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Supplementary Materials for

Phenotypic drug screen uncovers the metabolic GCH1/BH4 pathway as key regulator of EGFR/KRAS-mediated neuropathic pain and lung cancer

Shane J. F. Cronin *et al.*

Corresponding author: Shane J. F. Cronin, shane.cronin@imba.oeaw.ac.at;
Josef M. Penninger, josef.penninger@ubc.ca; Clifford J. Woolf, clifford.woolf@childrens.harvard.edu

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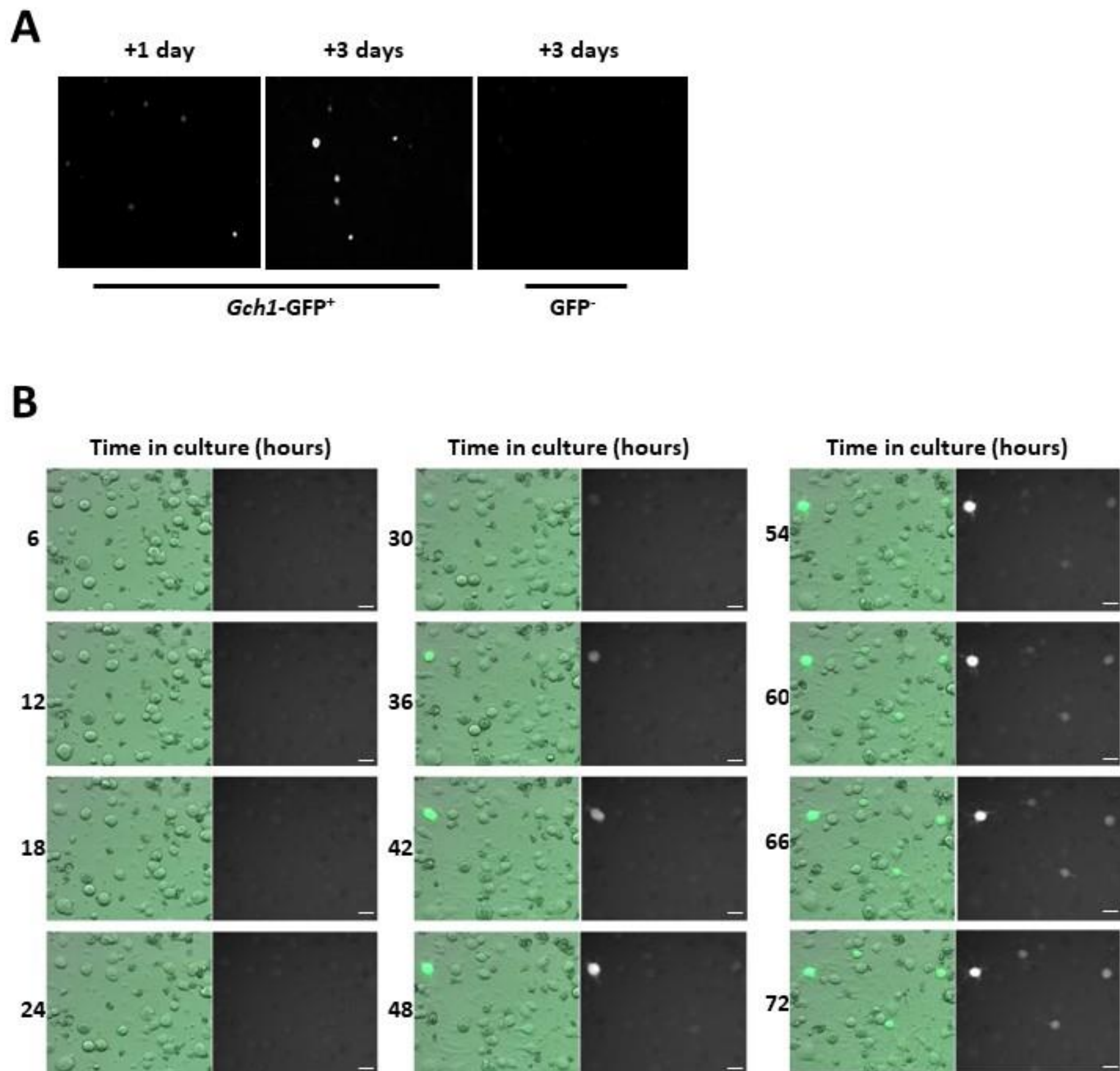
Legends for tables S1 to S7

Other Supplementary Material for this manuscript includes the following:

