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Supplementary Information for

Modified climbing fiber/Purkinje cell synaptic connectivity in the cerebellum of the neonatal phencyclidine model of schizophrenia.

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Supplementary material and methods
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Other supplementary materials for this manuscript include the following:

Dataset S1

Supplementary material and methods

Animals

All animal protocols were approved by the *Comité Regional d'Ethique en Experimentation Animale* (#21815) and animals were housed in authorized facilities of the CIRB (# C75 05 12). Phencyclidine (Phencyclidine hydrochloride, #P3029, Merck KGaA, Darmstadt, 10mg/kg diluted in saline solution) or vehicle (saline solution) was injected subcutaneously at P7, P9 and P11 in C57BL6/J mice. The NeuroD1Cre mouse line (Tg(Neurod1-cre)RZ24Gsat/Mmucd) was obtained from Pr. Mary Beth Hatten (The Rockefeller University, USA), and transgene detection was performed using the following primers: 5'TAG GAT TAG GGA GAG GGA GCT GAA 3' and 5' CGG CAA ACG GAC AGA AGC ATT 3'. The hM3Dq DREADD mouse line was obtained from the Jackson laboratory *(B6N;129-Tg(CAG-CHRM3*,-mCitrine)1Ute/J)*, and transgene detection was performed using the following primers: 5' CAG GTC GGC TCC AGC ATT 3' and 5' TCA CCA GTC ATT TCT GCC TTT G 3'. NeuroD1cre homozygous mice were crossed with hM3Dq DREADD heterozygous mice in order to obtain NeuroD1cre -hM3Dq double heterozygous and NeuroD1cre heterozygous/hM3Dq control (WT) mice in the same litters. Mice from both sexes were used in our experiments.

Antibodies

The following primary antibodies were used: mouse monoclonal anti-CABP (1:1000; Swant, Switzerland, #300), rabbit polyclonal anti-CABP (1:1000; Swant, #CB38), guinea pig polyclonal anti VGLUT1 (1:5000; Millipore, Massachusetts, USA, #AB5905), guinea pig polyclonal anti-VGLUT2 (1:5000; Millipore, #AB2251), goat anti-foxP2 (1:2000, Abcam, Cambridge, United kingdom, #ab1307), chicken anti-GFP (1:1000; Abcam, #ab13970), mouse anti-Parvalbumin (1:1000: Swant, #PV235), rabbit (1:1000, Abcam, #ab6556), and mouse anti-GAD65 (1:500, Abcam, #ab26113).

The following secondary antibodies were used: donkey polyclonal anti-guinea pig Alexa Fluor 594 (2µg/mL; Invitrogen, California, USA, #A11076), donkey anti-mouse Alexa Fluor 488 (2µg/mL; Invitrogen, #R37114), donkey polyclonal anti-Mouse Alexa Fluor 568 (2µg/mL; Invitrogen, #A10037), donkey polyclonal anti-Rabbit Alexa Fluor 488 (2µg/mL; invitrogen, #A21206).

Single molecule fluorescent in situ hybridization

Brains were extracted after intracardiac perfusion of mice with 4% paraformaldehyde (PFA) solution and post-fixed at 4°C for 24 hours. Brains were cryopreserved 24 hours in 10%, 20%, 30% sucrose/PBS at 4°C. Parasagittal sections were cut (30 µm-thick) using a freezing microtome and stored at -20°C in cryoprotectant until use. Slides mounted with sections were processed using the RNAscope Multiplex Fluorescent Assay (ACD, cat#323100) according to manufacturer instructions. SinglePlex *in situ* hybridization were done with the *Ctgf* RNAscope probe (ACD, cat# 314541). DAPI was used to stain nuclei and sections were mounted with Prolong Gold Antifade reagent. Images were taken using a spinning-disk confocal CSU-W1 microscope. Quantifications were performed using a custom-made java plugin for unbiased detection of individual RNA puncta inside each region of interest.

Immunohistochemistry

Brains were extracted after intracardiac perfusion of mice with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) solution. Brains were post-fixed in 4% PFA/PBS for one hour at 4°C, and then cryoprotected for 48h in a 30% sucrose/PBS solution at 4°C. After sectioning with a freezing microtome, 30µm-thick sections were washed and blocked with 4% normal donkey serum in PBS for 30 minutes at room temperature. The primary antibodies were diluted in PBS, 1% triton X-100, 1% donkey serum, and incubated overnight at 4°C under agitation. The sections were then washed three times for five minutes in PBS 1% triton X-100 and incubated for one hour at room temperature in the secondary antibody, diluted in PBS, 1% triton X-100, 1% donkey serum. The sections were then incubated for 15 minutes at room temperature with the nuclear marker Hoechst 33342 (0,2 mg/mL, Sigma, Gothenburg, Sweden, Cat#H6024) in PBS 0.2% triton. After washing, the sections were mounted with ProLong Gold Antifade Reagent (Invitrogen, #P36930).

