operant conditioning and self-administered cocaine for 10 days. *Plcb1*+/- mice were assessed for cocaine motivation, followed by 26 days of extinction and finally evaluated for cue-induced reinstatement of cocaine seeking. Gene expression alterations after reinstatement were assessed in medial prefrontal cortex (mPFC) and hippocampus (HPC) by RNAseq.

Key Results: *Plcb1*+/- mice showed normal behavior, although they had increased anxiety and impaired short-term memory. Importantly, after cocaine self-administration and extinction, we found a reduction in the cue-induced reinstatement of cocaine-seeking behavior in *Plcb1+/-* mice. After reinstatement, we identified transcriptomic alterations in the medial prefrontal cortex of *Plcb1*+/- mice, mostly related to pathways relevant to addiction like the dopaminergic synapse and long-term potentiation.

Conclusions and Implications: To conclude, we found that heterozygous deletion of the *Plcb1* gene decreases cue-induced reinstatement of cocaine seeking, pointing at PLCB1 as a possible therapeutic target for preventing relapse and treating cocaine addiction.

KEYWORDS: addiction, relapse, cocaine self-administration, RNAsequencing, transcriptomic, resilience, medial prefrontal cortex (mPFC)

INTRODUCTION

Cocaine is the most used psychostimulant illicit drug worldwide (UNODC, 2019), causing severe health problems that include the development of cocaine addiction in around 15-16% of cocaine users (O'Brien and Anthony, 2005). Cocaine addiction is a complex psychiatric disorder that results from the interaction of genetic, epigenetic, and environmental risk factors (Pierce et al., 2018). The heritability of cocaine addiction is one of the highest among psychiatric disorders, estimated around 65% for women (Kendler et al., 2000) and 79% for men (Kendler and Prescott, 1998). However, the genetic factors and mechanisms that underlie the transition from drug use to addiction and its establishment remain unknown.

In a previous study, we identified a single nucleotide polymorphism in the *PLCB1* gene associated with drug dependence (rs1047383), and especially with a subgroup of cocaine-addicted patients, that was replicated in an independent clinical sample (Cabana-Domínguez et al., 2017). Also, genetic variants in this gene were found nominally associated with an illegal substance and cocaine addiction in two GWAS performed in European-American samples (Drgon et al., 2012). On the other hand, we found that cocaine increased the expression of *PLCB1* both in human dopaminergic neuron-like cells (differentiated SH-SY5Y cells) after acute cocaine exposure and in postmortem samples of the nucleus accumbens (NAc) of cocaine abusers (Cabana-Domínguez et al., 2017). Interestingly, this gene was also found over-expressed in the same brain region in mice

66 after cocaine administration for 7 days and also during withdrawal (Eipper-Mains et al., 2013). All
67 this evidence suggest that *PLCB1* may play a role in cocaine addiction.

68 The *PLCB1* gene encodes phospholipase C beta 1, and it is highly expressed in the brain, mainly in
69 the frontal cortex, basal ganglia (caudate, putamen, and NAc) and hippocampus. These brain
70 regions are crucial for drug reward and the formation of drug-context associations, both
71 contributing to the development and maintenance of addiction (Bisagno et al., 2016; Castilla-
72 Ortega et al., 2016, 2017; Kutlu and Gould, 2016; Pitts et al., 2016; Cooper et al., 2017). Several
73 neurotransmitters activate this protein, including dopamine through DRD1 and DRD2 (Lee et al.,
74 2004; Rashid et al., 2007), serotonin by 5-HT2A and 2C receptors (Hagberg et al., 1998; Chang et
75 al., 2000) and glutamate by mGluR1 (Conn and Pin, 1997; Hannan et al., 2001). Thus, PLCB1 might
76 be a point of convergence of neurotransmitter systems that play an essential role in the
77 development of addiction (recently reviewed (Howell and Negus, 2014; Moretti et al., 2020)). The
78 activation of PLCB1 produces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into the
79 second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), responsible for
80 intracellular signal transduction. Alterations in PLCB1-mediated signaling in the brain have been
81 associated with other neuropsychiatric disorders such as epilepsy, schizophrenia, and bipolar
82 disorder (Yang et al., 2016).

83 Here we studied the contribution of the *PLCB1* gene to cocaine addiction and dissected its
84 participation in the different aspects of the addictive process. We used heterozygous knockout
85 (KO) mice (*Plcb1+/-*), as the homozygous KO (*Plcb1-/-*) showed seizure attacks and low viability
86 after birth (Kim et al., 1997). In addition, the use of a constitutive heterozygous KO mouse model
87 allowed us to assess *Plcb1* haploinsufficiency during neurodevelopment in mice similarly to
88 humans, where inherited genetic risk or protective variants can modulate the susceptibility to

89 addiction (Cabana-Domínguez et al., 2017). First, we performed a general phenotypic
90 characterization using a battery of behavioral tests, including memory, anxiety, locomotor activity,
91 coordination, food and water intake, and sucrose preference. Then, we evaluated cocaine operant
92 self-administration, extinction, and cue-induced reinstatement. Finally, we studied transcriptional
93 alterations to further understand the molecular mechanisms involved.

94

95 **RESULTS**

96 In the present work, we aimed to assess the contribution of the *PLCB1* gene in the different stages
97 of cocaine addiction using heterozygous knockout mice (*Plcb1+/-*). To do so, we performed a
98 general phenotypic characterization and, then we evaluated cocaine operant self-administration,
99 extinction, and cue-induced reinstatement in those animals. Also, we studied transcriptional
100 changes in mPFC and HPC to understand the mechanisms involved.

101 **Behavioral tests for phenotype characterization**

102 We first evaluated the effects of the heterozygous deletion of *Plcb1* on general behavioral
103 responses, including locomotor, cognitive and emotional responses, food, and water intake.
104 *Plcb1+/-* mice showed a lower discrimination index in the novel object recognition compared to
105 WT (*t*-test=3.21, $P<0.01$, Figure 1B), suggesting an impairment in short-term memory. This
106 difference was not influenced by exploration time, as this variable was equal between genotypes
107 (Figure 1C). Furthermore, no differences in locomotor activity were reported between genotypes,
108 discarding an involvement of the *Plcb1* heterozygous deletion in locomotion (Figure 1D). Mutant
109 mice showed increased exploratory activity with a higher number of rearings than the WT mice (*U*-
110 Mann Whitney=67.5, $P<0.01$, Figure 1E). An anxiogenic profile was revealed in the elevated plus

111 maze in mutants, as shown by the reduced time spent in the open arms (*t*-test=3.02, *P*<0.001,
112 Figure 1F) and the percentage of time (*t*-test=3.16, *P*<0.001, Figure 1G). Finally, the rota rod test
113 revealed that the heterozygous deletion of *Plcb1* did not affect motor coordination (Figure 1H-I).

114 Several consummatory and locomotor parameters were long-term monitored in the PheComp
115 boxes (Supplementary Figure 1A-H, n=6 per genotype). No differences between genotypes were
116 revealed during the whole experimental period in body weight, food, and water intake, levels of
117 sucrose preference, stereotyped movements, horizontal and vertical locomotor activity, indicating
118 no altered behavior in the *Plcb1+/-* mice.

119 **Operant conditioning maintained by cocaine**

120 Then, we investigated the effects of the heterozygous deletion of *Plcb1* in cocaine behavioral
121 responses related to its addictive properties. For this purpose, *Plcb1+/-* mice and their WT
122 littermates were trained for cocaine operant self-administration (0.5mg/kg/infusion) during FR1
123 and FR3, progressive ratio, extinction and cue-induced reinstatement (Figure 2A). Control mice
124 trained with saline were included for both genotypes. Results showed that the percentage of mice
125 reaching the criteria of operant conditioning was 100% for both genotypes trained with cocaine
126 and 33% for mice trained with saline: [*Chi-square* test=9.50; *P*<0.01, *Plcb1+/-* cocaine vs. *Plcb1+/-*
127 saline] and [*Chi-square* test=14.00; *P*<0.001, WT cocaine vs. WT saline], as expected.

