Using an engineered GluCl channel to silence sensory neurons and treat neuropathic pain at the source

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Supplemental information

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>β-only</th>
<th>αβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane conductance (nS)</td>
<td>6.25 ± 1.04</td>
<td>5.81 ± 0.87</td>
<td>5.39 ± 0.49</td>
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<tr>
<td>Resting membrane potential (mV)</td>
<td>-60.25 ± 1.62</td>
<td>-60.6 ± 1.91</td>
<td>-60.15 ± 1.43</td>
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<td>Rheobase (pA)</td>
<td>403.54 ± 105.76</td>
<td>330.32 ± 59.96</td>
<td>387.21 ± 73.16</td>
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<tr>
<td>Capacitance (pF)</td>
<td>35.15 ± 3.74</td>
<td>34.71 ± 3.68</td>
<td>33.29 ± 4.68</td>
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<tr>
<td>Number of cells</td>
<td>20</td>
<td>26</td>
<td>48</td>
</tr>
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</table>

Supplementary Table 1. Biophysical and evoked firing properties of untreated DRG neurons expressing GFP, GluClβ or GluClαβ. No statistical difference was observed for any measured parameter. One-way ANOVA comparing group vs control (GFP), followed by post-hoc Bonferroni test.
Supplementary Figure 1. GluCl silencing is effective in DRG of all sizes. (A) Representative fluorescent images of small, medium and large diameter GluCl^+ DRG neurons. Scale bar represents 25 µm. (B) Silencing efficacy of GluCl activation stratified by cell size with data derived from Fig 1F. Neuronal excitability was defined by the fold-increase in rheobase comparing pre- and post-IVM values: <3 (excitable), 3-10 (partial silencing) or >10-fold (full silencing). 5 nM IVM, n = 9 small, 8 medium and 7 large cells. 20 nM IVM, n = 9 small, 8 medium and 6 large cells.
Supplementary Figure 2. GluCl silencing is effective under conditions of high [Cl\textsuperscript{-}]. Patch clamp recording of spontaneous activity of a GluCl\textsuperscript{+} DRG while 20nM IVM is applied. Patch pipette was filled with an intracellular solution containing 80mM Cl\textsuperscript{-}. Under these recording conditions GluCl activation depolarizes the membrane potential, yet is still effective at reducing firing.

Supplementary Figure 3. GluCl activation prevents action potential firing in response to capsaicin. (A) Bright field image of experimental setup. Small diameter (15-25μM) neurons expressing GluCl were assessed for action potential firing during a 10s application of 1μM capsaicin (CAP) applied locally by a second pipette positioned adjacent to the cell. Scale bar represents 25μM. (B) Representative firing of GluCl\textsuperscript{+} neurons in response to CAP, before and 15 mins after treatment with 20 nM IVM. (C) Quantification of action potentials fired in
response to CAP pre- and 15 mins following 20 nM IVM. *P < 0.01, Student’s paired t-test comparing pre- and post-IVM responses (n = 6 for both groups). Data represents mean ± s.e.m.

**Supplementary Figure 4. Replacing YFP with Cerulean does not alter function of GluClα.**

(A) Endogenous cerulean and YFP signal in addition to immunostained β-III tubulin of DRG neurons electroporated with GluClα-mCerulean and GluClβ-mYFP. Scale bar, 30 μm. (B) Dose-response relation for IVM activation of GluCl expressed in HEK 293 cells. EC50 is 2.6 ± 0.2 nM for β-mYFP + α-mYFP and 2 ± 0.3 nM for β-mYFP + α-mCerulean expressing cells (P > 0.99, Student’s unpaired t-test). Each data point represents the mean ± s.e.m. of the input conductance of at least 5 cells at each IVM concentration. (C) Membrane conductance induced
by acute treatment of GluCl+ DRG with 5 and 20 nM IVM. ***$P < 0.001$, Student’s paired $t$-test comparing pre- and post-IVM levels (5 nM $n = 10$ cells, 20 nM $n = 6$ cells.) Data represents mean ± s.e.m. (D) Excitability status before and after IVM treatment. 5 nM $n = 10$ cells, 20 nM $n = 6$ cells.

Supplementary Figure 5. GluClα and β subunits co-localize following i.t delivery. (A) L4 DRG 4-weeks post-AAV delivery imaged for native cerulean/YFP fluorescence and immunostained NeuN. White arrows mark neurons only expressing a single subunit. Scale, 50 μm. (B) Quantification of co-expression. Data represents the mean ± s.e.m from three animals (total= 323 cells).
Supplementary Figure 6. GluCl expression is absent in brain and autonomic structures following i.t. AAV delivery. Whole brains and a range of ganglia were assessed from GluCl\(^+\) animals following i.t. delivery of AAV-GluCl (\(n = 3\)). No GluCl expression was observed in (A) brain (scale bar 500\(\mu\)m), (B) nodose ganglia, (C) Superior cervical ganglion, or (D) lumbar sympathetic chain neurons. Scale bar represents 50\(\mu\)m unless stated otherwise. TH-tyrosine hydroxylase.
**Supplementary Figure 7. GluCl\(^+\) afferents in skin.** Hind paw skin taken from GluCl AAV animals four weeks post AAV delivery. Representative images \((n = 3\) GluCl\(^+\) animals) showing immunostaining for YFP (green) and PGP 9.5 (red) in the skin and a transverse section of a deep dermal nerve fibre. Scale bar, 20 µm.
Supplementary Figure 8. Long-term expression of GluCl after a single i.t dose of GluCl\(\alpha\beta\)-AAV. Anatomical analysis of DRG taken from animals 7 months post i.t. delivery. (A) L4 DRG immunostained for NeuN (red) and YFP (green). Scale bar, 100 µm. (B) Total neurons that are GluCl\(^+\). Data represents mean ± s.e.m of four animals (with one animal from the cohort excluded for zero GluCl expression) and a total of 1762 cells.

Supplementary Figure 9. DRG ATF-3 expression and cutaneous afferent innervation in aged GluCl-AAV animals. Tissue derived from animals 7-months following i.t. delivery of GluCl-AAV. Naïve group represents age matched animals that did not undergo i.t. surgery and SNI group represents animals 4-weeks post SNI as a positive control for nerve injury. (A) Left, Representative images of β-III tubulin (blue) and ATF-3 (red) staining of L4 DRG sections. Right, Quantification of ATF-3 expression as a percentage of total neurons. ****\(P < 0.001\), one-way ANOVA followed by post-hoc Bonferroni test, data represents mean ± s.e.m. Naïve \(n = 3\), β-only \(n = 3\), αβ \(n = 4\), SNI \(n = 4\) animals. (B) Left, Representative images of DAPI (blue) and PGP9.5 (red) staining of hind paw glabrous skin of aged cohorts. Right, Quantification of intra-epidermal nerve fibre density (IENFD). \(P > 0.05\), one-way ANOVA.
followed by post-hoc Bonferroni test, data represents mean ± s.e.m. Naïve $n = 3$, β-only $n = 3$ and αβ $n = 4$ animals. Scale bars represent 50 µm.

Supplementary Figure 10. Mechanical thresholds of the contralateral paw of GluCl$^+$ animals following SNI. Time-course of contralateral paw mechanical sensitivity following SNI (related to Fig 5A) and dosing with IVM. Red arrows signal the time at which animals received a systemic dose of 5 mg/kg IVM. $^*P < 0.05$, RM-ANOVA followed by post-hoc Bonferroni test comparing post-SNI time-points with baseline, $n = 11$ animals per group.