Image acquisition and quantifications

Images for global cerebellar morphology were acquired using a Zeiss Axiozoom V16 macroscope, equipped with a digital camera (AxioCam HRm) using a 10x objective (pixel size: 0,650µm).
Images for synaptic quantifications in the PCP experiment were acquired using a Leica SP5 upright confocal microscope, using a 63x objective (1.4 NA, WD: 0.1mm), pixel size 57x57nm. The pinhole aperture was set to one Airy Unit and a z-step of 200 nm was used. Laser intensity and photomultiplier tube (PMT) gain was set in order to occupy the full dynamic range of the detector. Images were acquired in 16-bit range.
Images for synaptic quantifications in the NeuroD1cre/hM3Dq experiment were taken using a Zeiss Axioobserver Z1 inverted spinning disk microscope with a CSUW11 scan head and a 63x objective (1.4NA, WD:0.1mm, pixel size 103x103nm)
All the quantifications were done in blind condition, with the Fiji opensource software (1).
For VGLUT2, GAD65, and PV neuron quantifications, all the images were normalized using the quantile-based normalization plugin of Fiji (https://www.longair.net/edinburgh/imagej/quantile-normalization/). The intensity distribution of the images has been normalized using 256 quantiles for each staining.
The synaptic boutons were extracted from the background using the 3D Weka Segmentation plugin (https://imagej.net/Trainable_Weka_Segmentation) after manual selection of signal and background samples. The Fiji built-in plugin 3D object counter was then used in order to count and measure every object (cluster of VGLUT2 or GAD65 positive signal, or a whole PV+ molecular layer interneuron).
For VGLUT1 quantification, the background of the whole image was subtracted using the Fiji built-in subtract background plugin. The signal measured was the raw integrated density (the sum of the values of the pixels in the selection) divided by the area in µm², thus providing a measure of the mean intensity per µm².

Gene expression analysis

RNA extraction

The cerebellum and brainstem were dissected in cold Hanks balanced salt solution (HBSS). After removal of the meninges, the tissues were frozen in liquid nitrogen and stored at -80C°. Total RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Venlo, Netherlands, #74106) following tissue homogenization, according to manufacturer's instruction.

High throughput RTqPCR.

RNA quality and integrity were analyzed by capillary electrophoresis with the Fragment Analyzer (Agilent Technologies). The mean RNA Quality Number (defined on a scale ranging from 1 to 10) of the samples was 9.6. cDNAs were synthesized using 100ng total RNAs using Reverse Transcription Master Mix from Fluidigm™ on a Nexus Thermocycler (Eppendorf®) following the temperature protocol: 5min at 25°C, 30min at 42°C followed by heat-inactivation of the reverse transcriptase for 5min at 85°C and immediately cooled to 4°C. cDNA samples were diluted 5 times in low TE buffer [10mM Tris ; 0.1mM EDTA; pH = 8.0] (TEKNOVA®) and used for specific target multiplex pre-amplification using Fluidigm® PreAmp Master Mix and pooled TaqMan® Gene Expression assays (Life Technologies, ThermoFisher) with a final concentration of each assay of 180nM (temperature protocol: 95°C for 2min, followed by 18 cycles at 95°C for 15s and 60°C for 4min). Pre-designed Taqman Gene Expression assay are listed in Dataset S1. Quantitative PCR was performed using the high-throughput platform BioMark™ HD System and the 96.96 GE Dynamic Arrays (Fluidigm®). The expression of target genes was quantified in the samples by quantitative PCR on 96.96 microfluidic chips. The 5 times diluted pre-amplified cDNAs and assays were mixed inside the chip using HX IFC controller (Fluidigm). The loaded Dynamic Array was transferred to the Biomark™ real-time PCR instrument and subjected to PCR experiment (25°C for 30min and 70°C for 60min for thermal mix; 50°C for 2min and 95°C for 10min for hot start; 40 cycles at 95°C for 15s and 60°C for 1min). The parameters of the thermocycler were set with ROX as passive reference and single probe FAM-MGB as fluorescent detector. To determine the quantification cycle Ct, data were processed by automatic threshold for each assay, with linear derivative baseline correction using BioMark Real-Time PCR Analysis Software 4.0.1 (Fluidigm). The quality threshold was set at the default setting of 0.65.

