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Indirect effects of Halorhodopsin activation: potassium redistribution, non-specific inhibition and spreading depolarisation

1   Indirect effects of Halorhodopsin activation: potassium

2   redistribution, non-specific inhibition and spreading depolarisation

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Abstract

The movement of ions in and out of neurons can exert significant effects on neighboring cells. Here we report several experimentally important consequences of activation of the optogenetic chloride pump, Halorhodopsin. We recorded extracellular K^+ concentration, $[K^+]_{extra}$, in neocortical brain slices prepared from young adult mice (both sexes) which express Halorhodopsin in pyramidal cells. Strong Halorhodopsin activation induced a pronounced drop in $[K^+]_{extra}$, that persisted for the duration of illumination. Pharmacological blockade of K^+ channels reduced the amplitude of this drop, indicating that it represents K^+ redistribution into cells during the period of hyperpolarization. Halorhodopsin thus drives the inward movement of both Cl^- directly, and K^+ secondarily. When the illumination period ended, a rebound surge in extracellular $[K^+]$ developed over tens of seconds, partly reflecting the previous inward redistribution of K^+, but additionally driven by clearance of Cl^- coupled to K^+ by the potassium-chloride co-transporter, KCC2. The drop in $[K^+]_{extra}$ during light activation leads to a small (2-3mV) hyperpolarization also of other cells that do not express Halorhodopsin. Its activation therefore has both direct and indirect inhibitory effects. Finally, we show that persistent strong activation of Halorhodopsin causes cortical spreading depolarisations (CSDs), both *in vitro* and *in vivo*. This novel means of triggering CSDs is unusual, in that the events can arise during the actual period of illumination, when neurons are being hyperpolarized and $[K^+]_{extra}$ is low. We suggest that this fundamentally different experimental model of CSDs will open up new avenues of research to explain how they occur naturally.

Significance statement

Halorhodopsin is a light-activated electrogenic chloride pump, which has been widely used to inhibit neurons optogenetically. Here, we demonstrate three previously unrecognized consequences of its use: 1. Intense activation leads to secondary movement of K_+ ions into the cells. 2. The resultant drop in extracellular $[K^+]$ reduces excitability also in other, non-expressing cells. 3. Intense persistent Halorhodopsin activation can trigger cortical spreading

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73   depolarization (CSD). Halorhodopsin-induced CSDs can occur when neurons are hyperpolarized

74   and extracellular $[K^+]$ is low. This contrasts with the most widely used experimental models that

75   trigger CSDs with high $[K^+]$. Both models, however, are consistent with the hypothesis that CSDs

76   arise following net inward ionic movement into the principal neuron population.

Introduction

78   The complexity of neuronal behaviour derives in large measure from the fact that

79   neurons do not act in isolation. This is most apparent in the form of synaptic interactions, but

80   in certain circumstances, the neurons may also exert significant effects on neighbouring cells

81   through indirect means, arising from movement of ions in or out of the extracellular space

82   (Jefferys, 1995). Neuronal firing and synaptic activity leads to large ionic movement across the

83   neuronal membrane (Attwell and Laughlin, 2001; Hodgkin and Huxley, 1952), and if sufficiently

84   intense, can result in significant redistribution of key ions, with depletion of extracellular Na^+

85   and Ca^{2+} ions, and rises in extracellular K^+ ions.

86   The development of optogenetics has provided a new set of research tools for

87   investigating such matters, with novel ways to challenge neuronal networks (Deisseroth, 2011).

88   Optogenetic studies have mostly involved network level analyses, in which specific

89   subpopulations of neurons are either activated or inactivated to dissect out their roles in large

90   networks or whole animals. Cellular processes can also be examined using optogenetics, and in

91   this regard, the light activated ion pumps (Chow et al., 2010; Zhang et al., 2007) offer

92   particularly interesting ways to challenge the cells experimentally, either in isolation or in

93   tandem with ion channels. The light activated chloride pump, Halorhodopsin can be used to

94   drive chloride into neurons (Alfonsa et al., 2015; Raimondo et al., 2012), whereas chloride

95   extrusion can be achieved by coupling the action of the light activated proton pump and the

96   opening of a chloride channel, using a co-operative opsin called Cl-out (Alfonsa et al., 2016).

