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

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ABSTRACT

Background: 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.

Methods: 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.

Results: *Plcb1*+/- mice showed normal behavior, although they had increased anxiety and impaired short-term memory. A decreased cocaine seeking behavior in the cue-induced reinstatement test was revealed in *Plcb1*+/- mice. Transcriptomic alterations after reinstatement were selectively identified in mPFC of *Plcb1*+/- mice, mostly related to functions and pathways relevant to addiction.

Conclusion: We validated that heterozygous deletion of the *Plcb1* gene contributes to resilience to cocaine addiction. The decreased cocaine-seeking rate of the *Plcb1+/-* mice points at *Plcb1* as a possible therapeutic target for preventing relapse and treating cocaine addiction.

INTRODUCTION

Cocaine is the most used psychostimulant illicit drug worldwide (1), causing serious health problems that include the development of cocaine addiction in around 15-16% of cocaine users (2). Cocaine addiction is a complex psychiatric disorder that results from the interaction of genetic, epigenetic and environmental risk factors (3). The heritability of cocaine addiction is one of the highest among psychiatric disorders, estimated around 65% for women (4) and 79% for men (5). 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 (6). Also, genetic variants in this gene were found nominally associated with illegal substance and cocaine addiction in two GWAS performed in European-American samples (7). On the other hand, we found that cocaine increased the expression of *PLCB1* both in human dopaminergic neuron-like cells after acute cocaine exposure and in postmortem samples of the nucleus accumbens (NAc) of cocaine abusers (6). Interestingly, this gene was also found over-expressed in the same brain region in mice after cocaine administration during 7 days and also during withdrawal (8). All these evidences suggest that *PLCB1* may play a role in cocaine addiction.

The *PLCB1* gene encodes phospholipase C beta 1, and it is highly expressed in brain, mainly in frontal cortex, basal ganglia (caudate, putamen and NAc) and hippocampus. These brain regions are crucial for drug reward and for the formation of drug-context associations, both contributing to the development and maintenance of addiction (9–14). This protein is activated by several neurotransmitters including dopamine through DRD1 and DRD2 (15,16), serotonin by 5-HT2A and

2C receptors (17,18) and glutamate by mGluR1 (19,20). Thus, PLCB1 might be a point of convergence of neurotransmitter systems that play an important role in the development of addiction (recently reviewed (21,22)). The activation of PLCB1 produces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), responsible for intracellular signal transduction. Alterations in PLCB1-mediated signaling in the brain have been associated with other neuropsychiatric disorders such as epilepsy, schizophrenia and bipolar disorder (23).

In the present study, we validated the contribution of *PLCB1* to cocaine addiction to disentangle its participation in the different aspects of the addictive process. We used the heterozygous knockout (KO) mice (*Plcb1+/-*), as the homozygous KO (*Plcb1-/-*) showed seizure attacks and low viability after birth (24). First, we performed a general phenotypic characterization using a battery of behavioral tests, including memory, anxiety, locomotor activity, coordination, food and water intake and sucrose preference. Then, we evaluated cocaine operant self-administration, extinction and cue-induced reinstatement. Finally, we studied transcriptional alterations to further understand the molecular mechanisms involved.

METHODS

Animals

Male mice, 8 weeks old, were housed individually at temperature- and humidity-controlled laboratory conditions (21±1°C, 55±10%) maintained with food and water ad libitum. Mice were tested during the dark phase of a reverse light cycle (lights off at 8.00h and on at 20.00h). *Plcb1+/-* mice in a C57BL/6J background and their wild-type (WT) littermates were used (24). All

experimental protocols were performed in accordance with the guidelines of the European Communities Council Directive 2010/63/EU and approved by the local ethics committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, agreement N°9213). In agreement, maximal efforts were made to reduce the suffering and the number of mice used.

Behavioral tests for phenotype characterization

A group of 25 WT and 12 *Plcb1+/-* mice underwent different behavioral tests for phenotype characterization for 10 days, as described in Figure 1. Mice were first tested for short-term memory of a novel object recognition task on a V-maze apparatus. After this test, locomotor activity was evaluated in actimetry boxes. Anxiety-like behavior was then assessed by using the elevated plus maze test, and finally motor coordination was evaluated using the rota rod test. In addition, 6 animals per genotype were placed in experimental PHECOMP boxes (Panlab and Harvard Apparatus) to control several consummatory and locomotor parameters.

Operant conditioning maintained by cocaine

Cocaine self-administration experiments were performed as previously described (25,26). Each 2h daily self-administration session started with a priming injection of the drug. Cocaine was intravenously infused in 23.5µl over 2s (0.5 mg/kg per injection). Cue light, located above the active hole, was paired with the delivery of the reinforcer. Mice (WT n=36; *Plcb1+/-* n=26) were trained under a fixed ratio 1 schedule of reinforcement (FR1; one nose-poke lead to the delivery of one dose of cocaine) over 5 consecutive daily sessions and under a fixed ratio 3 (FR3) over 5 consecutive daily sessions. Control mice trained with saline were included for both genotypes (WT n=6; *Plcb1+/-* n=6). After the 10 FR sessions, animals were tested in a progressive ratio schedule of 4h where the response requirement to earn the cocaine escalated according to the following

series: 1–2–3–5–12–18–27–40–60–90–135–200–300–450–675–1000. Then, we proceed with the extinction phase, 2h daily sessions following the same experimental conditions than the cocaine self-administration sessions except that cocaine was not available, and cue-light was not presented. Finally, mice that accomplished extinction criterion were tested in the cue-induced reinstatement during a 2h session, to evaluate the reinstatement of cocaine-seeking behavior.