The raw data from gene expression analysis were extracted and analyzed using MATLAB. For the systematic fold change and statistical test analysis, a script was written using the R opensource software. For each dataset, a multiple t test comparison was performed on the 323 genes, assuming a normal distribution and the equality of variances. Benjamini-Hochberg procedure for multiple correction was performed. The delta Ct method (2−ΔCt) was used to determine the relative fold gene expression, using the formula $Q=2^{(deltaCT)}$, where (deltaCT) = CT(treated) - CT(untreated). The data were normalized to *Rpl13a*, which showed the most stable expression among all the samples.

Droplet Digital™ PCR (ddPCR™, BioRad)
Equivalent amounts of total RNA (100 ng) were reverse-transcribed using SuperScript® VILO™ cDNA Synthesis kit (Life Technologies, California, USA, Cat#11754-250), according to manufacturer's instructions. The cDNA samples diluted 5 times were mixed with the fluorescent probes FAM-labeled along with the VIC-labeled Rpl13a reference (pre-designed Taqman Gene Expression assay, Supplemental data 1) and ddPCR™ Supermix for Probes (No dUTP) (BioRad, California, USA Cat#1863023). The samples were then fractionated into >12.000 droplets in water–oil emulsion using the automatic droplet generator (Bio-Rad). The template molecules from each droplet were PCR amplified in a thermocycler (BioRad), following the protocol: one cycle of 95 °C for 10 minutes, 40 cycles of 94 °C for 30 seconds and 60 °C for one minute, and one cycle of 98 °C for 10 minutes. The fluorescence (FAM, VIC) in each droplet was then digitally counted using QX200 droplet reader (Bio-Rad). QuantaSoft analysis software (Bio-Rad) was used for data acquisition and analysis. For each assay, identical manual threshold was selected, and relative expression was determined doing the ratio of the number of copies of template molecules/µl of reaction to the number of copies of the reference/µl of reaction.

High density microelectrode array (MEA) analysis of Purkinje cell spiking
Acute cerebellar slices were obtained from P30 to P38 mice in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, KCl 2.5, D(+)Glucose 25, $NaHCO_3$ 25, NaH_2PO_4 1.25, $CaCl_2$ 2, and $MgCl_2$ 1, gassed with 5% CO_2/95% O_2. Parasagittal slices (320 µm) were cut at 30°C with a Campden Ci 7000 smz microtome at an advance speed of 0.03 mm/s and vertical vibration set to 0.1–0.3 µm. Slices were then transferred to a chamber filled with oxygenated ACSF at 37°C and allowed to recover for 1 h before recordings.
For recordings, the slices were placed over a high-density MEA of 4096 electrodes (electrode size, 21×21 µm; pitch, 42 µm; 64 × 64 matrix; Biocam X, 3Brain, Wädenswil, Switzerland), and constantly perfused with oxygenated ACSF at 37C°. Extracellular activity was digitized at 17 kHz and data were analyzed with the SpyKING CIRCUS software (2). Data were automatically sorted into individual units using the following parameters: cut off = 200hz (cut-off frequency for the butterworth filter), spike threshold (threshold for spike detection) = 6, N_t (width of the template) = 5 cc_merge = 0.95 (merging if cross-correlation similarity > 0.95). We excluded units presenting more than 5% of refractory period violation (refractory period between two spikes set to 3 ms). Firing pattern variability, or regularity, is defined as a measure of the consistency of time intervals between spikes [interspike interval (ISI) = seconds]. To quantify the average variability in firing patterns, the coefficient of variance of the ISI (CV) was calculated as the ratio of the standard deviation (SD) of ISIs to the mean ISI of a given cell. To measure rhythmicity of cells, CV2 was calculated. CV2 measures firing pattern variability within a short period of two ISIs [$CV2 = 2|ISI_{n+1} - ISI_n|/(ISI_{n+1} + ISI_n)$](3).