Tables S1 to S7

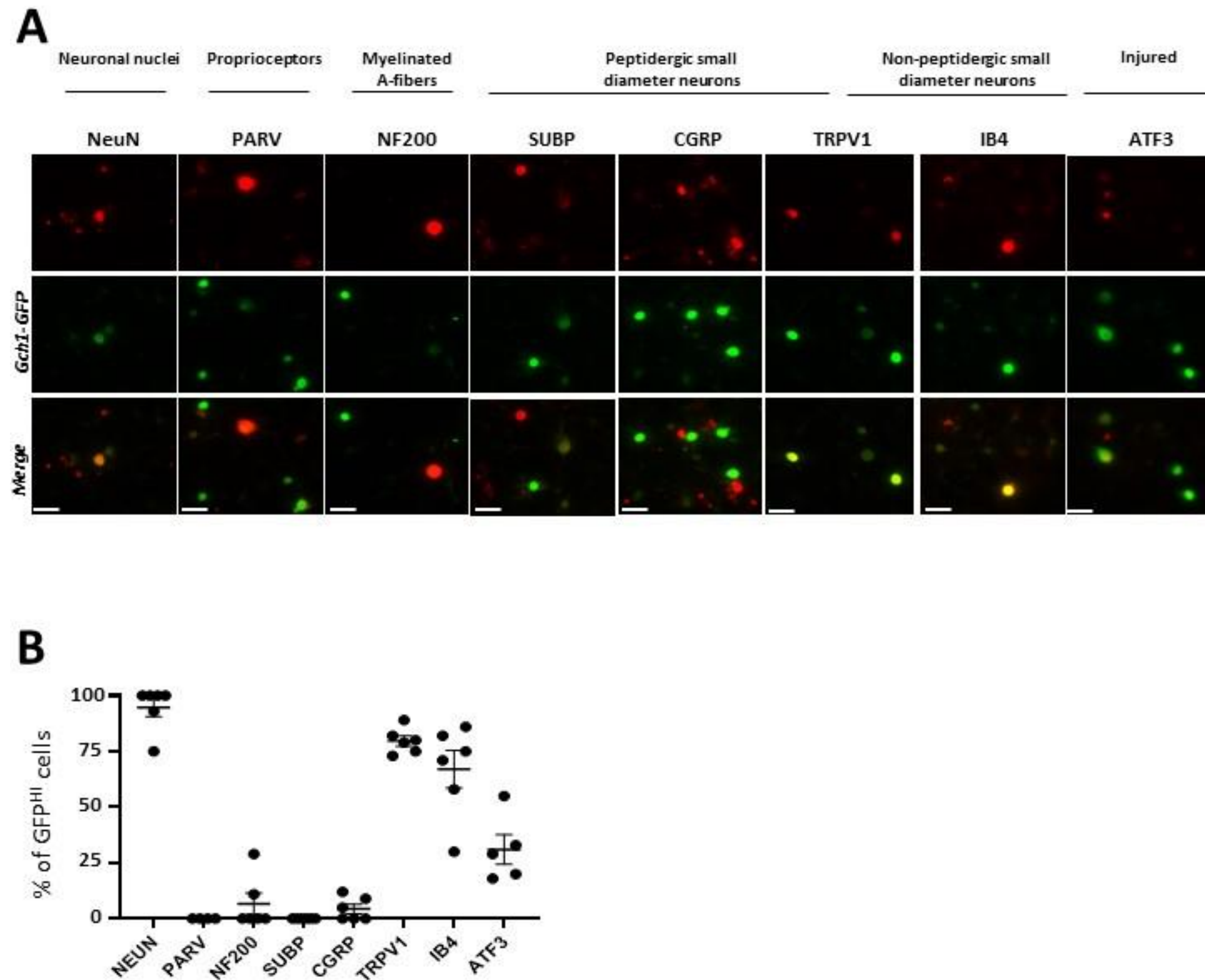
Supplementary Figures



Supplementary Figure 1. Kinetics of *Gch1*-GFP regulation in cultured DRG sensory neurons.

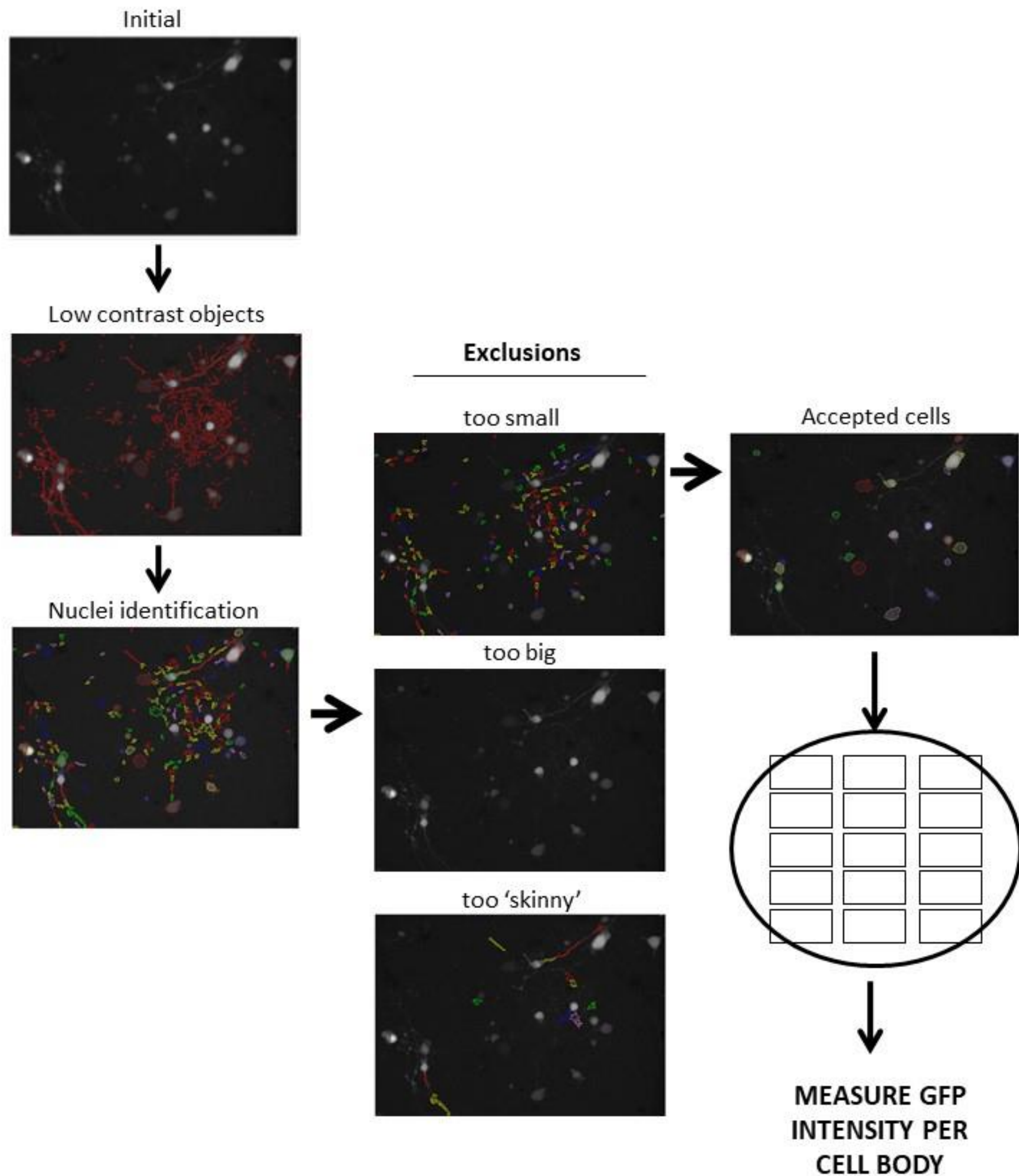
A, Representative GFP fluorescent images of GFP^+ dorsal root ganglion (DRG) sensory neurons isolated from a *Gch1*-GFP (*Gch1^{GFP}*) reporter mice cultured over 3 days. A wild type, GFP-