97   We reasoned that artificial chloride manipulation provides a further means of examining the

98   relationship between chloride entry and delayed extracellular $[K^+]$ rises via the potassium

99   chloride cotransporter (KCC2), that have to date only been examined by means of GABAergic

100   activation (Chang et al., 2018; Shiri et al., 2015; Viitanen et al., 2010). Specifically, we

101 hypothesized that Halorhodopsin-mediated ion redistribution would lead to increased KCl

102 efflux by KCC2, revealing itself as a surge in extracellular K^+ concentration, $[K^+]_{extra}$, after

103 illumination ends. To test this hypothesis, we utilized mice in which Halorhodopsin was

104 expressed in all pyramidal cells, under the Emx1 promoter (Gorski et al., 2002), and recorded

105 changes in $[K^+]_{extra}$ using ion-sensitive electrodes (Voipio et al., 1994).

106   These studies confirmed the relationship between intracellular $[Cl^-]$ and extracellular $[K^+]$

107 transients, but further revealed three other, previously unrecognized, consequences of

108 activation of optogenetic pumps. The first is that intense activation of Halorhodopsin leads to

109 redistribution of K^+ ions into neurons through K^+ ion channels, on account of the

110 hyperpolarization of a large population of neurons altering the balance between inward and

111 outward fluxes of ions; this is particularly evident for K^+ on account of it being highly permeant

112 at resting E_m, and its low basal extracellular concentration. An important consequence of the K^+

113 redistribution is that the lowering of $[K^+]_{extra}$ causes a hyperpolarisation of all neurons, not just

114 those that express the opsin; this represents a form of indirect inhibition by Halorhodopsin.

115 Finally, we report that Halorhodopsin activation can also trigger cortical spreading

116 depolarisation (CSD) events. Our initial observations of Halorhodopsin-triggered CSDs were of

117 events arising immediately after the end of illumination, but of particular note were other

118 instances, when CSDs arose even during the Halorhodopsin activation, when most neurons in

119 the network were strongly hyperpolarized and $[K^+]_{extra}$ was low. As such, this novel way of

120 triggering CSDs, by a hyperpolarizing photocurrent, is quite unlike most other known natural

121 and experimental triggers (e.g. ischaemia trauma, seizures (Somjen, 2004; Ullah et al., 2015))

122 where the common thread has been considered to be raised $[K^+]_{extra}$ and strong depolarization

123 (Tamim et al., 2021). The exception is the triggering of CSDs by application of hypotonic

124 solution, leading to the suggestion that osmotic stress may be the critical factor (Chebabo et al.,

125 1995; Somjen, 2004), a hypothesis that is consistent with the data we present here.

126 **Methods**

127 **Ethical Approval:** All procedures performed were in accordance with the guidelines of the

128 Home Office UK and Animals (Scientific Procedures) Act 1986 and approved by the Animal

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129   Welfare and Ethical Review Body at both Newcastle University and UCL. Male and female mice

130   were used for experimentation.