Patch-clamp recordings
Acute parasagittal cerebellar slices were obtained from PCP or vehicle-treated mice from P24 to P28. 200 µm-thick slices were cut at room temperature with a Campden Ci 7000 smz microtome in (in mM): Sucrose 120, NaCl 60, KCl 2.5, D(+)Glucose 25, $NaHCO_3$ 25, NaH_2PO_4 1.25, $CaCl_2$ 0.1, $MgCl_2$ 3, ascorbic acid 0.4, myo-inositol 3, NaPyruvate 2, pH=7.3-7.4. Slices were then transferred and allowed to recover for one hour at room temperature in the following solution (in mM): NaCl 125, KCl 2.5, D(+)Glucose 25, $NaHCO_3$ 25, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgCl_2$ 1, ascorbic acid 0.4, myo-inositol 3, NaPyruvate 2, pH=7.3-7.4, oxygenated. This solution was used to fill the stimulation pipette and, complemented with picrotoxin (100 µM) was used as external solution for

recordings. Borosilicate glass pipettes with 2-5 MΩ resistance were used for recordings and filled with the following internal solution (in mM): CsCl2 155, Hepes 10, EDTA 5, QX314 5, pH=7.35 adjusted with CsOH. Responses to CF stimulation were recorded at a holding membrane potential of −10 mV in Purkinje cells of lobule VI using a MultiClamp 700B amplifier (Molecular Devices, CA) and acquired using the freeware WinWCP written by John Dempster (https://pureportal.strath.ac.uk/en/datasets/strathclyde-electrophysiology-software-winwcp-winedr). Series resistance was compensated by 80–90% and cells were discarded if significant changes were detected. CF-mediated responses were identified by the typical all-or-none response and strong depression displayed by the second response elicited during paired pulse stimulations (20 Hz). Electrophysiological data were analyzed using the software Clampfit 10.7 (Molecular Devices).

Supplementary bibliography

1. J. Schindelin, *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).

2. P. Yger, *et al.*, A spike sorting toolbox for up to thousands of electrodes validated with ground truth recordings in vitro and in vivo. *eLife* **7**, e34518 (2018).

3. G. R. Holt, W. R. Softky, C. Koch, R. J. Douglas, Comparison of discharge variability in vitro and in vivo in cat visual cortex neurons. *J. Neurophysiol.* **75**, 1806–1814 (1996).

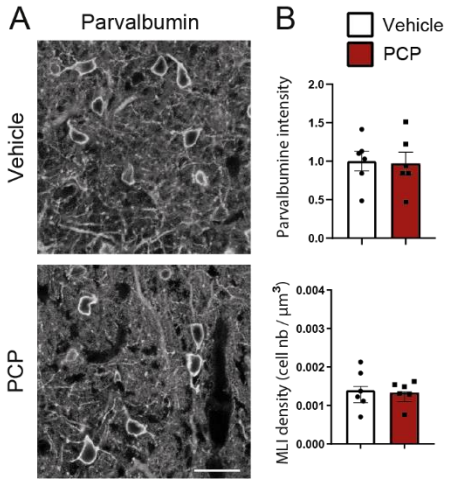

Fig. S1 Neonatal subchronic PCP treatment does not induce any changes in parvalbumin expression and molecular layer interneurons.
(A) Anti-parvalbumin immunostaining was used to reveal molecular layer interneurons (MLI) in cerebellar parasagittal sections of PCP-treated mice at P30. Scalebar = 20µm.
(B) Parvalbumin intensity, quantified using the ImageJ integrated density measurement (data normalized to vehicle), and MLI density are not changed in PCP mice compared to controls (Mean ± SEM; N=6 per condition. Unpaired Student's t-test).