negative control is also shown. **B,** Brightfield and fluorescent GFP images of identified cultured DRG neurons over time isolated from *Gch1*^{GFP} reporter mice of GFP-high-expressing, GFP-low-expressing and GFP-negative DRG neurons. Scale bar, 30µm.



Supplementary Figure 2. Characterization of *Gch1*-GFP high-expressing DRG neurons.

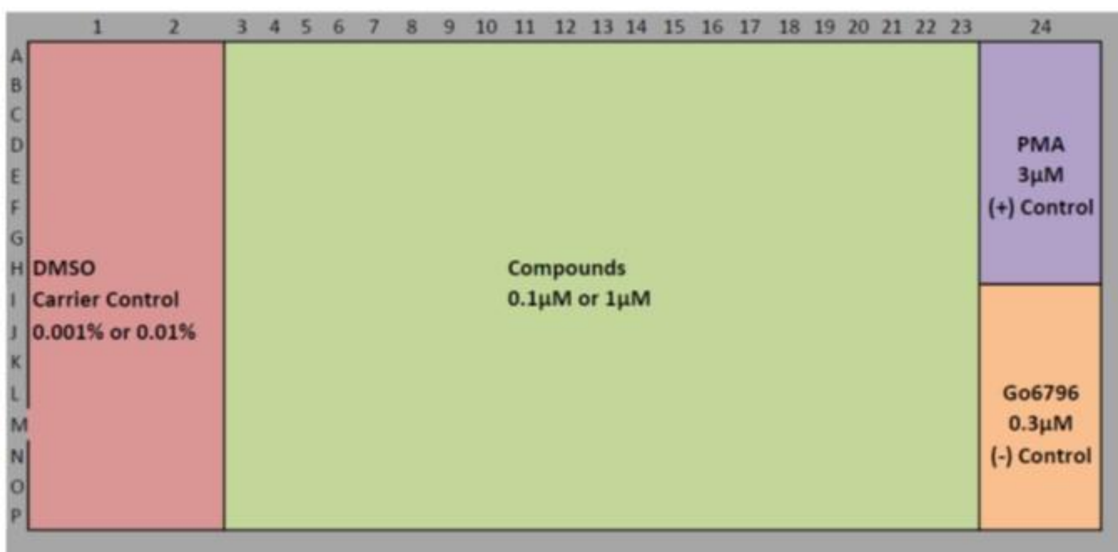
A,B, Representative immunofluorescent images (**A**) and quantification (**B**) using the various indicated markers for DRG neuronal subtypes to characterize those GFP high-expressing cultured DRG neurons on day 3 of culture. Scale bar, 50µm.



Supplementary Figure 3. Confocal-based screening for *Gch1* expression in DRG neurons.

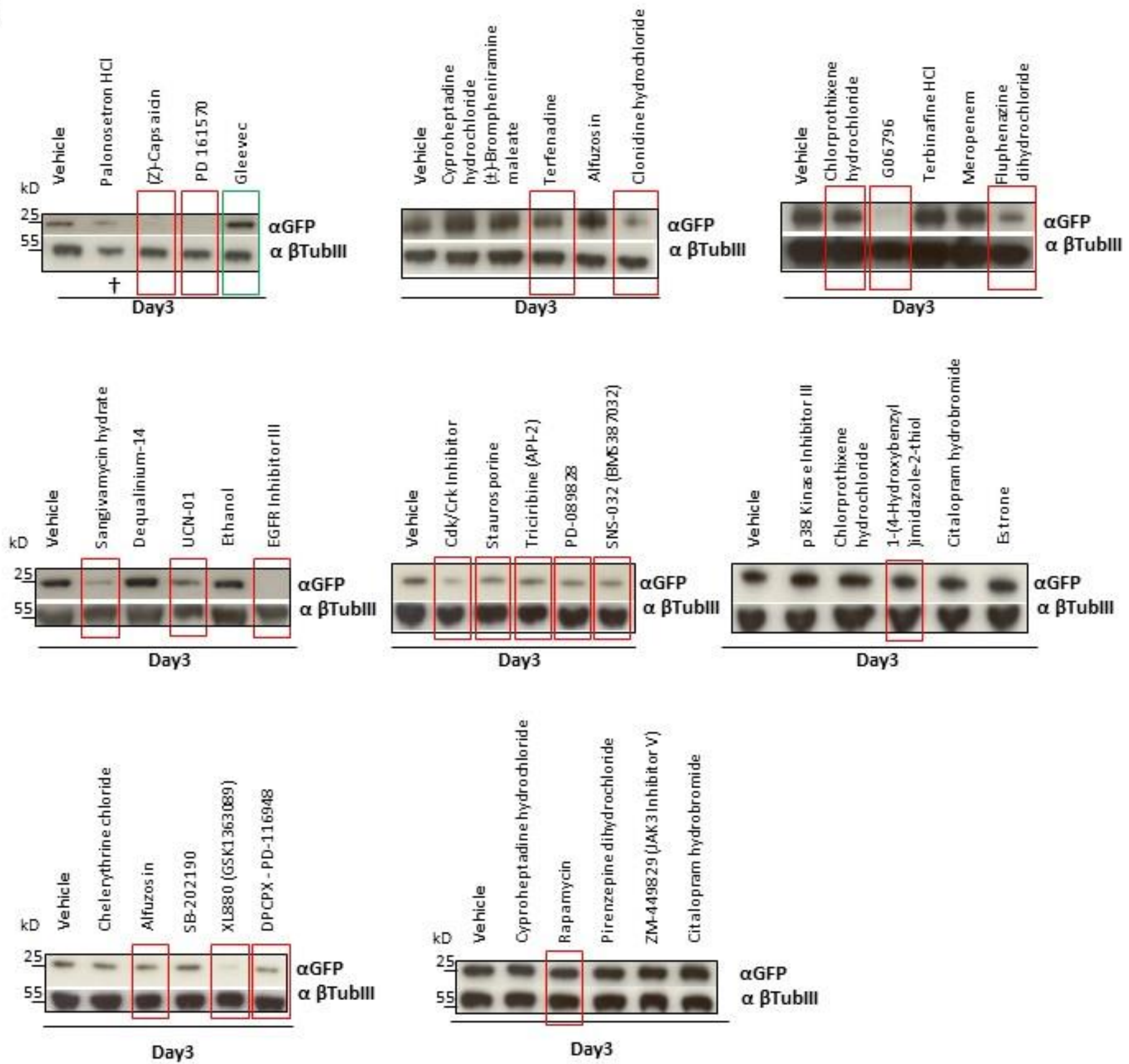
We employed the opera high content confocal screening platform to identify cultured DRG sensory neurons from *Gch1*^{GFP} reporter mice and measure GFP and PI fluorescent intensities. Images analysis was used to specifically identify neurons based on size and also to exclude material deemed too big or small of thin to be neuronal cell bodies. On those cells which