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

RNA extraction and RNA sequencing

Total RNA of 12 WT and 11 *Plcb1+/-* mice from mPFC and HPC were isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen Düsseldorf, Germany) according to the manufacturer's protocol. RNA samples were grouped in 4 pools consisting of 3 mice per pool for each experimental group.

RNA sequencing (RNAseq) was performed by the Centre de Regulació Genòmica (CRG, Barcelona, Spain). Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit_v2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol, and sequenced 2x75 on Illumina's HiSeq3000 system for both mPFC and HPC. The analysis of RNAseq was carried out by the Bioinformatics service of CRG. Briefly, FastQC v0.11.5(27) was used to inspect the reads quality and CutAdapt 1.7.1 (28) to clean the data. Then, reads were mapped to the *Mus musculus* genome of reference (GRCm38/mm10) with STAR 2.5.3a (29) and the differential expression analysis was done by DESeq2 (30) to compare WT and *Plcb1+/-*. Corrections for multiple testing were applied by adjusting the p-values with a 5% False Discovery Rate.

Functional annotation of RNAseq results

We performed a functional group enrichment of differentially expressed genes (DEGs) in mPFC using the DAVID Annotation Tool (http://david.abcc.ncifcrf.gov)(31) considering GO (Gene Ontology) biological processes and KEGG pathways (Kyoto Encyclopedia of Genes and Genomes). Then, we searched for over-represented transcription factor-binding sites (TFBS) using the information of MsigDB (https://www.gsea-msigdb.org/gsea/msigdb) integrated on WebGestalt2019 (http://www.webgestalt.org/)(32), and the default parameters, applying the weighted set cover method to reduce redundancy. In both analyses, the Benjamin–Hochberg procedure was performed for multiple testing. Finally, we investigated the existence of gene networks with Ingenuity Pathway Analysis 8.8 software (IPA, http://www.ingenuity.com/products/ipa; Ingenuity Systems, Redwood city, CA, USA) (33) after selecting genes with fold-change >|1.2|.

Statistical analysis

Three-way ANOVA with repeated measures was used to test the evolution over sessions or days. Sessions or days were used as within-subject factor and genotype (*Plcb1+/-* or WT) and drug (cocaine or saline) were used as between-subjects factors. Post-hoc analyses (Newman-Keuls) were performed when required. Comparisons between two groups were analyzed by Student *t*-test or *U*-Mann-Whitney depending on the distribution defined by the Kolmogorov-Smirnov normality test and the sample size. The chi-square analyses were performed to compare the percentage of mice that acquired operant learning criteria in the different experimental groups. Results are expressed as mean ± S.E.M or individual values with the median and the interquartile range specified in the figure legend. Differences were considered significant at $P<0.05$. The sample

size was calculated based on the power analysis. The statistical analyses were performed using the Statistical Package for Social Science tool SPSS®25.0 (SPSS Inc, Chicago, USA).

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

RESULTS

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

Behavioral tests for phenotype characterization

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

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

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

Operant conditioning maintained by cocaine

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

The primary reinforcing effects and the motivation for cocaine were similar in both genotypes (Figure 2B-C). During FR1, both genotypes similarly increased the number of cocaine infusions across sessions, whereas mice trained with saline remained steady (repeated measures ANOVA, interaction between genotype x drug x sessions, *P*<0.05, Figure 2B and Supplementary Table S1). The maximum number of infusions was reached on session 5 in both genotypes and was slightly

superior in *Plcb1+/-* mutants (36.35±1.47) than in WT (29.61±2.23) but post hoc analyses demonstrated that this difference was not significant (Figure 2B). Similar findings were observed for the total number of nose-pokes in which operant responding increased in both genotypes with cocaine but remained stable with saline (Supplementary Figure S2A). When the effort to obtain one dose of cocaine increased to FR3, the number of infusions was stable across sessions in WT and in *Plcb1*+/- mice. Similarly, operant responding was higher for all groups than in FR1 with stable higher levels of responding (Supplementary Figure S2A) and higher number of infusions (repeated measures ANOVA, main effect of drug, $P<0.001$, Figure 2B) in mice trained with cocaine than with saline, independently of the genotype.

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

Importantly, *Plcb1+/-* mice showed significantly reduced cue-induced reinstatement of cocaine-seeking compared to WT mice (*U* Mann-Whitney, $P<0.05$, Figure 2E), with 27.59% less active nose-pokes compared to WT mice trained with cocaine. Furthermore, mice trained with saline from both genotypes exhibited 55.84% reduction of active nose-pokes than WT mice trained with cocaine and 39.20% less than mutants trained with cocaine. No significant differences were obtained between genotypes in inactive nose-pokes during operant conditioning maintained by cocaine nor during extinction (Supplementary Figure S2B-C). Taken together, these data showed that *Plcb1+/-* resulted in a phenotype of resistance to cue-induced reinstatement with reduced cocaine seeking (Figure 2E).