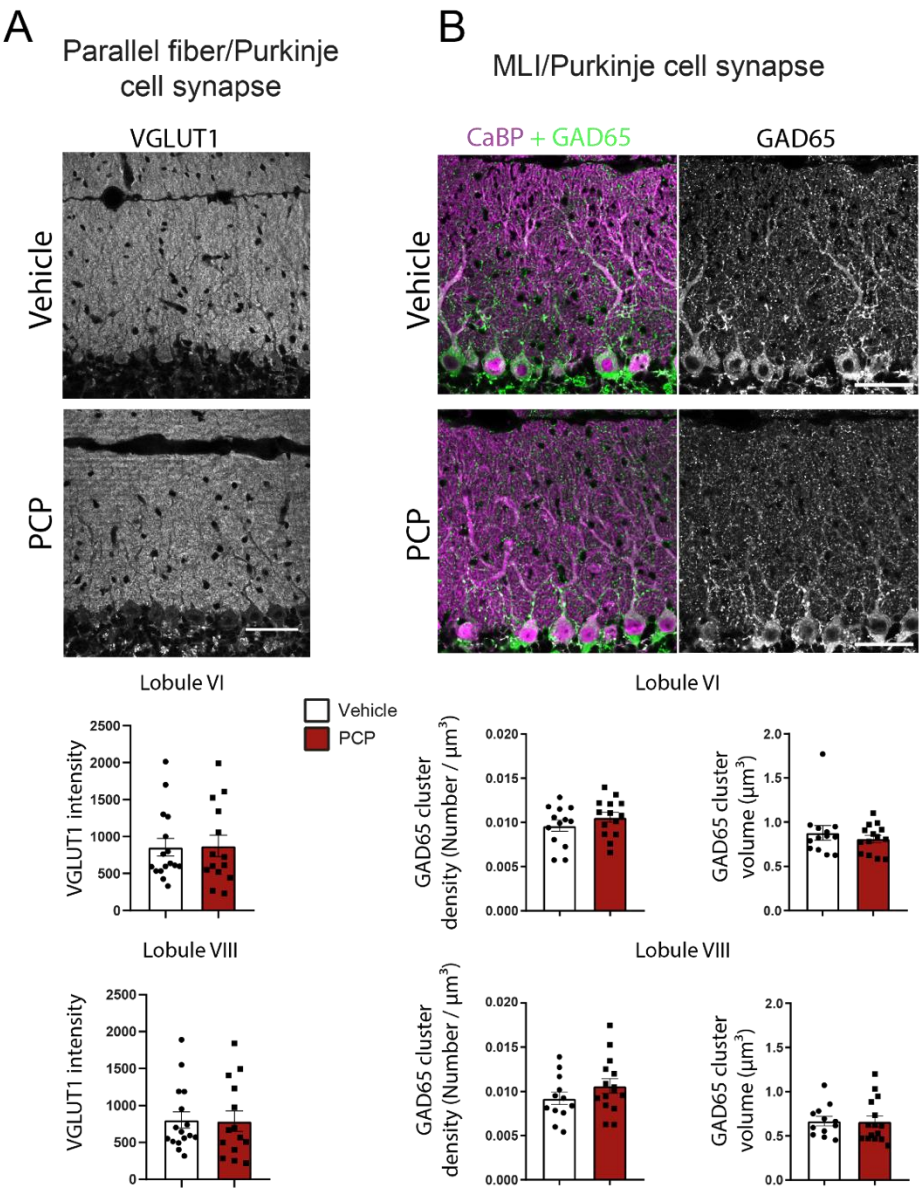

Fig. S2 Neonatal subchronic PCP treatment does not induce any changes in inhibitory synapses and parallel fiber synapses on cerebellar Purkinje cells.
(A) Parallel fiber presynaptic boutons were immunostained with an anti-VGLUT1 antibody, and an anti-calbindin antibody was used to stain Purkinje cells and its dendritic tree in parasagittal cerebellar sections from P30 vehicle and PCP mice. Scale bar: 50 µm.
Quantification of the mean VGLUT1 intensity in the molecular layer revealed no change in the PCP model, in both lobules VI and VIII (Mean ± SEM. Vehicle: n=16 animals, PCP n=14 animals. Unpaired Student's t-test).
(B) Molecular layer interneurons (MLI) presynaptic boutons were immunostained with an anti-GAD65 antibody (green), and an anti-calbindin antibody (magenta) was used to stain Purkinje cells and its dendritic tree in parasagittal cerebellar sections from P30 vehicle and PCP mice. Scale bar: 50 µm. Quantification of GAD65 puncta revealed normal presynaptic inhibitory boutons density and volumes in the molecular layer of lobules VI and VIII in PCP animals (Mean ± SEM. Vehicle: n=13 animals in lobule VI, n=12 in lobule VIII, PCP: n=14 in lobule VI, n=15 in lobule VIII. Unpaired Student's t-test for the density, and Welch's t-test for the mean volume).