128 The primary reinforcing effects and the motivation for cocaine were similar in both genotypes
129 (Figure 2B-C). During FR1, both genotypes similarly increased the number of cocaine infusions
130 across sessions, whereas mice trained with saline remained steady (repeated measures ANOVA,
131 interaction between genotype x drug x sessions, *P*<0.05, Figure 2B and Supplementary Table S1).
132 Thus, the evolution of operant responding was different in mice trained with cocaine and saline,

133 with an increased responding over nearly all sessions in cocaine-trained mice . This enhancement
134 in cocaine responding was more pronounced in mutants than WT mice. Indeed, WT mice showed a
135 decrease in cocaine intake in the second session. The maximum number of infusions was reached
136 on session 5 in both genotypes and was slightly superior in *Plcb1+/-* mutants (36.35±1.47) than in
137 WT (29.61±2.23) but post hoc analyses demonstrated that this difference in cocaine intake was
138 not significant (Figure 2B). Similar findings were observed for the total number of nose-pokes in
139 which operant responding increased in both genotypes with cocaine but remained stable with
140 saline (Supplementary Figure S2A). When the effort to obtain one dose of cocaine increased to
141 FR3, the number of infusions was stable across sessions in WT and *Plcb1*+/- mice. Similarly,
142 operant responding was higher for all groups than in FR1 with stable higher levels of responding
143 (Supplementary Figure S2A) and a higher number of infusions (repeated measures ANOVA, the
144 main effect of the drug, $P<0.001$, Figure 2B) in mice trained with cocaine than with saline,
145 independently of the genotype.

146 Motivation for cocaine was evaluated in a progressive ratio schedule, and no significant
147 differences were obtained between genotypes (Figure 2C). The levels of extinction of the operant
148 behavior were similar between genotypes and decreased progressively across sessions (repeated
149 measures ANOVA, interaction genotype x sessions, $P<0.001$, Figure 2D). The percentage of mice
150 reaching cocaine-seeking extinction criteria was similar in *Plcb1+/-* (73%) and WT (78%) mice.

151 Importantly, *Plcb1+/-* mice showed significantly reduced cue-induced reinstatement of cocaine-
152 seeking compared to WT mice (*U* Mann-Whitney, $P<0.05$, Figure 2E), with 27.59% less active nose-
153 pokes compared to WT mice trained with cocaine. Furthermore, mice trained with saline from
154 both genotypes exhibited 55.84% reduction of active nose-pokes than WT mice trained with
155 cocaine and 39.20% less than mutants trained with cocaine. No significant differences were

156 obtained between genotypes in inactive nose-pokes during operant conditioning maintained by
157 cocaine nor during extinction (Supplementary Figure S2B-C). Both genotypes trained with cocaine
158 acquired the reinstatement criterion (double nose pokes in the active hole than the number of
159 nose pokes during the 3 consecutive days when the mice acquired the extinction criteria) showing
160 their capability to maintain this conditioning learning task. These data showed that *Plcb1+/-*
161 resulted in a phenotype of resistance to cue-induced reinstatement with reduced cocaine-seeking
162 (Figure 2E).

163 **Brain transcriptomic analysis after the reinstatement of cocaine-seeking behavior**

164 To further understand the role of PLCB1 in the molecular mechanisms involved in this cocaine
165 relapse-related phenotype, we analyzed the transcriptomic profiles of mPFC and HPC immediately
166 after the reinstatement of cocaine-seeking behavior in WT and *Plcb1*+/- mice trained with cocaine.
167 We identified 2,115 protein-coding genes differentially expressed (DEGs) in mPFC (1,231
168 downregulated and 943 upregulated) and only 12 in HPC, when comparing *Plcb1+/-* and WT
169 animals (Supplementary Tables S2-3). In accordance, PCA analysis and heatmap plots revealed that
170 individuals with the same genotype plotted together only in mPFC, but not in HPC (Supplementary
171 Figure S3). This suggested a more prominent role of mPFC, so further studies were carried out only
172 with DEGs of this brain area. Analysis of functional group over-representation identified several
173 processes previously related to cocaine addiction, including dopaminergic synapse, learning, long-
174 term potentiation (LTP), neurotransmitter secretion, and axon guidance, as well as other relevant
175 signaling pathways such as MAPK, mTOR, and neurotrophin (Figure 3A-B and Supplementary
176 Tables S3-5). We focused on the dopaminergic synapse pathway (enriched in the mPFCs DEGs;
177 P_{Adj}<0.05), in which phospholipase c (such as Plcb1) is directly participating in signal transmission
178 (Figure 4). Interestingly, many genes coding for proteins in this pathway are differentially

179 expressed in *Plcb1+/-* mice after cue-induced reinstatement (Figure 4 and Supplementary Table
180 S6). Furthermore, we found that several TFBS were over-represented in the DEGs of mPFC,
181 including YY1, MYOD, NRF1, ERR1, FREAC2, NFY and E4F1 (complete list of TFBS in Supplementary
182 Table S7). Then, we filtered the DEGs on mPFC based on fold-change (FC>|1.2|) and obtained a list
183 of 238 genes that we used for gene network construction. This analysis showed a highly scored
184 network (score=62, Figure 3C) that includes 31 DEGs involved in "cellular development, cellular
185 growth and proliferation, nervous system development and function".

186

187 **DISCUSSION**

188 Here we investigated, for the first time, the contribution of the *PLCB1* gene to cocaine addictive
189 properties using *Plcb1+/-* mice. We found that the heterozygous *Plcb1* genotype resulted in a
190 phenotype of resistance to cue-induced reinstatement of cocaine-seeking behavior. Furthermore,
191 we found relevant transcriptomic differences in *Plcb1+/-* mice compared to WT after cue-induced
192 reinstatement of cocaine seeking in mPFC, a brain area essential in relapse (Jasinska et al., 2015;
193 Moorman et al., 2015). This study supports a role for *PLCB1* in cocaine addiction, confirming
194 previous findings in humans (Cabana-Domínguez et al., 2017), and suggests it may be relevant in
195 relapse to cocaine addiction.

196 The phenotype of *Plcb1+/-* mice was characterized at different levels to evaluate consummatory
197 and general behavior. In general, mutant mice presented normal levels of body weight, food and
198 water intake, locomotor activity, and motor coordination, demonstrating that this animal model is
199 valid to study the effects of the heterozygous deletion of the *Plcb1* gene in other behavioral
200 responses. *Plcb1+/-* showed increased anxiety in the elevated plus maze, reduced short-term

201 memory in the novel object recognition paradigm, without any sign of depressive-like behavior in
202 the anhedonia sucrose preference test. Thus, the single targeting of the *Plcb1* gene has an impact
203 on selective emotional and cognitive responses.

204 The short-term memory impairment observed in the mutants did not affect the acquisition of
205 operant associative conditioning nor the instrumental learning that drives the goal-directed action,
206 as shown by similar levels of operant cocaine self-administration and extinction learning than WT.
207 In agreement, both genotypes trained with cocaine accomplish cue-induced reinstatement
208 criterion with high enhancement of responding during this test compared to extinction. Thus,
209 different brain circuits are involved in each kind of learning, with perirhinal-hippocampal
210 structures (Winters et al., 2004) participating in short-term memory in the novel object
211 recognition paradigm and mPFC-dorsal striatum pathway in cue-associated seeking. Also
212 differences in each paradigm such as the acute retrieval or repetitive exposure involved
213 respectively in each task may have a crucial influence in cognition. Hence, cocaine seeking can be
214 multidimensional, involving different types of associative learning that together lead to an
215 extensive repertoire of conditioned and instrumental responding.