Brain transcriptomic analysis after the reinstatement of cocaine seeking behavior

To further understand the molecular mechanisms involved in this cocaine relapse-related phenotype we analyzed the transcriptomic profiles of mPFC and HPC after reinstatement of cocaine seeking behavior in WT and *Plcb1*+/- mice. We identified 2,115 protein-coding genes differentially expressed (DEGs) in mPFC (1,231 downregulated and 943 upregulated) and only 12 in HPC, when comparing *Plcb1+/-* and WT animals (Supplementary Tables S2-3). In accordance, PCA analysis and heatmap plots revealed that individuals with same genotype plotted together only in mPFC, but not in HPC (Supplementary Figure S3). This suggested a more prominent role of mPFC, so further studies were carried out only with DEGs of this brain area. Analysis of functional group over-representation identified several processes previously related to cocaine addiction, including dopaminergic synapse, learning, long-term potentiation (LTP), neurotransmitter secretion and axon guidance, as well as other relevant signaling pathways such as MAPK, mTOR and neurotrophin (Figure 3A-B and Supplementary Tables S3-5). We focused on the dopaminergic synapse pathway (enriched in the mPFCs DEGs; $P_{Adj}<0.05$), in which phospholipase c (such as Plcb1) is directly participating in signal transmission (Figure 4). Interestingly, many genes coding for proteins in this pathway are differentially expressed in *Plcb1+/-* mice after cue-induced reinstatement (Figure 4 and Supplementary Table S6). Furthermore, we found that several TFBS were over-represented in the DEGs of mPFC, including YY1, MYOD, NRF1, ERR1, FREAC2, NFY and E4F1 (complete list of TFBS in Supplementary Table S7). Then, we filtered the DEGs on mPFC based on fold-change (FC>|1.2|) and obtained a list of 238 genes that we used for gene network construction. This analysis showed a highly scored network (score=62, Figure 3C) that includes 31

DEGs involved in "cellular development, cellular growth and proliferation, nervous system development and function".

DISCUSION

Here we investigated, for the first time, the contribution of the *PLCB1* gene to cocaine addictive properties using *Plcb1+/-* mice. We found that the heterozygous *Plcb1* genotype resulted in a phenotype of resistance to cue-induced reinstatement of cocaine seeking behavior. Furthermore, we found relevant transcriptomic differences in *Plcb1+/-* mice compared to WT after cue-induced reinstatement of cocaine seeking in mPFC, a brain area essential in relapse (34,35). This study supports a role for *PLCB1* in cocaine addiction, confirming previous findings in humans (6), and suggests it may be relevant in relapse to cocaine addiction.

The phenotype of *Plcb1+/-* mice was characterized at different levels to evaluate consummatory and general behavior. In general, mutant mice presented normal levels of body weight, food and water intake, locomotor activity and motor coordination, demonstrating that this animal model is valid to study the effects of the heterozygous deletion of the *Plcb1* gene in other behavioral responses. *Plcb1+/-* showed increased anxiety in the elevated plus maze, reduced short-term memory in the novel object recognition paradigm, without any sign of depressive-like behavior in the anhedonia sucrose preference test. Thus, the single targeting of the *Plcb1* gene has an impact on selective emotional and cognitive responses.

The short-term memory impairment observed in the mutants did not affect the acquisition of operant associative conditioning, nor the instrumental learning that drives the goal-directed action as shown by similar levels of operant cocaine self-administration and extinction learning than WT.

This can be explained by the different brain circuits involved in each kind of learning, perirhinal-hippocampal structures (36) for short-term memory in the novel object recognition paradigm and mPFC-dorsal striatum pathway for cue-associated seeking (Figure 5), as well as by the acute retrieval or repetitive exposure involved respectively in each task. Thus, cocaine seeking can be multidimensional, involving different types of associative learning that together lead to a large repertoire of conditioned and instrumental responding.

The anxiogenic profile of *Plcb1+/-* is in accordance to the results previously reported in the *Plcb4-/-* mice, which were associated with alterations in cholinergic activity of the medial septum (37). However, the selective knock-down of *Plcb1* in the mPFC did not replicate this phenotype in a previous study (38), suggesting that other areas may be involved. The anxiogenic profile of *Plcb1+/-* mice had no effect on cocaine self-administration since the acquisition and extinction of this operant behavior was not modified in the mutants. In addition, mutants showed a protection against cue-induced reinstatement of cocaine seeking, instead of the expected cocaine seeking promoted by an anxiogenic phenotype (39). Therefore, the association of an anxiogenic profile with a resilient phenotype to cocaine seeking in the *Plcb1+/-* mice suggests modifications in specific brain areas involved in cocaine relapse, such as the mPFC. Recently, a resilient phenotype to develop food addiction has been associated with an increased strength of pyramidal glutamatergic synaptic transmission in the mPFC related to decreased compulsivity in face of negative consequences (40) in mice with an anxiogenic profile (41). With respect to cue-induced cocaine seeking, the mPFC has a key role and the network of glutamatergic projections from the prelimbic mPFC to the dorsal striatum participates in this phenotype (42). In our model, *Plcb1* haploinsufficiency is linked to a reduced cue-induced cocaine seeking possibly associated to modifications in this top-down corticolimbic brain network (Figure 5). Furthermore, these glutamatergic projections from mPFC to dorsal striatum receive mesocortical dopaminergic inputs

from the VTA that could be crucially involved in the protective effects of *Plcb1,* since this gene plays an important role in the dopaminergic signal transmission in the mPFC and many of the genes encoding for proteins in this pathway are differentially expressed in *Plcb1+/-* mice after cue-induced reinstatement (Figure 4).

Transcriptomic analyses performed after the cue-induced reinstatement of cocaine seeking behavior revealed some of the molecular underpinnings of the behavioral alterations observed in the mutant mice. Interestingly, differences in gene expression between *Plcb1+/-* and WT were predominantly found in the mPFC, whereas almost no differences were observed in the HPC. This highlights mPFC as a key region to explain these differences in cocaine seeking reinstatement observed in *Plcb1+/-* mice (Figure 5A). Consistently, in DEGs in mPFC we found an enrichment on pathways that are essential for the development of addiction, including the dopaminergic neurotransmission (Figures 3-4) (43). *PLCB1* is part of the dopamine-DARPP-32 signaling pathway (Figure 4), which plays a key role on cocaine reward (44,45). In a previous study we also found this pathway enriched in DEGs in frontal cortex and ventral striatum of mice that showed frustrated expected reward, produced by the cue in absence of expected reward after a high level of effort in a progressive ratio schedule of reinforcement with palatable food (46). Importantly, *Plcb1* was upregulated in the frontal cortex of those frustrated mice with increased responses to obtain the reward (palatable food). This is in line with our findings in which decreased expression of *Plcb1* (*Plcb1*+/- mice) results in decreased cue-induced responses to obtain cocaine after extinction. All these results support the participation of *Plcb1* in cue-induced cocaine seeking revealed in the present study and the involvement of mPFC (Figure 5).