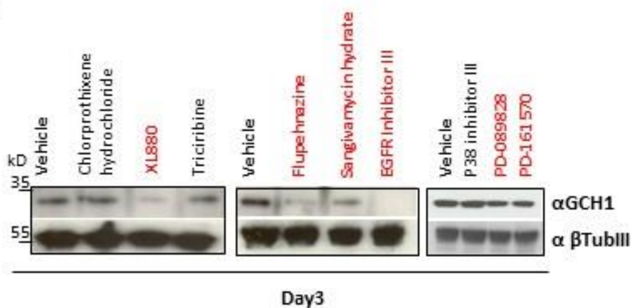
remained, fluorescent intensities of GFP and PI were measured in all cells in each section. Each well of the 384-well plate was subdivided into 15 sections and section parameters were then combined to get a complete overview of each well.



Supplementary Figure 4. Plate layout for screening.

For each 384 flat well blackened plate, 1000 cultured primary DRG sensory neurons were plated. Compounds were tested at two concentrations depending on the compound set used (1 and 10 µM for GPCR/ion channels/epigenetic modifier collection, 0.1 µM and 1.0 µM for kinase collection; in the schematic kinase concentrations are presented). To reduce plate-to-plate variability, each plate had its own set of control compounds - vehicle treatment (48 wells) as well as the positive control PMA (12 wells) and negative control GO6796 (12 wells). 156 individual compounds were tested on each 384-well plate.

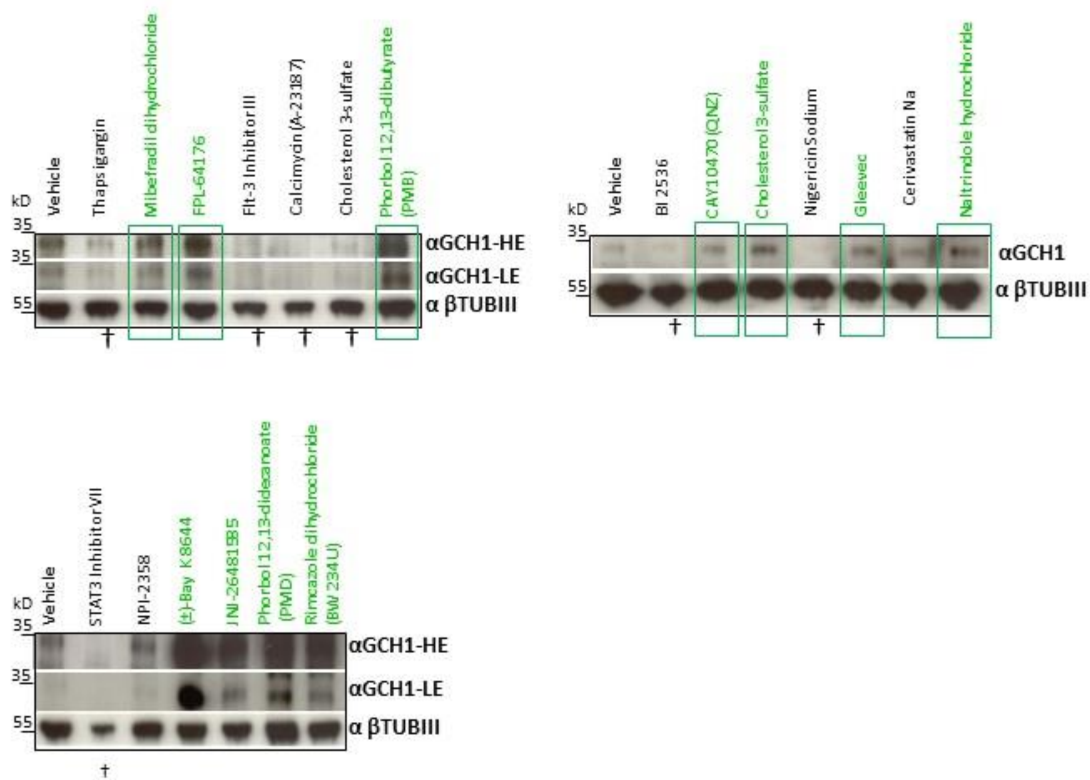




Supplementary Figure 5. Western blot confirmation of candidate "decreaser compounds".