216 The anxiogenic profile of *Plcb1+/-* is in accordance with the results previously reported in the
217 *Plcb4-/-* mice, which were associated with alterations in the cholinergic activity of the medial
218 septum (Shin et al., 2009). However, the selective knock-down of *Plcb1* in the mPFC did not
219 replicate this phenotype in a previous study (Kim et al., 2015), suggesting that other areas may be
220 involved. The anxiogenic profile of *Plcb1+/-* mice had no effect on cocaine self-administration since
221 the acquisition and extinction of this operant behavior was not modified in the mutants. Besides,
222 mutants showed protection against cue-induced reinstatement of cocaine seeking, instead of the
223 expected cocaine seeking promoted by an anxiogenic phenotype (Mantsch et al., 2016).

224 Therefore, the association of an anxiogenic profile with a phenotype resilient to cocaine seeking in
225 the *Plcb1+/-* mice suggests modifications in specific brain areas involved in cocaine relapse, such
226 as the mPFC. Recently, a phenotype resilient to develop food addiction has been associated with
227 increased strength of pyramidal glutamatergic synaptic transmission in the mPFC related to
228 decreased compulsivity in the face of negative consequences (Domingo-Rodriguez et al., 2020) in
229 mice with an anxiogenic profile (Lutz et al., 2015). Concerning cue-induced cocaine seeking, the
230 mPFC has a crucial role and the network of glutamatergic projections from the prelimbic mPFC to
231 the dorsal striatum participates in this phenotype (Lüscher et al., 2020). In our model, *Plcb1*
232 haploinsufficiency is linked to reduced cue-induced cocaine seeking possibly associated with
233 modifications in this top-down corticolimbic brain network. Furthermore, these glutamatergic
234 projections from mPFC to dorsal striatum receive mesocortical dopaminergic inputs from the VTA
235 that could be crucially involved in the protective effects of *Plcb1,* since this gene plays an essential
236 role in the dopaminergic signal transmission in the mPFC and many of the genes encoding for
237 proteins in this pathway are differentially expressed in *Plcb1+/-* mice after cue-induced
238 reinstatement (Figure 4).

239 Transcriptomic analyses performed after the cue-induced reinstatement of cocaine-seeking
240 behavior revealed some of the molecular underpinnings that underlie the cocaine relapse-related
241 phenotype observed in the mutant mice. Interestingly, differences in gene expression between
242 *Plcb1+/-* and WT were predominantly found in the mPFC, whereas almost no differences were
243 observed in the HPC. This evidence highlights mPFC as a key region to explain these differences in
244 cocaine-seeking reinstatement observed in *Plcb1+/-* mice. Consistently, in DEGs in mPFC, we found
245 enrichment on pathways that are essential for the development of addiction, including the
246 dopaminergic neurotransmission (Figures 3-4) (Wise and Robble, 2020). *PLCB1* is part of the
247 dopamine-DARPP-32 signaling pathway (Figure 4), which plays a key role in cocaine reward

248 (Borgkvist and Fisone, 2007; Nishi and Shuto, 2017). In a previous study, we also found this
249 pathway enriched in DEGs in the frontal cortex and ventral striatum of mice that showed
250 frustrated expected reward, produced by the cue in the absence of expected reward after a high
251 level of effort in a progressive ratio schedule of reinforcement with palatable food (Martín-García
252 et al., 2015b). Notably, *Plcb1* was upregulated in the frontal cortex of those frustrated mice with
253 increased responses to obtain the reward (palatable food). These data are in line with our findings
254 in which decreased expression of *Plcb1* (*Plcb1*+/- mice) results in decreased cue-induced responses
255 to obtain cocaine after extinction. All these results support the participation of *Plcb1* in cue-
256 induced cocaine seeking revealed in the present study and the involvement of mPFC .

257 Our transcriptomic analyses also found enrichment in genes related to learning and memory
258 processes, such as LTP in the mPFC. The transition from drug use to drug addiction is a
259 maladaptive process that directly affects learning and memory (Torregrossa et al., 2011; Goodman
260 and Packard, 2016). LTP produces long-lasting activity-dependent synaptic modifications that
261 underlie memory, and drugs of abuse alter this mechanism in several brain regions such as mPFC
262 (Huang et al., 2007; Lu et al., 2010), mesocorticolimbic system (Thomas et al., 2008), VTA (Placzek
263 et al., 2016) and hippocampus (Keralapurath et al., 2017; Preston et al., 2019), among others.
264 Importantly, genes related to the glutamatergic system such as *Gria2-4* (glutamate ionotropic
265 receptor AMPA2-4 (alpha 2-4)) and *Grik2* (ionotropic glutamate receptor kainate 2 (beta 2)) were
266 found to be upregulated in *Plcb1+/-* mice. Meanwhile, *Grin1, 2c, and 2d* (glutamate ionotropic
267 receptor NMDA1 (zeta 1, epsilon3 and epsilon4)) were downregulated, suggesting an increase in
268 AMPAR/NMDAR ratio in cocaine-experienced and abstinent *Plcb1 +/-* mice as compared to WT,
269 one of the key indicators of LTP induction and increased synaptic strength (Lüscher and Malenka,
270 2011). Furthermore, we found enrichment in other neuroplasticity (synapse organization, neuron
271 projection development, and axon guidance) (Bahi and Dreyer, 2005; O'Brien, 2009; Cooper et al.,

272 2017; Dong et al., 2017) and signaling pathways (MAPK, mTOR, and neurotrophin) (Li and Wolf,
273 2015; García-Pardo et al., 2016) related to addiction. The assessment of gene expression in the
274 mPFC of the *Plcb1+/-* mice highlighted alterations in relevant pathways for the addictive process,
275 which could contribute to the results observed in the cue-induced reinstatement.

276 Nowadays, there are few effective pharmacological treatments available for cocaine use disorders,
277 and frequently, psychosocial interventions in combination with pharmacotherapy are needed
278 (Kampman, 2019). Studies performed in mice have pinpointed multiple potential therapeutic
279 approaches for the reinstatement of cocaine-seeking behavior, targeting the reward circuit
280 (Buchta et al., 2020). In humans, treatment with bupropion, topiramate, or disulfiram has been
281 widely used. Bupropion, a non-tricyclic antidepressant that inhibits dopamine and norepinephrine
282 reuptake, is effective in reducing craving (Frishman, 2007). Topiramate, a GABA/glutamatergic
283 medication, has also been used to treat cocaine use disorder as it reduces the activity of the
284 mesocorticolimbic dopaminergic system (Kampman, 2019). A completely different strategy is the
285 use of disulfiram which potentially inhibits the oxidoreductase dopamine β-hydroxylase (DβH,
286 encoded by the *DBH* gene), which converts dopamine to norepinephrine (Kampangkaew et al.,
287 2019). Significantly, a genetic variation in the *SLC6A3* gene (encoding DAT) has been associated
288 with disulfiram treatment for cocaine addiction, with patients with higher DAT levels having better
289 treatment outcomes than those with lower DAT levels (Kampangkaew et al., 2019). Thus, further
290 studies and new therapeutic targets are needed to obtain effective treatment for cocaine
291 addiction. The results obtained in the present study underscore the relevance of the *Plcb1* gene in
292 the cue-induced reinstatement of cocaine seeking after extinction. Together with previous findings
293 in humans (Drgon et al., 2012; Cabana-Domínguez et al., 2017) and mice (Eipper-Mains et al.,
294 2013), *PLCB1* merits to be further evaluated as a promising novel therapeutic target for preventing
295 relapse and treating cocaine addiction .