Our transcriptomic analyses also found enrichment in genes related to learning and memory processes, such as LTP in the mPFC. The transition from drug use to drug addiction is a

maladaptive process that directly affects learning and memory (47,48). LTP produces long-lasting activity-dependent synaptic modifications that underlie memory, and drugs of abuse alter this mechanism in several brain regions such as mPFC (49,50), mesocorticolimbic system (51), VTA (52) and hippocampus (53,54), among others. Importantly, genes related to the glutamatergic system such as *Gria2-4* (glutamate ionotropic receptor AMPA2-4 (alpha 2-4)) and *Grik2* (glutamate ionotropic receptor kainate 2 (beta 2)) were found to be upregulated in *Plcb1+/-* mice. Meanwhile *Grin1, 2c and 2d* (glutamate ionotropic receptor NMDA1 (zeta 1, epsilon3 and epsilon4)) were downregulated, suggesting increase in AMPAR/NMDAR ratio in cocaine-experienced and abstinent *Plcb1 +/-* mice as compared to WT, one of the key indicators of LTP induction and increased synaptic strength (55). Furthermore, we found enrichment in other neuroplasticity (synapse organization, neuron projection development and axon guidance) (14,56–58) and signaling pathways (MAPK, mTOR and neurotrophin) (59,60) related to addiction. The assessment of gene expression in the mPFC of the *Plcb1+/-* mice highlighted alterations in relevant pathways for the addictive process, which could contribute to the results observed in the cue-induced reinstatement (Figure 5A).

Nowadays, there are few effective pharmacological treatments available for cocaine use disorders and frequently, psychosocial interventions in combination with pharmacotherapy are needed (61). Studies performed in mice have pinpointed multiple potential therapeutic approaches for the reinstatement of cocaine seeking behavior, targeting the reward circuit (62). In humans, treatment with bupropion, topiramate or disulfiram have been widely used. Bupropion, a non-tricyclic antidepressant that inhibits dopamine and norepinephrine reuptake, has been shown to be effective in reducing craving (63). Topiramate, a GABA/glutamatergic medication, has also been used to treat cocaine use disorder as it reduces the activity of the mesocorticolimbic dopaminergic system (61). A completely different strategy is the use of disulfiram, that potentially inhibits the

oxidoreductase dopamine β-hydroxylase (DβH, encoded by the *DBH* gene), which converts dopamine to norepinephrine (64). Importantly, a genetic variation in the *SLC6A3* gene (encoding DAT) has been associated to disulfiram treatment for cocaine addiction, with patients with higher DAT levels having better treatment outcomes than those with lower DAT levels (64). Thus, further studies and new therapeutic targets are needed to obtain an effective treatment for cocaine addiction. The results obtained in the present study underscore the relevance of the *Plcb1* gene in the cue-induced reinstatement of cocaine seeking after extinction. Together with previous findings in humans (6,7) and mice (8), *PLCB1* merits to be further evaluated as a promising novel therapeutic target for preventing relapse and treating cocaine addiction (Figure 5B).

The experimental approach used in our study, the *Plcb1+/-* mouse model, allowed us to better reproduce the phenotype observed in humans in comparison to the use of a complete KO mouse (65), as these animals preserved, at least, half of the expression of *Plcb1*. However, the haploinsufficiency of *Plcb1* during neurodevelopment in these animals could produce alterations that may contribute to the phenotype observed in the present study. Nevertheless, this may also be similar in humans with genetic risk variants that decrease *PLCB1* expression. Therefore, this approach is appropriate to study a specific genetic alteration that confers susceptibility to drug addiction, and to delineate the precise contribution of *PLCB1.*

To sum up, we studied, for the first time, the contribution of the *PLCB1* gene to cocaine addictive properties using *Plcb1+/-* mice. Previous studies have revealed an up-regulation of *Plcb1* in brain areas related to the reward circuit after cocaine exposure in animals (8) and in human cocaine abusers (6). These changes together with our results suggest that cocaine increases the expression of *Plcb1* and this mechanism plays an important role in cocaine addiction, as revealed now by the resistance to cue-induced reinstatement of cocaine seeking behavior exhibited by *Plcb1+/-* mutant

mice. These results highlight the importance of the *Plcb1* gene in the development of cocaine addiction and relapse and pinpoint PLCB1 as a promising therapeutic target for cocaine addiction and perhaps other types of addiction.

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J.C-D, B.C. and N.F.-C. and 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 analyses and graphs with the supervision of R.M., B.C. and N.F.-C.; A.G.R. collaborated with the extraction of the samples; J.C.-D. performed the RNA extractions, RNA sequencing and the bioinformatic analyses supervised by B.C. and N.F.-C.; E.M.-G. and J.C.-D. wrote the manuscript and N.F.C., R.M. and B.C. provided critical review of the manuscript with inputs from all the other authors.