A, Western blot of GFP in 3-day old cultures of sensory DRG neurons isolated from *Gch1*^{GFP} reporter mice. Those compounds identified as "decreasers" from the primary and retests

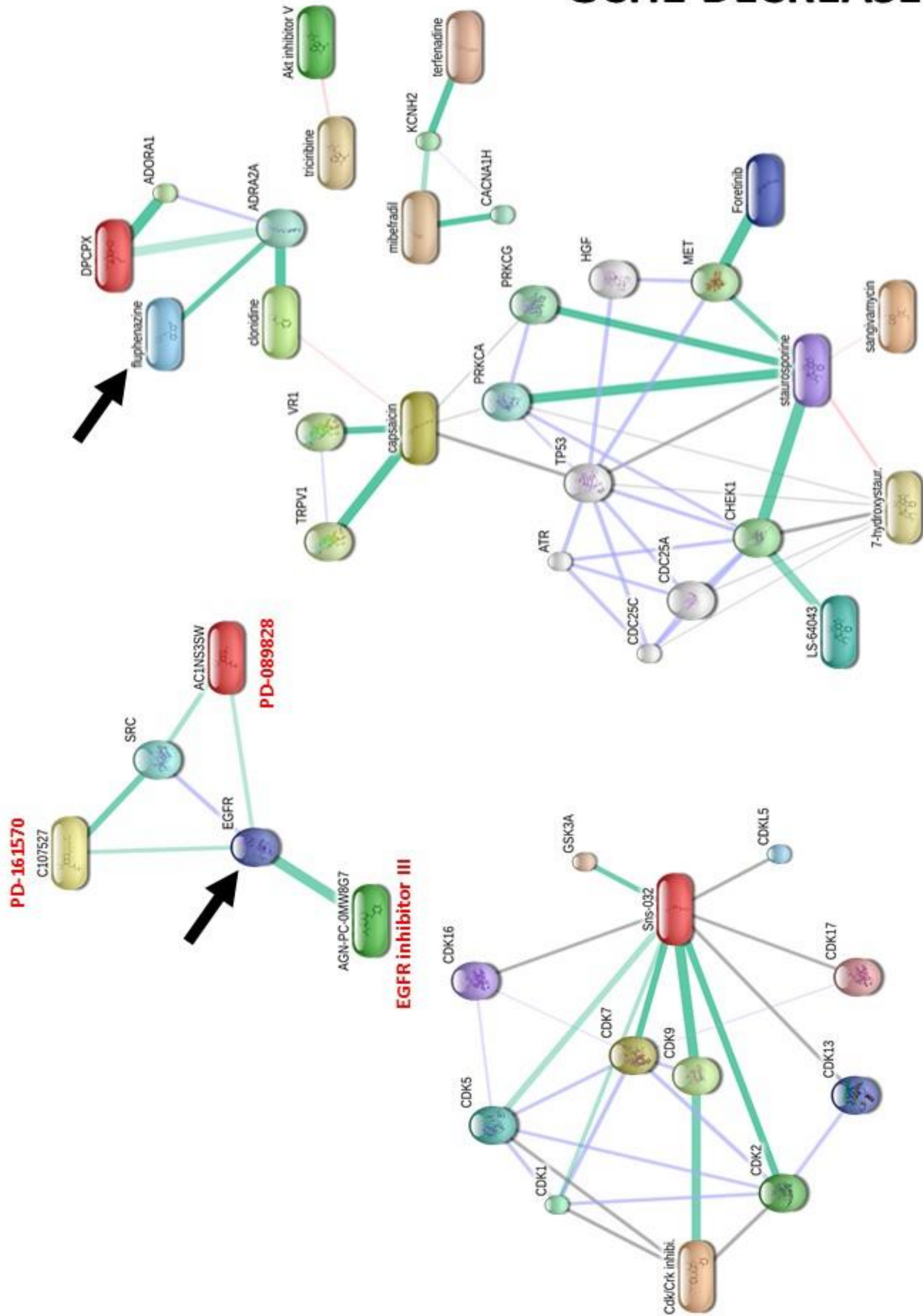
were applied as well as vehicle (DMSO or ethanol) treatment. βTubIII was used as a loading control. Cross symbol (†) indicates toxicity of the compound on the neurons as determined by visual microscopic analysis and low βTUBIII amounts. Those compounds highlighted in red boxes were selected as hits which decrease GFP/GCH1 protein by 20% or more. Quantification of those hits are presented in Figure 2F. **B,** Western blot of endogenous GCH1 protein in 3-day old cultures of sensory DRG neurons isolated from wild type mice. βTubIII was used as a loading control. Quantification of those hits are presented in Figure 4A.



Supplementary Figure 6. Western blot confirmation of candidate "increaser compounds".

Western blot of endogenous GCH1 protein in 3-day old cultures of sensory DRG neurons isolated from wild type mice. Those compounds identified as "increasers" from the primary and retests were applied as well as vehicle treatment. βTubIII was used as a loading control. Cross symbol (†) indicates toxicity of the compound on the neurons as determined by visual microscopic analysis and low βTUBIII amounts. Those compounds highlighted in green boxes