296 The experimental approach used in our study, the *Plcb1+/-* mouse model, allowed us to reproduce
297 better the molecular context observed in humans in comparison to the use of a complete KO
298 mouse (Hall et al., 2013), as these animals preserved, at least, half of the expression of *Plcb1*.
299 However, the haploinsufficiency of *Plcb1* during neurodevelopment in these animals could
300 produce alterations that may contribute to the phenotype observed in the present study.
301 Nevertheless, this may also be similar in humans with genetic risk variants that decrease *PLCB1*
302 expression. Therefore, this approach is appropriate to study a specific genetic alteration that
303 confers susceptibility to drug addiction and to delineate the precise contribution of *PLCB1.*

304 To sum up, we studied, for the first time, the contribution of the *PLCB1* gene to cocaine addictive
305 properties using *Plcb1+/-* mice. Previous studies have revealed an up-regulation of *Plcb1* in brain
306 areas related to the reward circuit after cocaine exposure in animals (Eipper-Mains et al., 2013)
307 and in human cocaine abusers (Cabana-Domínguez et al., 2017). These changes, together with our
308 results, suggest that cocaine increases the expression of *Plcb1,* and this mechanism plays an
309 essential role in cocaine addiction, as revealed now by the resistance to cue-induced
310 reinstatement of cocaine-seeking behavior exhibited by *Plcb1+/-* mutant mice. These results
311 highlight the importance of the *Plcb1* gene in the development of cocaine addiction and relapse
312 and pinpoint PLCB1 as a promising therapeutic target for cocaine addiction and perhaps other
313 types of addiction.

314

315 **METHODS**

316 **Animals**

317 Male mice, 8 weeks old, were housed individually at temperature- and humidity-controlled
318 laboratory conditions (21±1°C, 55±10%) maintained with food and water ad libitum. Mice were
319 tested during the dark phase of a reverse light cycle (lights off at 8.00h and on at 20.00h). *Plcb1+/-*
320 mice in a C57BL/6J background and their wild-type (WT) littermates were used (Kim et al., 1997).
321 All experimental protocols were performed in accordance with the guidelines of the European
322 Communities Council Directive 2010/63/EU and approved by the local ethics committee (Comitè
323 Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, agreement
324 N°9213). In agreement, maximal efforts were made to reduce the suffering and the number of
325 mice used.

326 **Genotyping of transgenic mice**

327 *Plcb1+/-* mice in a C57BL/6J background were kindly provided by H.-S. Shin (Kim et al., 1997). Very
328 briefly, the knockout animals were generated by the replacement of exons that encode aminoacid
329 residues 50–82 by a Neomycin cassette that was used both for gene disruption and for positive
330 selection.

331 To genotype the mice, genomic DNA from ear punches or tail tips was extracted as previously
332 described (Gómez-Grau et al., 2017). Genotyping was carried out by PCR on extracted genomic
333 DNA using three specific PCR primers: a sense primer, 5′-GTTAAGTCCTCAGGCAAACACC-3', and
334 two antisense primers, 5′-ACCTTGGGAGCTTTGGCGTG3' and 5′-CTGACTAGGGGAGGAGTAGAAG-
335 3', that allowed us to amplify a 180bp *Plcb1+/+* band and a 290bp *Plcb1-/-* band (Filis et al., 2009).
336 Fragments were separated by 2.5% agarose gel electrophoresis. Known WT and *Plcb1+/-* controls
337 were always used to validate genotyping results. The genotype of all animals used was confirmed
338 after the cue-induced reinstatement.

339 **Behavioral tests for phenotype characterization**

340 A group of 25 WT and 12 *Plcb1+/-* mice underwent different behavioral tests for phenotype
341 characterization for 10 days, as described in Figure 1:

342 *Short-term memory.* To measure short-term memory, the novel object recognition (NOR) test was
343 used. This test was performed in a V-maze apparatus (40 cm per side, Panlab, Spain) as previously
344 described by us and others (Bura et al., 2010; Planaguma et al., 2015). On day 1 mice were
345 habituated for 9 minutes in the V-maze. On day 2, mice were put back into the V-maze for 9
346 minutes; two identical objects were presented, and the time the mice spent exploring each object
347 was recorded. After a retention phase of 3 hours, the mice were placed for another 9 minutes into
348 the V-maze; in each paradigm, one of the familiar objects was replaced with a novel object and the
349 total time spent exploring each object (novel and familiar) was registered. Object exploration was
350 defined as the orientation of the nose to the object at a distance of less than 2 cm. A
351 discrimination index was calculated as the difference in the time spent exploring the novel and the
352 time spent exploring the familiar object divided by the total time exploring both objects. A higher
353 discrimination index is considered to reflect more excellent memory retention for the familiar
354 object.

355 *Locomotor activity.* Locomotor activity was assessed in locomotor activity boxes (9×20×11 cm;
356 Imetronic, Passac, France), equipped with 2 rows of photocell detectors, and placed in a low-
357 luminosity environment (20–25 lux), as previously described (Martin-Garcia et al., 2013). The
358 locomotor activity was recorded for 2 hours, and 2 variables were measured: horizontal activity as
359 horizontal displacement and vertical activity as vertical exploration/elevation.

360 *Elevated plus maze test.* This test was performed as previously reported (Planaguma et al., 2015).
361 The test measures the conflict between the natural tendency of mice to avoid an illuminated and
362 elevated surface, and the natural tendency to explore new environments. It consisted of a black

363 plastic cross with arms 40 cm long and 6 cm wide placed 50 cm above the floor. Two opposite
364 arms were surrounded by walls (15 cm high, closed arms, 10 lux), while the two other arms were
365 devoid of such walls (open arms, 200 lux). A central platform connected the four arms. At the start
366 of the session, the mouse was placed at the end of a closed arm facing the wall. During the 5-
367 minutes trial, the number of entries and the time spent in each arm were recorded. Anxiety was
368 assessed as both the time spent avoiding the open arms and the number of entries into them.

369 *Motor coordination.* Rota rod apparatus was used to assess motor coordination. Precisely, this test
370 measures the ability of the mouse to remain on a rotating rod in which the speed of rotation is
371 gradually increased and evaluate general motor coordination. The device is a round drum (5 cm
372 diameter) suspended between 2 plexiglass walls. The drum is suspended at 24 cm from a soft mat
373 covered tabletop. The speed of rotation is controlled by an electric engine with a digital revolution
374 per min (rpm) display. The test consisted in five trial sessions. Each trial started with the mouse
375 being placed on the rotating rod at 4.0 rpm, and then every 3 s the speed increased by 1.0 rpm
376 until 20.0 rpm. The trial terminated when the mouse fell from the rod or after 90 s, whichever
377 occurred first. There was a 10 s interval between trials. The average maximum rota rod speed and
378 time to fall was calculated for each mouse (Martin-Garcia et al., 2013).

379 *Experimental procedure for food and drink monitoring.*

380 *Habituation.* Six animals per genotype were taken from their home cages and placed in
381 experimental chambers named PHECOMP boxes (Panlab and Harvard Apparatus) equipped with a
382 food, drink and motor activity monitoring system
383 (https://www.panlab.com/en/products/phecomp-system-panlab) for two weeks of habituation.
384 Animals were monitored in these boxes 24 h a day during the whole experimental period.

385 *Sucrose preference test.* After a period of habituation, a sucrose preference test was performed on
386 the third week of PheComp cage housing during one week, as previously described (Bura et al.,
387 2010; Planaguma et al., 2015). Two bottles of water, one with 2% sucrose and the other without,
388 were placed in the cage. The position of bottles were exchanged everyday, and the consumption
389 was measured after a 24h interval. The preference for sucrose was calculated as the relative
390 amount of water with sucrose versus total liquid (water with and without sucrose) consumed by
391 the mice (Bura et al., 2010).

392 **Operant conditioning maintained by cocaine**

393 *Operant self-administration apparatus.* The self-administration experiments were conducted in
394 mouse operant chambers (Model ENV-307A-CT; Med Associates Inc., Georgia, VT, USA) equipped
395 with two holes, one randomly selected as the active hole and the other as the inactive hole as
396 previously described (Gutierrez-Cuesta et al., 2014).