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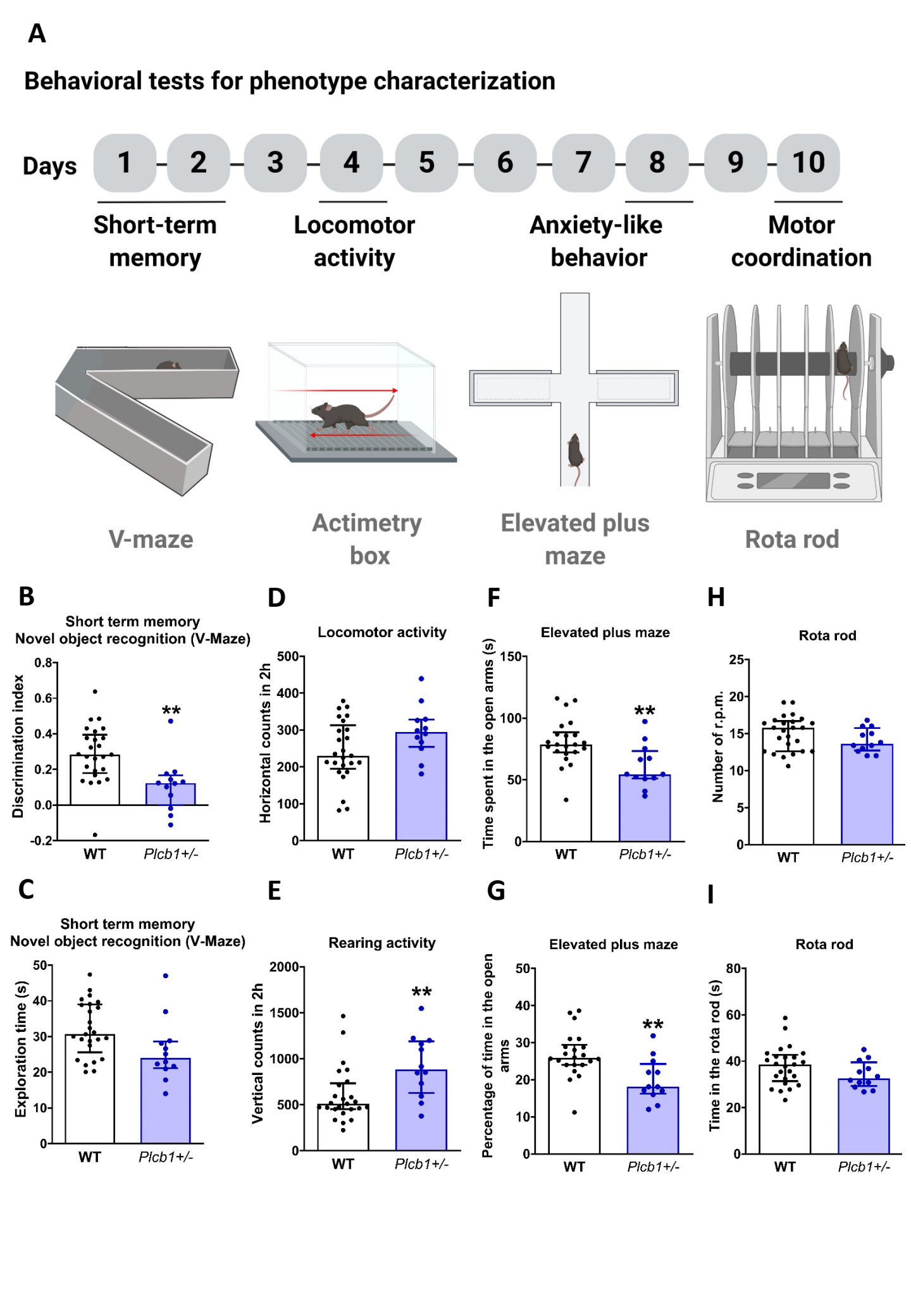
A
Behavioral tests for phenotype characterization
Days
1 2 3 4 5 6 7 8 9 10
Short-term memory
Locomotor activity
Anxiety-like behavior
Motor coordination
V-maze
Actimetry box
Elevated plus maze
Rota rod
B
Short term memory
Novel object recognition (V-Maze)
Discrimination index
0.8
0.6
0.4
0.2
0.0
-0.2
**
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).