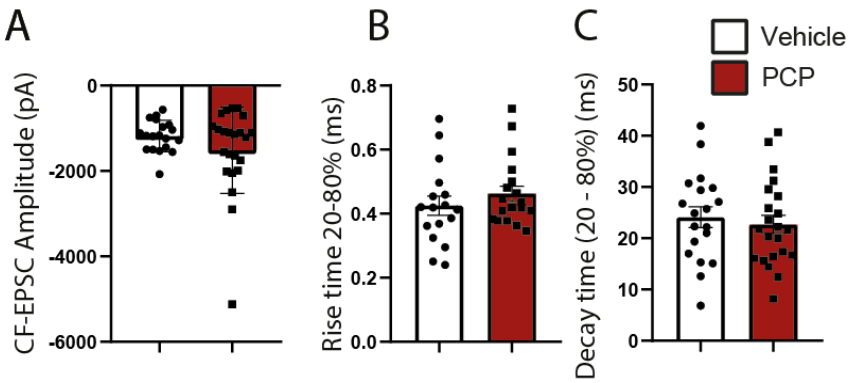

Fig. S3 Properties of climbing fiber/Purkinje cell transmission in PCP-treated mice.
(A) The mean amplitude of climbing fiber (CF)- elicited EPSC is not changed in PCP mice compared to vehicle. Note that the variance is increased in PCP treated animals (vehicle: n=19 cells/ 9 mice; PCP n=23 cells/ 9 mice. Welch's t test).
(B) Mean rise time (20 – 80%) is not changed in PCP mice compared to vehicle (vehicle: n=17 cells/ 9 mice, PCP n=19 cells/ 8 mice. Unpaired Student's t-test).
(C) Decay time (20-80% is not changed in PCP mice compared to controls (vehicle: n=17 cells/ 9 mice; PCP: n=19 cells/ 8 mice; Unpaired Student's t-test).

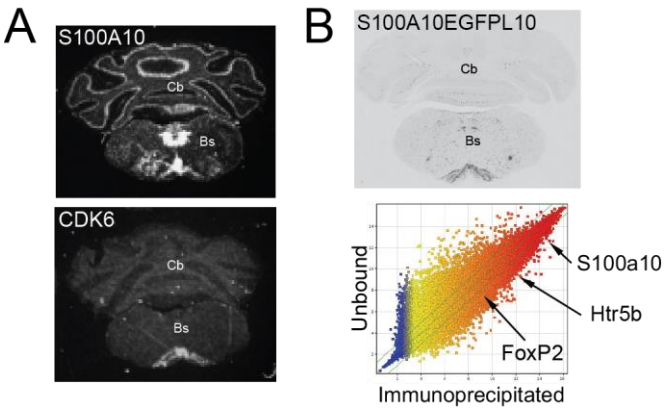

Fig S4 *S100a10* and *Cdk6* regulatory elements can be used as drivers for expression in inferior olivary neurons
(A) In situ hybridization for S100A10 and *Cdk6,* the two genes used as drivers for EGFPL10A in the bacTRAP experiments (BGEM data from gensat.org).
(B) EGFPL10A expression in the S100A10EGFPL10A line (data from gensat.org) and identification of known ION markers in the immunoprecipitated fraction after bacTRAP purification (enrichment shown compared to the unbound fraction). Abbreviations: Bs = brainstem, Cb = cerebellum.