397 *Operant conditioning maintained by cocaine.* Cocaine self-administration experiments were
398 performed as previously described (Gutierrez-Cuesta et al., 2014; Martín-García et al., 2015a).
399 Mice were deeply anesthetized by intraperitoneal injection of a mixture of ketamine (75mg/kg;
400 Imalgène; Merial Laboratorios S.A., Barcelona, Spain) and medetomidine (1mg/kg; Domtor;
401 Esteve, Barcelona, Spain). After surgery, anesthesia was reversed by subcutaneous injection of the
402 synthetic α2 adrenergic receptor antagonist, atipamezole (2.5mg/kg; Revertor; Virbac, Barcelona,
403 Spain) indicated for the reversal of the sedative and analgesic effects of medetomidine (α2
404 adrenergic receptor agonist). In addition, mice received an intraperitoneal injection of gentamicin
405 (1 mg/kg; Genta-Gobens; Laboratorios Normon, S.A., Madrid, Spain) along with subcutaneous
406 administration of the analgesic meloxicam (2 mg/kg; Metacam; Boehringer Ingelheim, Rhein,
407 Germany). Each 2h daily self-administration session started with a priming injection of the drug.

408 Cocaine (obtained from The Spanish Agency of Drugs, Madrid, Spain) was intravenously infused in
409 23.5µl over 2s (0.5 mg/kg per injection). Cue light, located above the active hole, was paired with
410 the delivery of the reinforcer. Mice (WT n=36; *Plcb1+/-* n=26) were trained under a fixed ratio 1
411 schedule of reinforcement (FR1; one nose-poke lead to the delivery of one dose of cocaine) over 5
412 consecutive daily sessions and under a fixed ratio 3 (FR3) over 5 consecutive daily sessions. Control
413 mice trained with saline were included for both genotypes (WT n=6; *Plcb1+/-* n=6). The timeout
414 period after infusion delivery was 10 s. Responses on the inactive hole and all responses elicited
415 during the 10-s timeout period were also recorded. Responses during the 10-s timeout period
416 were considered a measure of impulsivity reflecting the inability to stop motor behavior once it is
417 initiated. The criteria for self-administration behavior were achieved when all of the following
418 conditions were met: (1) mice maintained stable, responding with 20% deviation from the mean of
419 the total number of reinforcers earned in three consecutive sessions (80% of stability); (2) at least
420 75% of mice responding on the active hole; and (3) a minimum of 10 reinforcers per session.

421 After the 10 FR sessions, animals were tested in a progressive ratio schedule of 4h where the
422 response requirement to earn the cocaine escalated according to the following series: 1–2–3–5–
423 12–18–27–40–60–90–135–200–300–450–675–1000. Then, we proceed with the extinction phase,
424 2h daily sessions following the same experimental conditions than the cocaine self-administration
425 sessions except that cocaine were not available, and cue-light was not presented. Mice were given
426 2-h daily sessions until they achieved the extinction criterion with a maximum of 26 sessions. The
427 criterion for extinction was achieved when, during 3 consecutive sessions, mice completed a mean
428 number of nose pokes in the active hole consisting of 30% of the mean responses obtained during
429 the 3 days to achieve the acquisition criteria for cocaine self-administration training. On day 27,
430 only mice that accomplished extinction criterion were tested in the cue-induced reinstatement
431 during a 2h session, to evaluate the reinstatement of cocaine-seeking behavior. The test for cue-

432 induced reinstatement was conducted under the same conditions used in the training phase
433 except that cocaine was not available. The reinstatement criterion was achieved when nose pokes
434 in the active hole were double the number of nose pokes in the active hole during the 3
435 consecutive days when the mice acquired the extinction criteria.

436 The catheter was flushed daily with heparinized saline (30 USP units/ml). The patency of
437 intravenous catheters was evaluated after the last cocaine self-administration session and
438 whenever the behavior appeared to deviate dramatically from that observed previously by
439 infusion of thiopental through the catheter. If prominent signs of anesthesia were not apparent
440 within 3s of the infusion, the mouse was removed from the experiment. The success rate for
441 maintaining patency of the catheter (mean duration of 11 days) until the end of the cocaine self-
442 administration training was 90%.

443 After the cue-induced reinstatement, animals were euthanized by decapitation, brains were
444 quickly removed, and the medial prefrontal cortex (mPFC) and hippocampus (HPC) were dissected.
445 Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice and stored
446 at −80°C for later RNA isolation and transcriptomic analyses.

447 **RNA extraction and RNA sequencing**

448 Total RNA of 12 WT and 11 *Plcb1+/-* mice from mPFC and HPC were isolated using the RNeasy
449 Lipid Tissue Mini Kit (Qiagen Düsseldorf, Germany) according to the manufacturer's protocol. RNA
450 concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop
451 Technologies, Wilmington, DE, USA), and integrity was evaluated using the Bioanalyzer2100
452 platform (Agilent Technologies, Santa Clara, CA, USA). RNA samples were grouped in 4 pools
453 consisting of 3 mice per pool for each experimental group, except for 1 group in the *Plcb1+/-* mice

454 were only 2 animals were pooled. The pools were organized to homogenize the average number
455 of nose pokes in the different pools. The pooled individuals were the same for both mPFC and
456 HPC.**RNA sequencing**

457 RNA sequencing (RNAseq) was performed by the Centre de Regulació Genòmica (CRG, Barcelona,
458 Spain). Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit_v2 (Illumina,
459 San Diego, CA, USA) according to the manufacturer's protocol and sequenced 2x75 on Illumina's
460 HiSeq3000 system for both mPFC and HPC. The Bioinformatics service of CRG carried out the
461 analysis of RNAseq. Briefly, FastQC v0.11.5(Andrews S., 2010) was used to inspect the reads
462 quality and CutAdapt 1.7.1 (Martin, 2011) to clean the data of adapters and low-quality reads.
463 Then, reads were mapped to the *Mus musculus* genome of reference (GRCm38/mm10) with STAR
464 2.5.3a (Dobin et al., 2013), and the differential expression analysis was done by DESeq2 (Love et
465 al., 2014a) to compare WT and *Plcb1+/-*. Corrections for multiple testing were applied by adjusting
466 the p-values with a 5% False Discovery Rate.

467 RNAseq data of mPFC and HPC were explored on a principal component analysis (PCA) plot using
468 the "plotPCA" method from the DESeq2 package (Love et al., 2014b) and log2 gene expression
469 data. The PCAs were performed with the 500 genes showing the highest variance among the
470 samples to calculate the distance among them. The heatmaps were performed using the
471 "heatmap" function on R, and the hierarchical clustering considered the euclidean distance
472 between the samples considering all the genes or only those with corrected p-value < 1e-05.

473 **Functional annotation of RNAseq results**

474 We performed a functional group enrichment of differentially expressed genes (DEGs) in mPFC
475 using the DAVID Annotation Tool (http://david.abcc.ncifcrf.gov)(Jiao et al., 2012) considering GO
476 (Gene Ontology) biological processes and KEGG pathways (Kyoto Encyclopedia of Genes and

477 Genomes). Then, we searched for over-represented transcription factor-binding sites (TFBS) using
478 the information of MsigDB (https://www.gsea-msigdb.org/gsea/msigdb) integrated on
479 WebGestalt2019 (http://www.webgestalt.org/)(Liao et al., 2019), and the default parameters,
480 applying the weighted set cover method to reduce redundancy. In both analyses, the Benjamin–
481 Hochberg procedure was performed for multiple testing. Finally, we investigated the existence of
482 gene networks with Ingenuity Pathway Analysis 8.8 software (IPA,
483 http://www.ingenuity.com/products/ipa; Ingenuity Systems, Redwood City, CA, USA) (Krämer et
484 al., 2014) after selecting genes with fold-change >|1.2|.