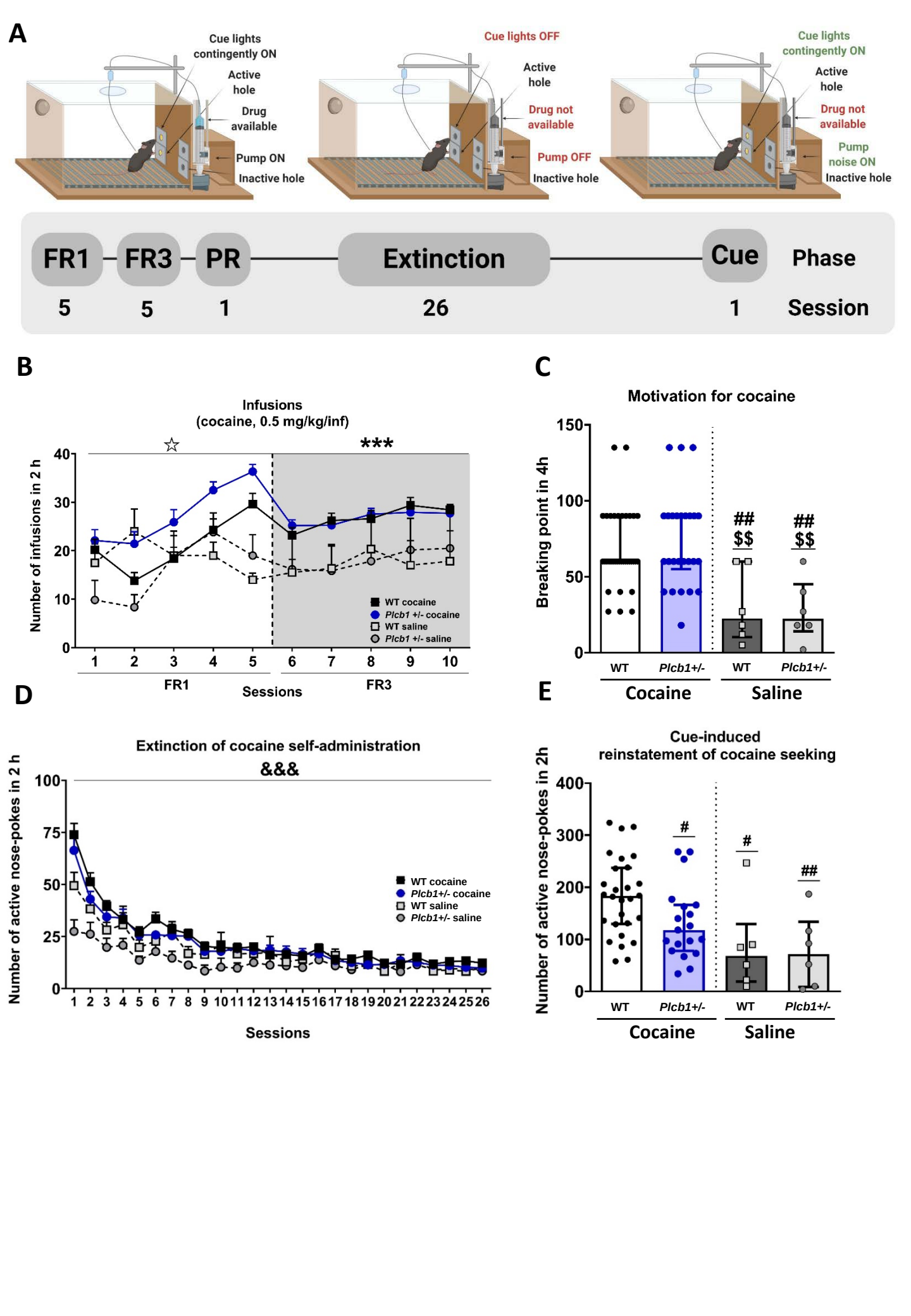
A
Cue lights contingently ON
Active hole
Drug available
Pump ON
Inactive hole
Cue lights OFF
Active hole
Drug not available
Pump OFF
Inactive hole
Cue lights contingently ON
Active hole
Drug not available
Pump noise ON
Inactive hole
FR1
FR3
PR
Extinction
Cue
Phase
5
5
1
26
1
Session
B
Infusions (cocaine, 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.