131   **Slice Preparation:** We used both wild-type C57BL/6 mice, and also mice expressing eNpHR3.0

132   within the pyramidal cell population, generated by cross-breeding homozygous Emx1-cre mice

133   (Jackson Laboratory, Stock 005628) with mice containing a floxed STOP cassette in front of an

134   eNpHR3.0/EYFP domain (Jackson Laboratory, Stock 014539; both maintained on the C57BL/6

135   background). Emx1-promoter yields gene expression only in pyramidal cells in neocortex, in

136   adult mice, although it can drive gene expression in glia in other brain areas, and early in

137   development (Gorski et al., 2002). Experiments were performed on mice aged 1 - 8 months, of

138   both sexes. Mice were sacrificed by Schedule 1 method, brains were removed and placed in

139   cold cutting solution containing (in mM): 3 MgCl2; 126 NaCl; 2.6 NaHCO3; 3.5 KCl; 1.26

140   NaH2PO4; 10 glucose. 400 μm horizontal sections were made on a Leica VT1200 vibratome

141   (Leica Microsystem, Germany). Slices were stored at room temperature, in an interface holding

142   chamber for 1 – 4 hours prior to experimentation. Solutions were bubbled with carbogen (95%

143   O_2 and 5% CO_2) in artificial cerebro-spinal fluid (aCSF) containing (in mM): 2 $CaCl_2$; 1 $MgCl_2$; 126

144   NaCl; 26 $NaHCO_3$; 3.5 KCl; 1.26 NaH_2PO_4; 10 glucose.

145   *In vitro* **Extracellular recordings:** Extracellular recordings were performed using an interface

146   recording chamber. Slices were placed in the recording chamber and perfused with aCSF

147   supplemented in different experiments with various combinations of the following drugs: 1μM

148   Tetrodotoxin (TTX) (Abcam), 10μM VU 0463271 (Tocris Bioscience), 10mM

149   tetraethylammonium (TEA) (Sigma), 2mM Ba^{2+} (Fisons plc, England), as indicated in the results

150   section. Recordings were obtained using aCSF-filled ~1-3MΩ borosilicate glass microelectrodes

151   (GC120TF-10; Harvard apparatus, Kent) placed in deep layers of neocortex. Extracellular

152   potassium, $[K^+]_{extra}$, was measured using single-barrelled K^+-selective microelectrodes. The

153   pipettes were pulled from nonfilamented borosilicate glass (Harvard Apparatus, Kent, UK), and

154   the glass was exposed to vapor of dimethyl-trimethyl-silylamine (Sigma-Aldrich), baking at 200^0

155   C for 40 minutes. The pipettes were then backfilled with aCSF. A short column of the K^+ sensor

156   (Potassium ionophore I, cocktail B; Sigma-Aldrich, #99373) was taken into the tip of the

157   salinized pipette by using slight suction. The recordings through the K^+-sensor electrode were

158 referenced to a second electrode filled with aCSF. From the differential signal from a custom

159 build amplifier, we calculated the $[K^+]_{extra}$ from calibration recordings made in an open bath,

160 using sudden increments in $[K^+]_{extra}$. This provided a scaling factor S, of 55-59mV, where the K^+

161 concentration at a given moment in time, t, was calculated from the differential voltage, V(t), as

162 follows:

163 $[K] = [K]_{baseline} \, 10^{V(t)/S}$

164 $[K]_{baseline}$ for our experiments was 3.5 mM. The temperature of the chamber and perfusate was

165 maintained at 33-36^{0}C using a closed circulating heater (FH16D, Grant instruments, Cambridge,

166 UK). The solutions were perfused at the rate of 3 ml/min by a Watson Marlow 501U peristaltic

167 pump (Watson-Marlow Pumps Limited, Cornwall UK). The direct current local field potential

168 signal was unfiltered and amplified to a 10X output with a custom build amplifier. These

169 waveform signals were digitized with a Micro 1401-3 ADC board (Cambridge Electronic Design,

170 UK) and Spike2 version 7.10 software (Cambridge Electronic Design, UK). Signals were sampled

171 at 10 kHz. Recordings were analysed using a custom-written code in MATLAB (Mathworks, MA,

172 USA).

173 ***In vitro* patch clamp recordings:** Brain slices were prepared, from the same mice strain (Halo^{Fl} x