485 **Statistical analysis**

486 Three-way ANOVA with repeated measures was used to test the evolution over sessions or days.
487 Sessions or days were used as within-subject factors and genotype (*Plcb1+/-* or WT) and drug
488 (cocaine or saline) were used as between-subjects factors. Post-hoc analyses (Newman-Keuls)
489 were performed when required. Comparisons between two groups were analyzed by Student *t*-
490 test or *U*-Mann-Whitney depending on the distribution defined by the Kolmogorov-Smirnov
491 normality test and the sample size. The chi-square analyses were performed to compare the
492 percentage of mice that acquired operant learning criteria in the different experimental groups.
493 Results are expressed as mean ± SEM or individual values with the median and the interquartile
494 range specified in the figure legend. Differences were considered significant at $P<0.05$. The sample
495 size was calculated based on power analysis. The statistical analyses were performed using the
496 Statistical Package for Social Science tool SPSS®25.0 (SPSS Inc, Chicago, USA).

497 See supplementary information for more details of genotyping of transgenic mice, drugs,
498 behavioral tests of phenotype characterization, operant conditioning maintained by cocaine, RNA
499 extraction and sequencing.

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J.C-D, B.C. and N.F.-C. obtained and maintained the mutant mice (*Plcb1+/-*); E.M.-G. and R.M. conceived and designed the behavioral studies with input from J.C.-D, B.C. and N. F.-C; E.M.-G. and J.C.-D. performed the behavioral phenotype characterization of mutant mice. E.M.-G. performed the surgery for i.v. catheterization and the operant conditioning maintained by cocaine. A.G.-R. collaborated in extinction and cue-induced reinstatement. E.M.-G. and J.C.-D. performed statistical

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529 **DISCLOURES**

530 The authors have no conflicts of interest.

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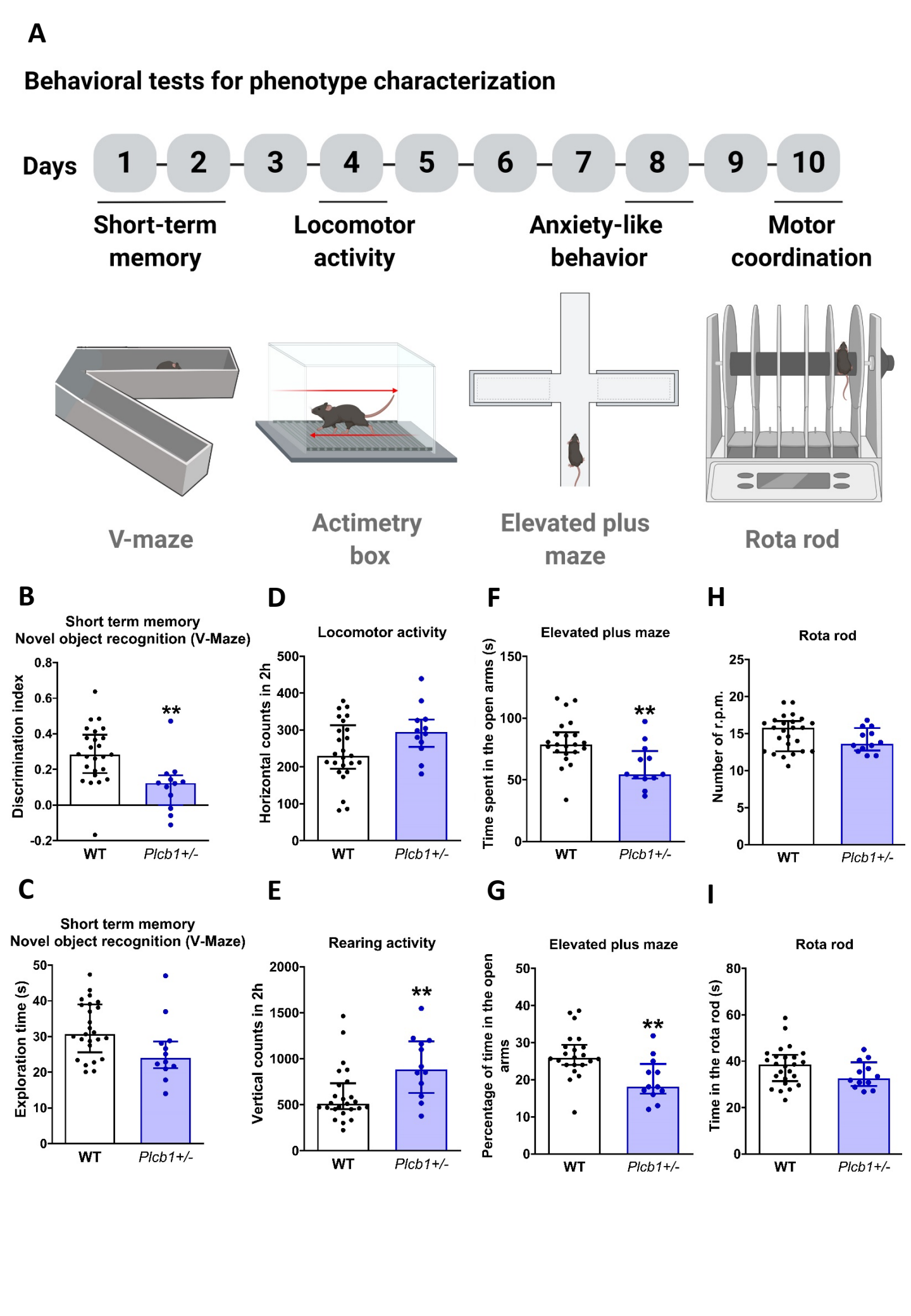
A
Behavioral tests for phenotype characterization
Days
1 2 3 4 5 6 7 8 9 10
Short-term memory
Locomotor activity
Anxiety-like behavior
Motor coordination
V-maze
Actimetry box
Elevated plus maze
Rota rod
B
Short term memory
Novel object recognition (V-Maze)
Discrimination index
0.8
0.6
0.4
0.2
0.0
-0.2
**
WT
Plcb1+/-
C
Short term memory
Novel object recognition (V-Maze)
Exploration time (s)
50
40
30
20
10
0
WT
Plcb1+/-
D
Locomotor activity
Horizontal counts in 2h
500
400
300
200
100
0
WT
Plcb1+/-
E
Rearing activity
Vertical counts in 2h
2000
1500
1000
500
0
**
WT
Plcb1+/-
F
Elevated plus maze
Time spent in the open arms (s)
150
100
50
0
**
WT
Plcb1+/-
G
Elevated plus maze
Percentage of time in the open arms
50
40
30
20
10
0
**
WT
Plcb1+/-
H
Rota rod
Number of r.p.m.
25
20
15
10
5
0
WT
Plcb1+/-
I
Rota rod
Time in the rota rod (s)
80
60
40
20
0
WT
Plcb1+/-

F**igure 1. Behavioral tests for phenotype characterization.** (A) Timeline of the experimental sequence. (B,C) Short-term memory measured in the V-maze for novel object recognition task, (B) discrimination index and (C) exploration. (D) Locomotor and (E) rearing activity measured in actimetry boxes. (F) Anxiety-like behavior measured by the time spent in the open arms and (G) the percentage of time in the open arms in the elevated plus maze. Motor coordination measured in the rota rod test by the (H) number of r.p.m. that the mice performed in the average trials or the (I) mean time they remain in the apparatus. All data are expressed in median and interquartile range and individual data are shown (WT n=25; Plcb1+/- n=12).