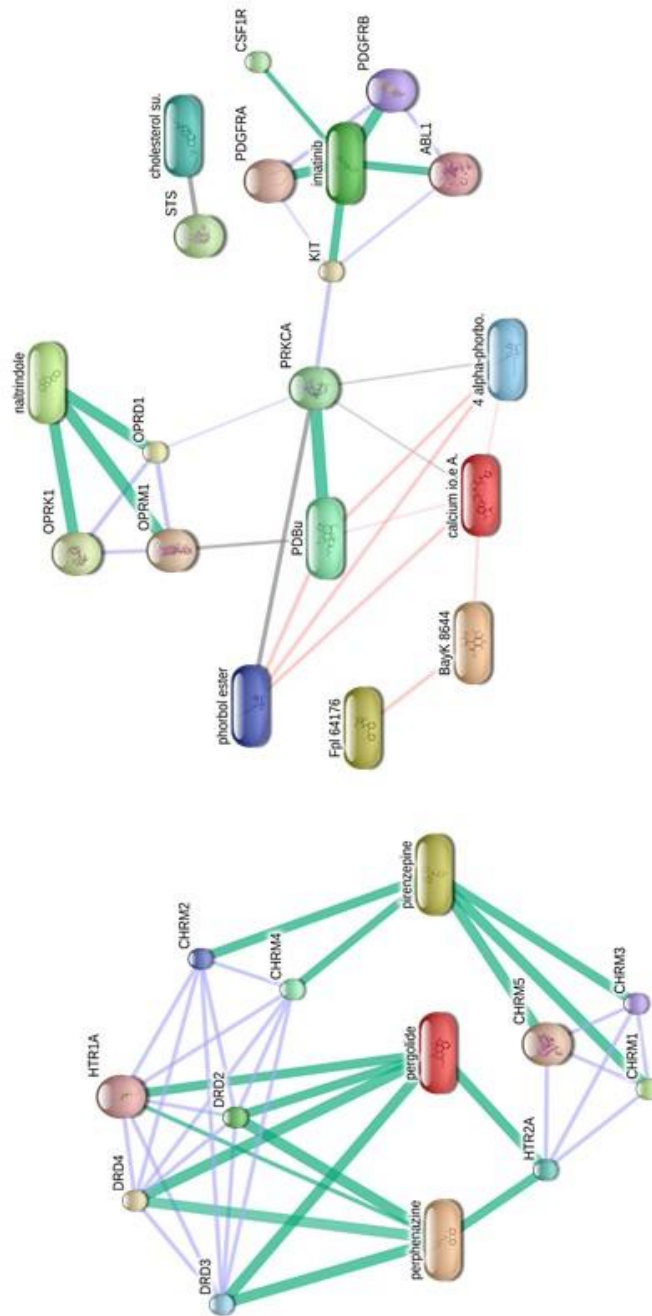
were selected as hits which increase GCH1 protein by 50% or more and are quantified in Figure 2H. HE, high exposure; LE, low exposure.



Supplementary Figure 7. STITCH compound analysis of "decreaser compounds"

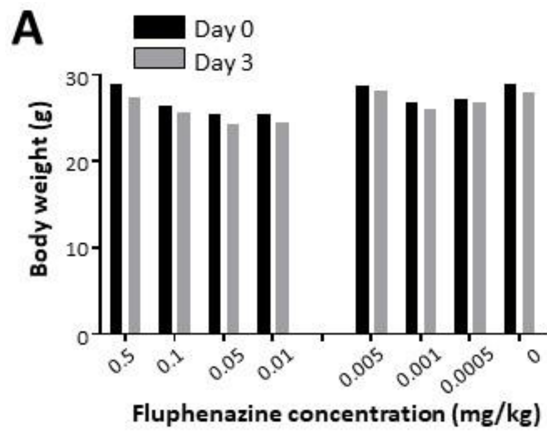
Interaction networks of those compounds which were validated by Western blot to reduce GCH1 protein and their known protein targets. Black arrows indicate EGFR as a central node of particular interest as well as the anti-psychotic drug, fluphenazine hydrochloride. Compounds are represented as cylindrical shaped whereas protein targets are rounded. The images represent confidence views where stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, whereas chemical-protein interactions in green. Small nodes indicate protein of unknown 3D structure while large nodes indicate protein of known or partially known 3D structure.

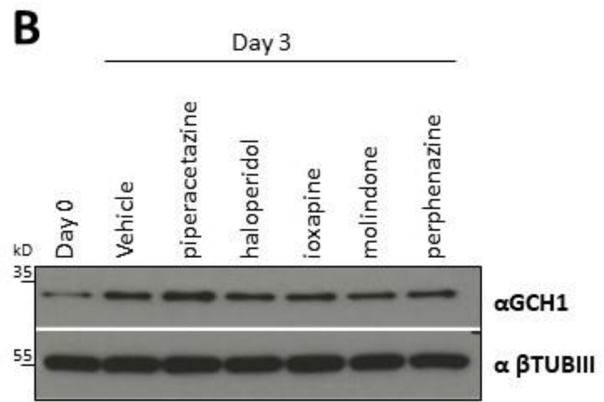
GCH1 INCREASE

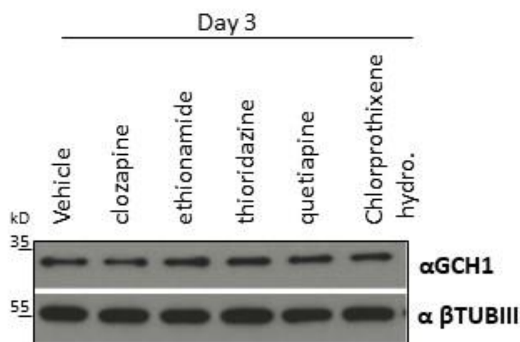


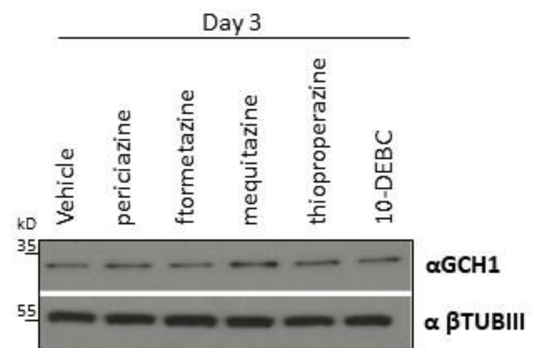
Supplementary Figure 8. STITCH compound analysis of “increaser compounds”

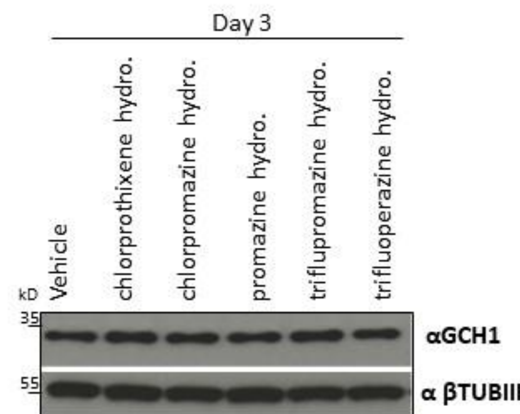
Interaction networks of those compounds which were validated by Western blot to increase GCH1 protein and their known protein targets. Compounds are represented as cylindrical shaped whereas protein targets are rounded. The images represent confidence views where stronger associations are represented by thicker lines. Protein-protein interactions are shown in blue, whereas chemical-protein interactions in green and interactions between chemicals in red. Small nodes indicate protein of unknown 3D structure while large nodes indicate protein of known or partially known 3D structure.