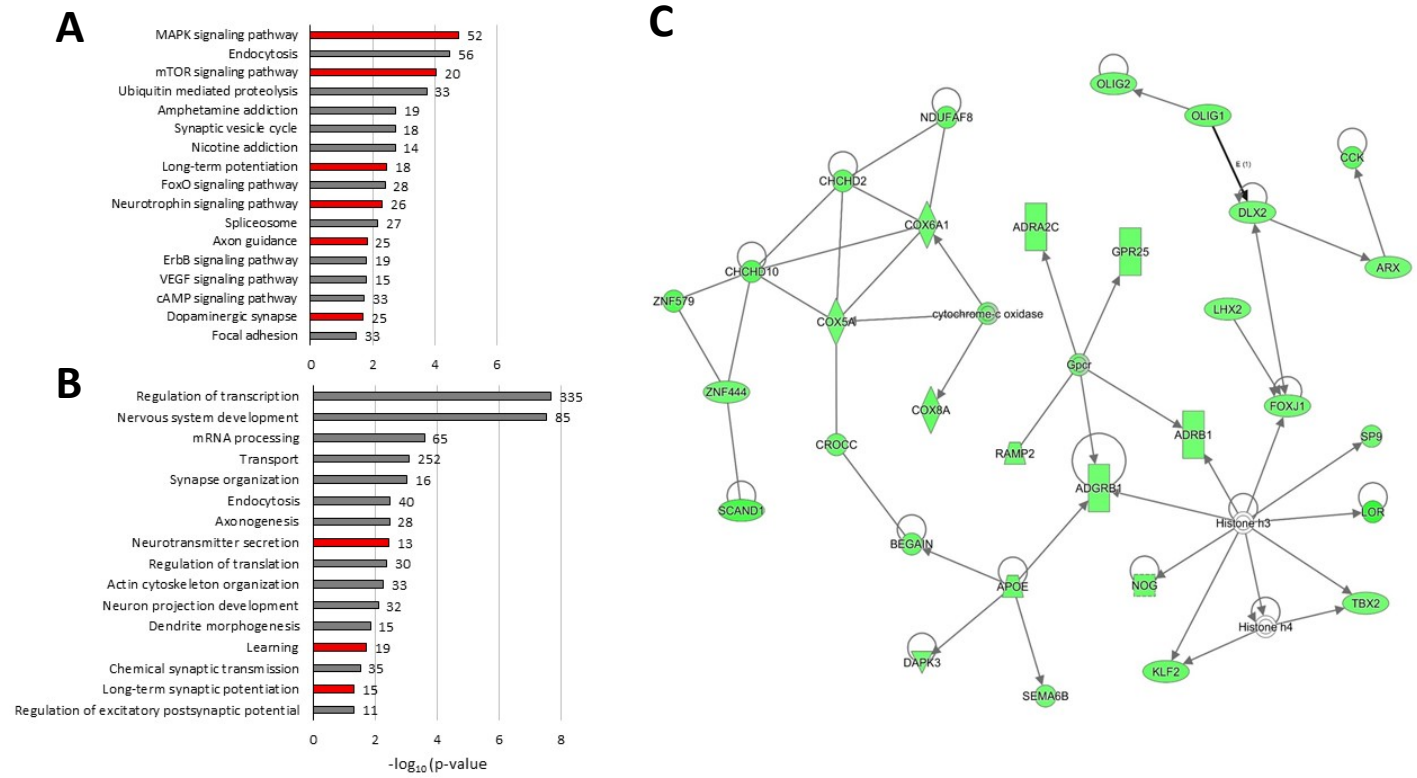

Figure 3. Gene expression changes in mPFC after cue-induced reinstatement of cocaine seeking comparing Plcb1+/- versus wild-type (WT) mice. (A) Selection of over-represented KEGG pathways (Kyoto Encyclopedia of Genes and Genomes) and (B) GO (Gene Ontology) identified by DAVID software among the differentially 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.

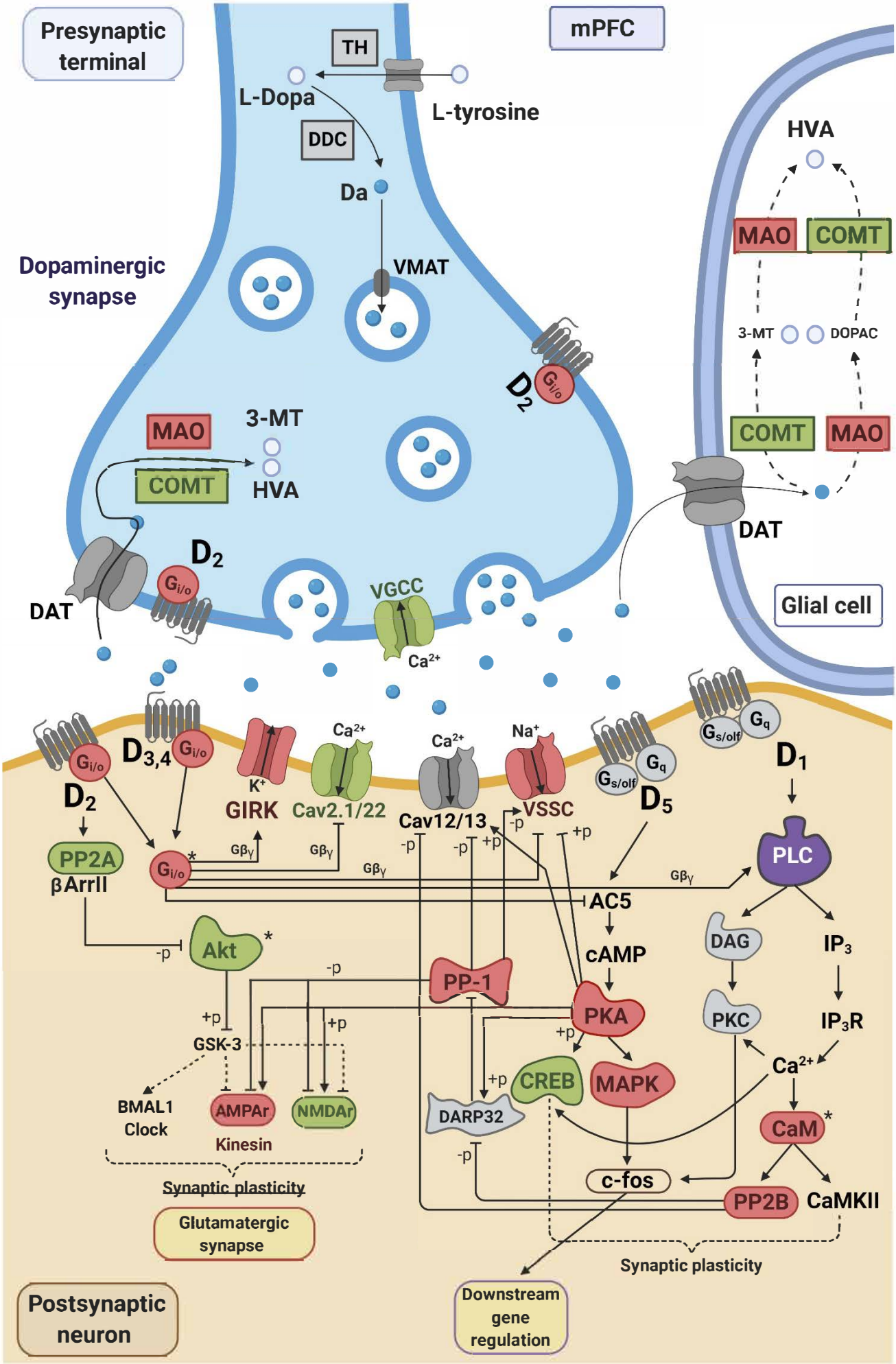
Presynaptic terminal
mPFC
TH
L-Dopa
L-tyrosine
DDC
Da
VMAT
Dopaminergic synapse
HVA
MAO
COMT
3-MT
DOPAC
COMT
MAO
DAT
Glial cell
D2
Gi/o
3-MT
MAO
COMT
HVA
D2
Gi/o
DAT
VGCC
Ca2+
D3,4
Gi/o
D2
Gi/o
K+
GIRK
Ca2+
Cav2.1/22
Ca2+
Cav12/13
Na+
VSSC
Gs/olf
Gq
D5
Gs/olf
Gq
D1
PP2A
βArrII
Gi/o
Gβγ
Gβγ
Gβγ
Gβγ
PLC
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.