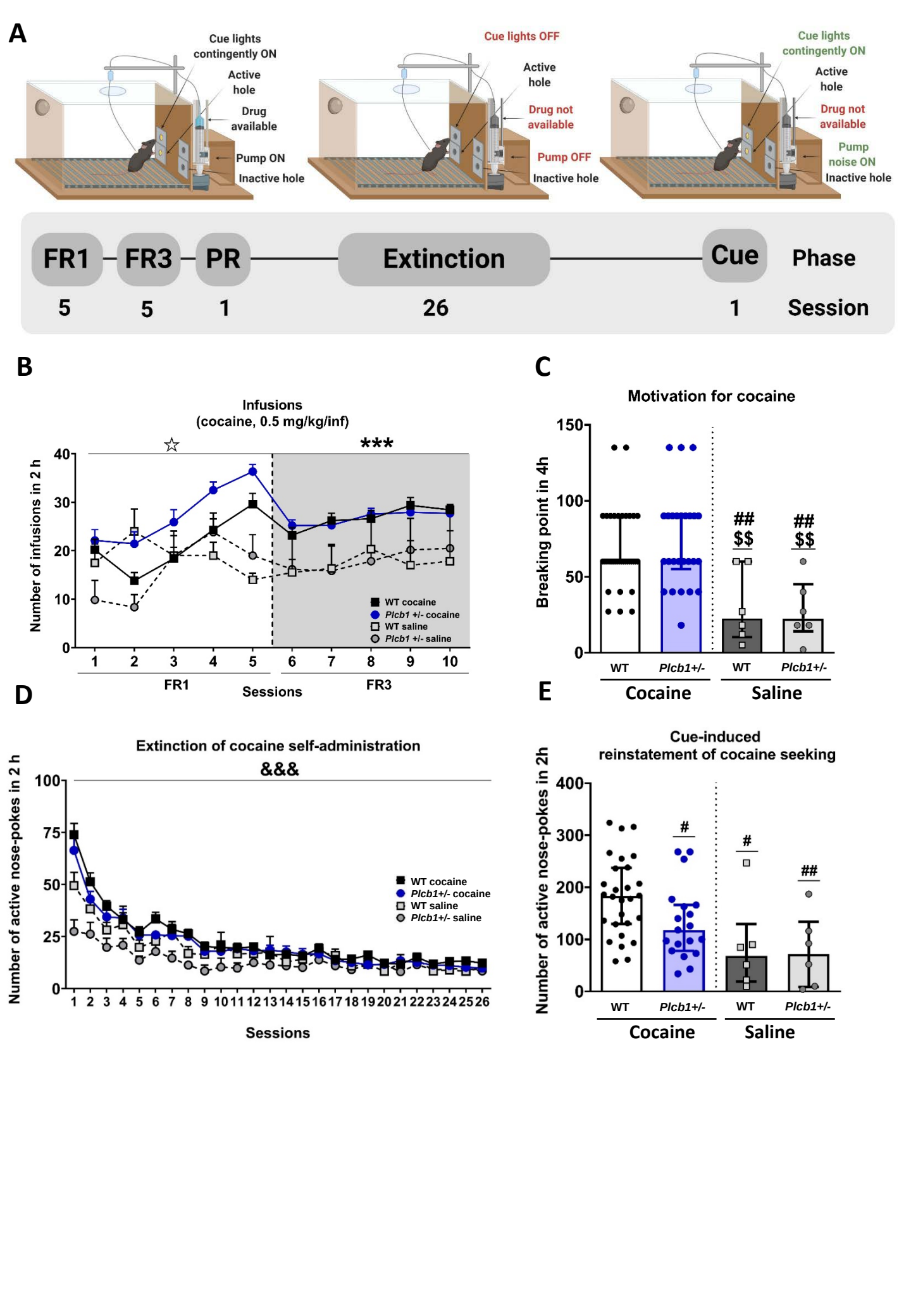
A
Cue lights contingently ON
Active hole
Drug available
Pump ON
Inactive hole
Cue lights OFF
Active hole
Drug not available
Pump OFF
Inactive hole
Cue lights contingently ON
Active hole
Drug not available
Pump noise ON
Inactive hole
FR1
FR3
PR
Extinction
Cue
Phase
5
5
1
26
1
Session
B
Infusions (cocaine, 0.5 mg/kg/inf)
Number of infusions in 2 h
WT cocaine
Plcb1 +/- cocaine
WT saline
Plcb1 +/- saline
FR1
Sessions
FR3
C
Motivation for cocaine
Breaking point in 4h
WT
Plcb1+/-
Cocaine
Saline
D
Extinction of cocaine self-administration
Number of active nose-pokes in 2 h
WT cocaine
Plcb1+/- cocaine
WT saline
Plcb1+/- saline
Sessions
E
Cue-induced reinstatement of cocaine seeking
Number of active nose-pokes in 2h
WT
Plcb1+/-
Cocaine
Saline

Figure 2. Operant conditioning maintained by cocaine in Plcb1+/-, wild-type (WT) and corresponding control saline mice. (A) Timeline of the experimental sequence. (B) The number of cocaine infusions (0.5 mg/kg/infusion) in both genotypes increased progressively across sessions during FR1 (repeated measures ANOVA, interaction between genotype x drug x sessions, P<0.05) and remained stable during FR3 and higher in mice trained with cocaine (repeated measures ANOVA, main effect of drug, ***P<0.001). The maximum number of infusions was reached on session 5 in both genotypes and was slightly superior in Plcb1+/- (36.35±1.47) than in WT (29.61±2.23) but post hoc analyses demonstrated that this difference was not significant. (C) The motivation for cocaine measured by the breaking point achieved in the progressive ratio schedule of reinforcement (4h) was equivalent in both genotypes trained with cocaine but higher than those trained with saline independently of the genotype (individual data with interquartile range, U Mann-Whitney, ##P<0.01 vs WT cocaine, $$P<0.01 vs Plcb1+/- cocaine). (D) Both genotypes also showed similar levels of extinction that decreased during sessions, but the curve was more pronounced in WT mice than in mutants (repeated measures ANOVA, interaction genotype x sessions, &&&P<0.001). (E) Decreased cue-induced reinstatement of cocaine seeking in Plcb1+/- mice was obtained compared to WT (individual data with interquartile range, U Mann-Whitney, #P<0.05, ##P<0.01 vs WT cocaine). All data are expressed in mean ± SEM when sessions are represented or median and interquartile range when individual data are shown (WT cocaine n=28-36; Plcb1+/- cocaine n=19-26; saline n=6 per genotype). Statistical details are included in Supplementary Table S1.