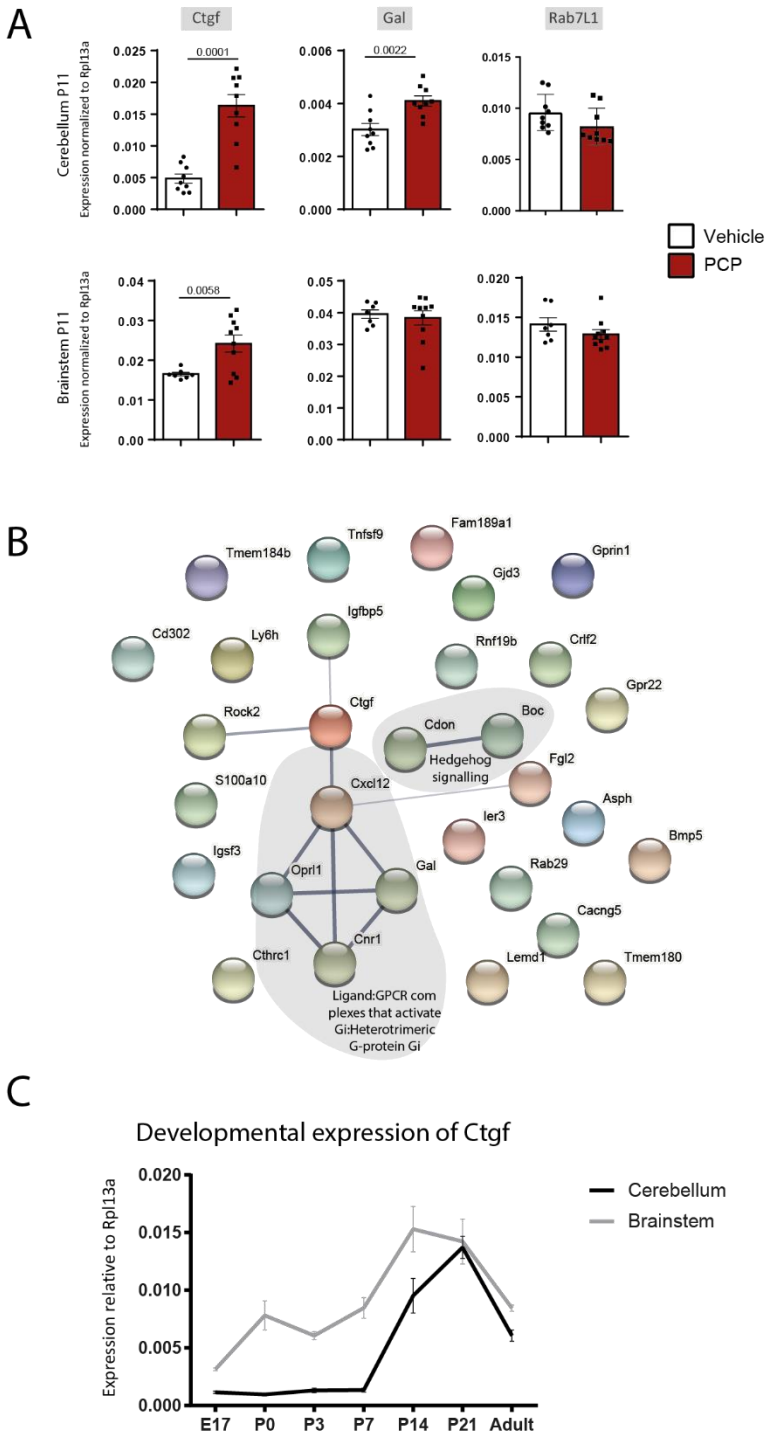

Fig. S5 *Ctgf* expression is increased by PCP and during postnatal development

(A) Molecular changes detected by high throughput RTqPCR in cerebellar and brainstem extracts were confirmed using ddPCR on mRNA extracts from P11 animals treated with PCP or vehicle at P7, P9 and P11. PCP treatment leads to a significant increase in *Ctgf* expression in both tissues, and to a significant increase in *Gal* expression in cerebellum but not brainstem tissue compared to vehicle treatment. Gene expression is normalized to *Rpl13a*. Data are presented as Mean ± SEM. Student's *t*-test with Welsch's correction when variances are significantly different; Cerebellum: vehicle: n=9, PCP: n=9; Brainstem: vehicle: n=7; PCP: n=10. N=2 to 3 independent litters.

(B) The 30 genes found to be transiently misregulated at P11 in the cerebellum and brainstem were mapped in the String-database to represent known and predicted functional interactions between proteins. Line thickness indicates the strength of data support collected from curated databases, extracted from PubMed abstracts or from gene fusion or co-expression databases. *Ctgf* is part of a network of several misregulated genes, including *Igfbp5*, *Rock2*, and *Cxcl12*.

(C) Expression of *Ctgf* was assessed at different stages of postnatal mouse brain development on mRNA extracts from brainstem and cerebellum. Gene expression is normalized to *Rpl13a*. Data are presented as Mean ± SEM; n=3 to 4 animals per timepoint.

A

Spontaneous locomotion at P11 after PCP injection				
Time after injection (hrs)	0.25-0.5	1	2	>4
Limb clonus and deficiency in surface righting	+++	++	++	-
Head waving	+++	++	+	-
Circling	+	+++	++	-