174 Emx1-Cre), as follows. Mice were anaesthetised by intraperitoneal injection of ketamine (75

175 mg kg^{-1}, Ketalar™ Injection, Pfizer Ltd., Sandwich, UK) combined with medetomidine (1 mg kg^{-1},

176 Domitor®, Janssen Animal Health, Basingstoke, UK), and perfused through the heart using a

177 chilled and aerated "sucrose aCSF" (in mM: 228 sucrose; 26 NaHCO$_3$; 4 MgCl$_2$; 3 KCl; 1.25

178 NaH$_2$PO$_4$; 10 glucose) for 1 minute, before removing the brain, and making 350µm coronal

179 brain slices in the same sucrose solution. Slices were then incubated in a submerged chamber

180 in conventional 1mM Mg^{2+} / 2mM Ca^{2+} aCSF, at room temperature. For the recordings, slices

181 were transferred to a recording chamber held on a movable top plate (Scientifica) with

182 mounted electrode micromanipulators (Scientifica). Slices were visualized, and illuminated,

183 through a UMPlanFL N 40x, 0.5 NA objective (Olympus, UK), using a Scientifica upright

184 microscope fitted with a spinning disk confocal attachment (Visitech, UK) and a Hamamatsu

185 C9100 EM camera (Hamamatsu Photonics, Japan) and coordinated using Visitech software. The

186 temperature in the recording chamber was maintained at 32-34°C by warming the perfusate

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187 (delivered at 3-5mls/min) using a heated sleeve immediately prior to the recording chamber,

188 and with the additional aid of a plate heater (Warner Instruments). Whole cell recordings of

189 neurons from the temporal association area were made using 4-7MΩ pipettes. Pipettes were

190 filled with a KMeSO$_4$-based internal solution containing (mM): 125 KMeSO$_4$, 6 NaCl, 10 HEPES,

191 2.5 Mg-ATP, 0.3 Na$_2$-GTP. Osmolarity and pH of all the internal solutions used were adjusted to

192 284mOsm and 7.4. The offset of the patch clamp amplifier was zeroed prior to patching the

193 cells, and we adjusted the subsequent measurement of membrane potential, therefore, by the

194 liquid   junction   potential,   which   was   calculated   using   LJPcalc   software

195 (https://swharden.com/LJPcalc) to be 9.6mV, according to the stationary Nernst–Planck

196 equation (Marino et al., 2014). The eNPHR3.0 was conjugated to an eYFP fluorescent marker,

197 allowing us to discriminate between cells that expressed the opsin (pyramidal cells), and those

198 that did not (presumptive interneurons and some glia). After going whole cell, we recorded in

199 current clamp mode, first testing the optogenetic response to a short (200ms) illumination with

200 568nm light, and then the firing response to a current injected through the patch electrode.

201 We then assessed the response to a more intense, and sustained illumination with both 488nm

202 and 568nm wavelength light. We used this combination because previous work

203 indicated that concurrent illumination with both yellow and blue light yields a more sustained

204 eNPHR3.0 photocurrent, because the blue light refreshes the opsin (Han and Boyden, 2007). In

205 non-expressing, fast-spiking interneurons, we finally tested the firing response to 200pA

206 current either in darkness, or under steady-state illumination with 488nm and 568nm light.

207 Patch clamp data was collected using a Multiclamp 700B amplifier (Axon Instruments,

208 Molecular Probes), digitized using a 1401 AD converter and recorded onto a Dell Precision

209 Desktop computer.   Recordings were analysed offline, using custom-written codes in

210 Matlab2018b. The input-output functions were fitted with sigmoidal equation, as follows:

$$F = \frac{F_{max}}{(1 + e^{(\sigma - I)/N})}$$

211   Where F is the firing rate, I is the current, and the fitting procedure used a non-linear least

212 squares process to derive the other three parameters: maximal firing rate (F_{max}), the current

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213 giving half-maximal firing (σ) and a parameter which dictates the slope (*N*; note, high values

214 give shallower slopes).