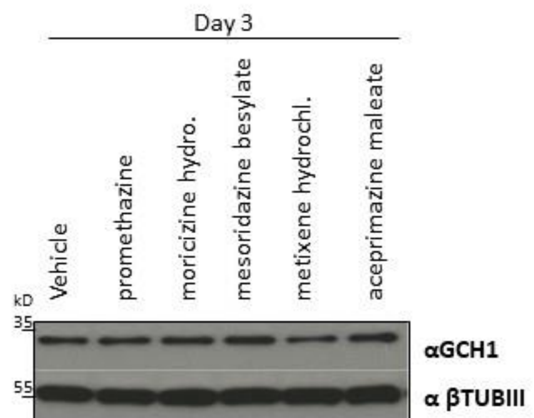

A
Day 0
Day 3
Body weight (g)
Fluphenazine concentration (mg/kg)

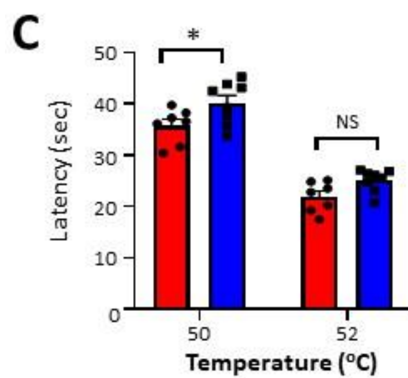

B
Day 3
Day 0
Vehicle
piperacetazine
haloperidol
ioxapine
molindone
perphenazine
αGCH1
α βTUBIII

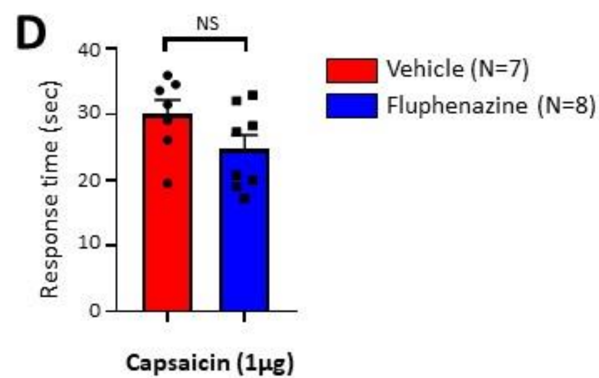

Day 3
Vehicle
clozapine
ethionamide
thioridazine
quetiapine
Chlorprothixene hydro.
αGCH1
α βTUBIII


Day 3
Vehicle
periciazine
ftormetazine
mequitazine
thioproperazine
10-DEBC
αGCH1
α βTUBIII


Day 3
Vehicle
chlorprothixene hydro.
chlorpromazine hydro.
promazine hydro.
triflupromazine hydro.
trifluoperazine hydro.
αGCH1
α βTUBIII