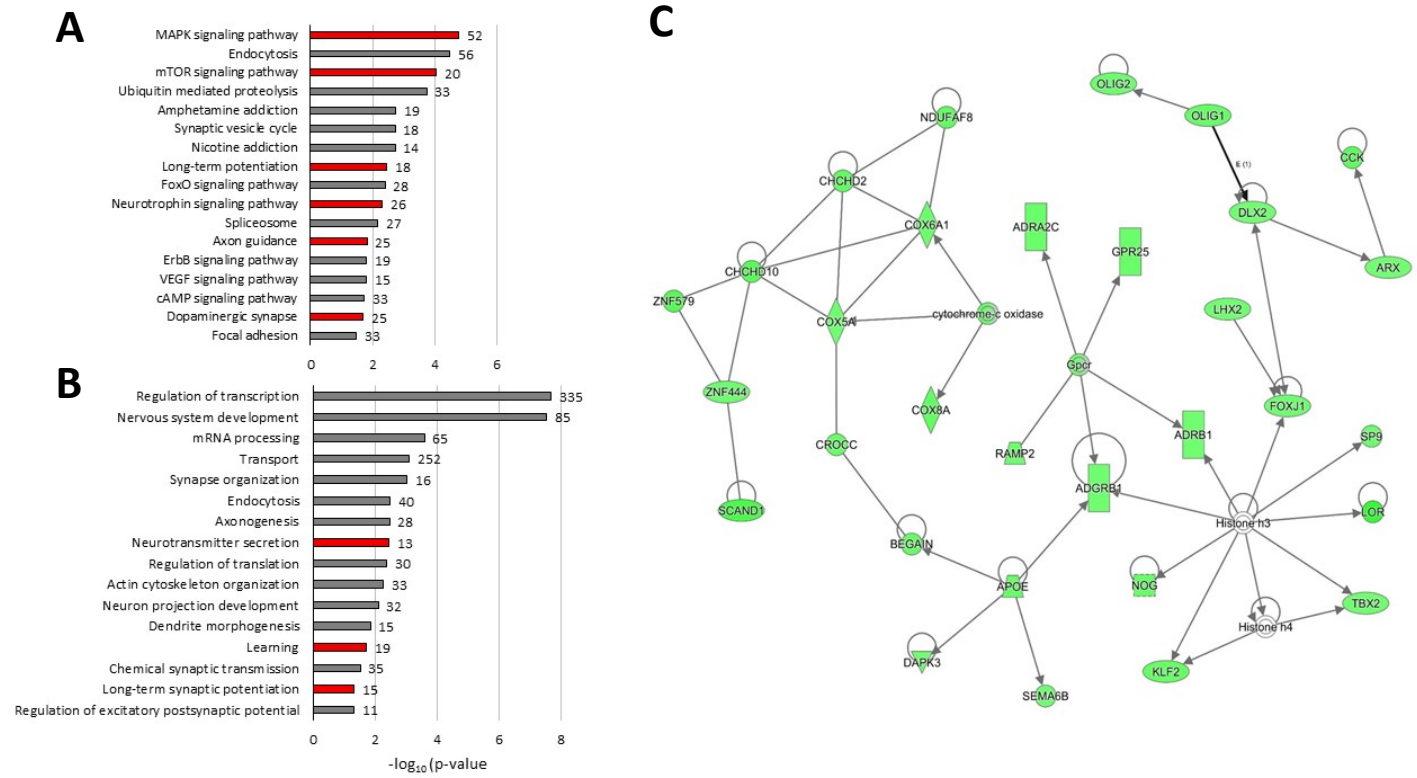

Figure 3. Gene expression changes in mPFC after cue-induced reinstatement of cocaine seeking comparing Plcb1+/- versus wild-type (WT) mice. (A) Selection of over-represented KEGG pathways (Kyoto Encyclopedia of Genes and Genomes) and (B) GO (Gene Ontology) identified by DAVID software among the differentially expressed genes. The number of genes with altered expression included in each category is indicated on the right side of the bar. In red, relevant pathways for the addictive process. (C) Gene network involved in cellular development, cellular growth and proliferation, nervous system development and function (score = 62). The green nodes in the pathway indicate genes with downregulated expression in Plcb1+/- identified in RNAseq.

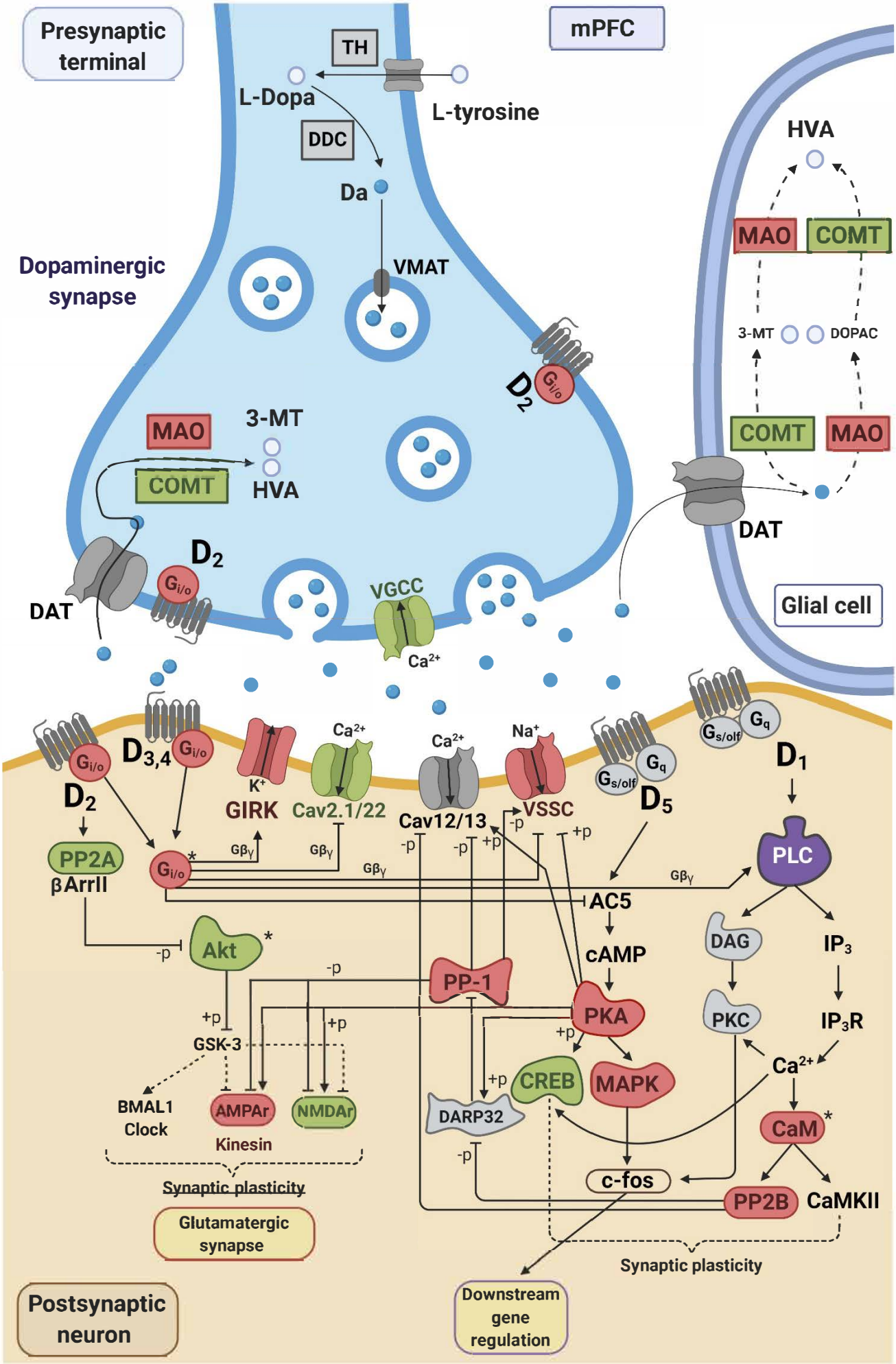
Presynaptic terminal
mPFC
TH
L-Dopa
L-tyrosine
DDC
Da
VMAT
Dopaminergic synapse
HVA
MAO
COMT
3-MT
DOPAC
COMT
MAO
DAT
Glial cell
D2
Gi/o
3-MT
MAO
COMT
HVA
D2
Gi/o
DAT
VGCC
Ca2+
D3,4
Gi/o
D2
Gi/o
K+
GIRK
Ca2+
Cav2.1/22
Ca2+
Cav12/13
Na+
VSSC
Gs/olf
Gq
D5
Gs/olf
Gq
D1
PP2A
βArrII
Gi/o
Gβγ
Gβγ
Gβγ
Gβγ
PLC
AC5
Akt
-p
cAMP
DAG
IP3
PP-1
PKA
PKC
IP3R
GSK-3
+p
+p
+p
Ca2+
CREB
MAPK
BMAL1
Clock
AMPAr
NMDAr
DARP32
CaM
Kinesin
c-fos
Synaptic plasticity
PP2B
CaMKII
Glutamatergic synapse
Synaptic plasticity
Postsynaptic neuron
Downstream gene regulation

Figure 4. Alterations in expression in the dopaminergic synapse in mPFC of Plcb1+/- mice after cue-induced reinstatement of cocaine seeking. Adapted from KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (mmu04728). Enriched pathway in DEGs in mPFC, 25 out of 131 genes in the pathway were differently expressed (Praw=2e-03; Pajd=0.02). In red, upregulated genes and in green, downregulated genes in mPFC. *Protein complexes including upregulated and downregulated genes. Correspondence between genes and proteins can be found in Supplementary Table S6.