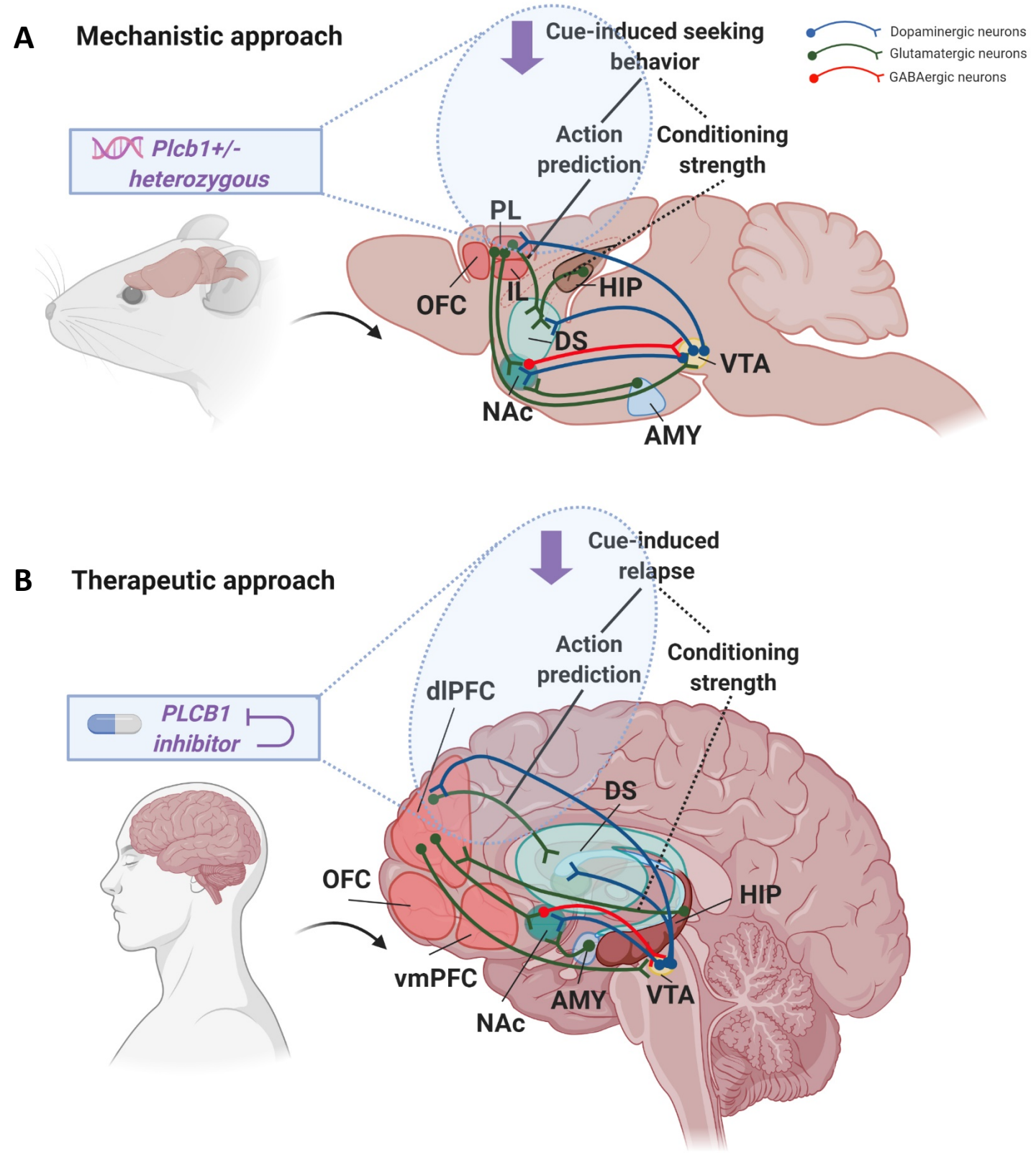
A
Mechanistic approach
Cue-induced seeking behavior
Dopaminergic neurons
Glutamatergic neurons
GABAergic neurons
Plcb1+/- heterozygous
Action prediction
Conditioning strength
PL
OFC
IL
HIP
DS
VTA
NAc
AMY
B
Therapeutic approach
Cue-induced relapse
Action prediction
Conditioning strength
PLCB1 inhibitor
dlPFC
DS
OFC
HIP
vmPFC
AMY
VTA
NAc

Figure 5. Schematic representation of brain circuits and mechanisms of Plcb1 in brain neural circuits that are engaged in drug seeking and in the transition to addiction. (A) Mechanistic approach of Plcb1 heterozygous deletion effect on reduced cue-induced reinstatement of cocaine seeking. We propose that mPFC (PL) has a key role and the network of glutamatergic projections from the prelimbic mPFC to the dorsal striatum (DS) could participate. Furthermore, mesocortical dopaminergic inputs from the VTA to mPFC could be crucially involved in the protective effects of Plcb1, reducing cocaine seeking. (B) Possible therapeutic approach in humans for cocaine addiction treatment. Targeting PLCB1 and reducing its activity could have a crucial effect in the same top-down network from mPFC (dlPFC) to dorsal striatum (DS), regulated by dopaminergic inputs from VTA, and reduce relapse to cocaine. Connections underlying drug-seeking behavior are shown in sagittal section of mouse (upper panel) and human (lower panel). Blue indicates dopamine, green indicates glutamate and red indicates GABA. Abbreviations: AMY, amygdala; dlPFC, dorsolateral prefrontal cortex; DS, dorsal striatum; HIP, hippocampus; IL, infralimbic; mPFC, medial prefrontal cortex, NAc, nucleus accumbens; PL, prelimbic; OFC, orbitofrontal cortex; vmPFC, ventral medial prefrontal cortex; VTA, ventral tegmental area.