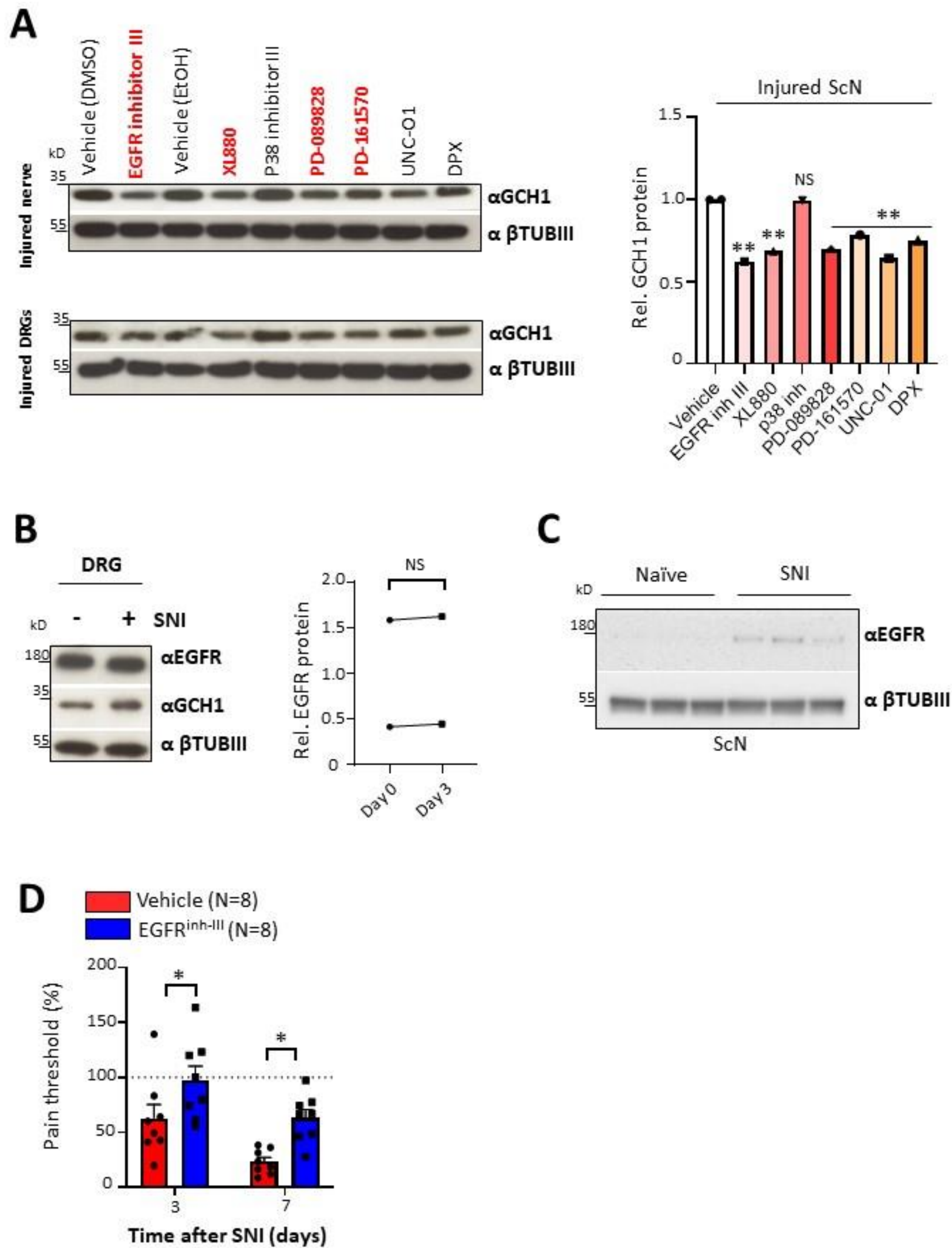

Day 3
Vehicle
promethazine
moricizine hydro.
mesoridazine besylate
metixene hydrochl.
aceprimazine maleate
αGCH1
α βTUBIII


C
Latency (sec)
NS
Temperature (°C)


D
NS
Response time (sec)
Capsaicin (1μg)
Vehicle (N=7)
Fluphenazine (N=8)

Supplementary Figure 9. Effect of fluphenazine and fluphenazine-like compounds on GCH1 protein and nociceptive behavior.

A, Body weights of mice before and after fluphenazine treatment. **B,** Western blot of endogenous GCH1 protein in 3-day old cultures of sensory DRG neurons isolated from wild type mice. Compounds tested are those with a similar chemical structure to fluphenazine hydrochloride. βTubIII was used as a loading control. **C, D,** Latency of wildtype mice treated for two consecutive days with vehicle or fluphenazine (0.1mg/kg) on the hotplate at indicated temperatures (**C**) and response time to intradermal capsaicin (1μg) injection (**D**). Data are shown as means ± s.e.m. Individual mice for each genotype are shown. $^*P < 0.05$; NS, not significant (Student's t-test).



Supplementary Figure 10. In vivo confirmation of candidate decreasers on GCH1 protein from SNI-treated animals.

A, Western blot of GCH1 in injured sciatic nerve and injured L4/5 DRG tissue 3 days after SNI in wild type animals treated with doses of indicated compounds on day 0.5, 1.5 and 2.5 intraperitoneally. βTubIII was used as a loading control. Red font indicates the compounds

which target EGFR. Relative (to vehicle treatments) quantification of GCH1 protein in injured nerve after 3 days of SNI in wild type animals. Data are shown as means ± s.e.m. Individual mice for each treatment are shown. **P < 0.01; NS, not significant (One-way ANOVA with Dunnett's multiple comparison test). B, Representative Western blot of EGFR and GCH1 as well as relative quantification of EGFR in the DRG of naïve and 3-day SNI-treated wild type animals. βTubIII was used as a loading control. Data are pooled from two independent experiments. Individual mice for each condition are shown. NS, not significant (Paired t-test). C, Western blot of EGFR in naïve (n=3 mice) and day 3 SNI-treated (n=3 mice) sciatic nerve (ScN) tissue from wild type mice. βTubIII was used as a loading control. Quantiifcation is presented in Figure 4E. **D,** Percent change of mechanical thresholds on day 3 and day 7 post-injury compared to baseline values of SNI wild type animals treated with vehicle and EGFR$^{inh-III}$ (10mg/kg). Treatment was administered i.p. for two days before each behavioral testing. Dotted line indicates baseline values set at 100%. Data are shown as means ± s.e.m. Individual mice for each genotype are shown. *P < 0.05; (Two-way ANOVA with Sidak's multiple comparison test).

Table S1 – Table S7 (provided as separate Excel files)

Table S1. Compounds screened.

Compounds used in the screen with each unique RL number as well as their CAS number and ordering details.

Table S2. Effect of the compound screen on injured *Gch1*-GFP sensory neurons.

The "Mean GFP intensity", "Number of GFP^{HI} neurons" and "%GFP^{HI} alive/total alive nuclei" of all the compounds used. Also included are their unique RL number, the concentration used, plate and well locations as well as the DMSO, PMA, and G06976 control compounds. PI intensity allowed the exclusion of dead cells.

Table S3. Compounds that decreased *Gch1*-GFP expression.

Compounds ranked for efficacy in reducing *Gch1*-GFP expression in injured neurons.

Table S4. Compounds that increased *Gch1*-GFP expression.

Compounds ranked for efficacy in increasing *Gch1*-GFP expression in injured neurons.

Table S5. Dose-response of *Gch1*-GFP decreasers.

List of validated compounds and details of their biological activities, which reduced *Gch1*-GFP expression. The compounds are ranked according to their optimal GFP reduction compared to DMSO control.

Table S6. Dose-response of *Gch1*-GFP increasers.

List of validated compounds and details of their biological activities which increased *Gch1*-GFP expression. The compounds are ranked according to their optimal GFP increase compared to DMSO control.

Table S7. Validated compounds which regulate GCH1 protein

Selected compound candidates were scored for their effect on increasing (labelled in green) or decreasing (labelled in red) GCH1 protein in cultured DRG cells from wild type animals. They were ranked by the effect on protein increase/decrease relative to vehicle treatment.