B

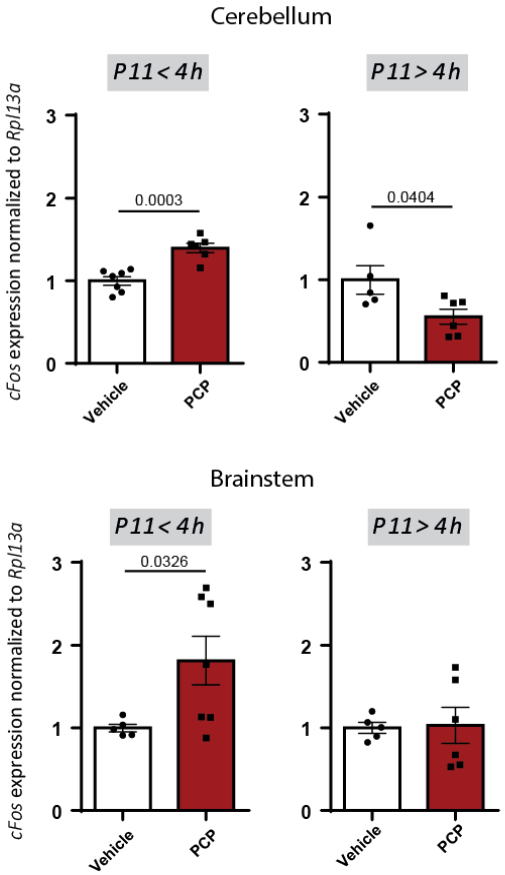

Fig. S6 Transient effect of PCP on P11 mice locomotion and *cFos* expression
(A) Observation of the spontaneous locomotion of P11 mice after PCP or vehicle treatment reveals a transient hyperactivity of PCP-treated animals starting 15 minutes after PCP injection that disappears after 4 hours compared to vehicle-treated mice.
(B) cFos expression in cerebellar and brainstem extracts was assessed using ddPCR on mRNA extracts from P11 animals treated with PCP or vehicle at P11, either less or more than 4 hrs after the treatments. PCP treatment leads to a significant increase in *cFos* expression in both tissues when analysed less than 4 hrs after the injection. When samples are obtained more than 4 hrs after the injection, the increase in *cFos* expression is not observed anymore, but rather a decrease is observed in the cerebellum, while no difference is found in the brainstem. Gene expression is normalized to *Rpl13a*. Data are presented as Mean ± SEM. Student's *t*-test with Welsch's correction when variances are significantly different; < 4hrs of treatment: Cerebellum: vehicle: n=7, PCP: n=6; Brainstem: vehicle: n=5; PCP: n=7; > 4hrs of treatment: Cerebellum: vehicle: n=5, PCP: n=6; Brainstem: n=5; PCP: n= 6.

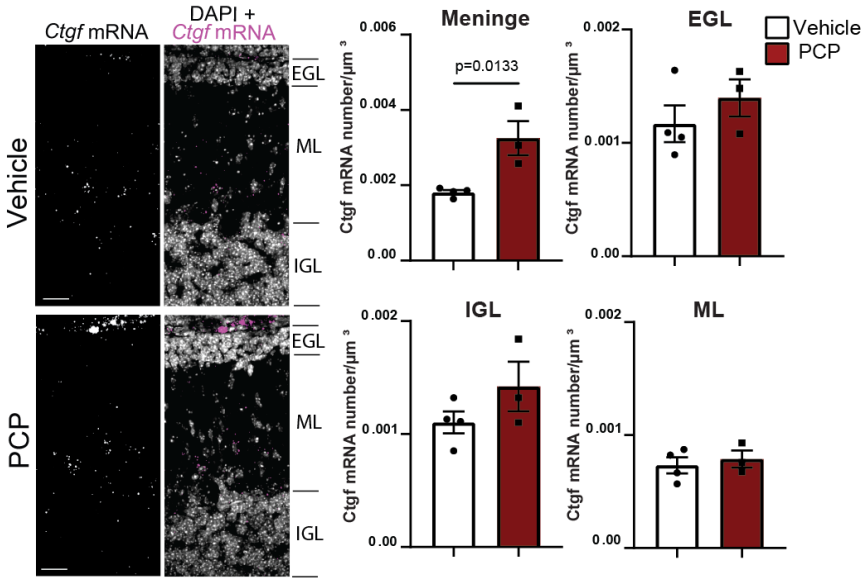

Fig. S7 Cellular expression of *Ctgf* mRNA in the cerebellum of vehicle- and PCP-treated mice. SmFISH for *Ctgf* mRNA (*Ctgf*, magenta; DAPI, gray) in parasagittal cerebellar sections from vehicle- or PCP-treated mice at P11. Scale bar: 20µm. The number of fluorescent puncta per μm^3 were quantified in meninges (M), external granular layer (EGL), molecular layer (ML), internal granular layer (IGL) in lobule Vi and VIII (Mean ± SEM. Vehicle: n=4 animals, PCP: n=3 animals; 4 images per animals. Unpaired Student's t-test).