215 *In vivo* **Extracellular recordings:** Animals were anaesthetised with urethane (20% w/v in saline

216 for injection at 0.1ml/gram of mouse) and placed on a stereotaxic frame with heating pad,

217 monitored with a rectal probe to maintain body temperature. A cranial window was created on

218 the left hemisphere and an LFP electrode was placed into the somatosensory cortex. DC LFP

219 recordings were performed as in the *in vitro* experiments, with the electrode about 1mm deep

220 into the tissue. No mice were used past 4 hours from urethane injection.

221 **Optogenetic illumination for extracellular recordings:** Optogenetic illumination was performed

222 using a Fiber-coupled LED light at 565 nm (Thorlabs, M565F3) and driven by an LED driver

223 (Thorlabs, LEDD1) placed in trigger mode. Light output was measured at an average of 9 mW.

224 The LED light was position just above the superficial layers of the neocortex for the slice

225 experiments and pointed toward the deep layers, where the extracellular recordings were

226 performed. For the *in vivo* experiments, the LED was placed in the somatosensory cortex, just

227 above the tissue and about 2mm from the recording electrode. Optogenetic activation was

228 from 90 seconds to 5 minutes depending on the experiment.

229 *In vivo* **recordings in awake head-fixed mice using graphene micro-transistor arrays:**

230 C57BL/6 mice (~3-4 months of age), were injected with 500nl of rAAV5-hSyn-eNpHR3.0-eYFP

231 (UNC vector core) unilaterally into the primary motor cortex (M1) and headbars attached.

232 Three weeks later, we initiated rounds of habituation to the Neurotar fixation frame (Neurotar,

233 Finland). Once the animal was fully habituated, a second surgery was performed to allow a

234 craniotomy over the ipsilateral somatosensory and visual cortex. Upon recovery from this

235 surgery (3-4hrs), the animal was placed in the neurotar frame, a 16-channel graphene micro-

236 transistor array positioned over the craniotomy (dura intact), and a fiber optic cannula,

237 connected to a Thorlabs green (594nm) LED positioned over a small bur hole made over the M1

238 area. Optogenetic or pinprick induction of CSD was induced in the motor cortex and

239 electrographically recorded using the graphene transistor arrays over ipsilateral somatosensory

240 and visual cortex - see (Masvidal-Codina et al., 2021) for full surgical, experimental and

241 graphene transistor data analysis details).

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242 **Statistics:** Statistical analysis of electrophysiology was performed using GraphPad Prism

243 (GraphPad Software, Inc., La Jolla, CA, USA). Data was analysed with a Mann-Whitney test, a

244 Kruskal-Wallis with a Dunn's multiple comparisons test, or a paired t-test where appropriate.

245 Figures of electrophysiology traces were created in Matlab2015b or 2018b (Mathworks, MA,

246 USA). Statistics are expressed as the mean $\pm$ standard error of mean (s.e.m.).

247 **Results**

248 **Activation of Halorhodopsin causes secondary redistribution of K^+ ions**

249 Previous work showed that intense GABAergic activation is followed by a surge in $[K^+]_{extra}$

250 (Chang et al., 2018; Shiri et al., 2015; Viitanen et al., 2010). The explanation for this is that Cl^-

251 ions which moved into neurons through the GABA receptors are then extruded coupled to K^+

252 via KCC2. This hypothesized mechanism predicts that a similar surge in $[K^+]_{extra}$ should occur if

253 neurons are artificially loaded with Cl^- using the optogenetic chloride pump, Halorhodopsin

254 (Alfonsa et al., 2015; Raimondo et al., 2012). We prepared brain slices from adult mice which

255 expressed Halorhodopsin in all pyramidal cells, under the Emx1 promoter, and measured

256 $[K^+]_{extra}$ during and after a 90s illumination epoch (Figure 1). In all slices, we recorded large

257 increases in $[K^+]_{extra}$, peaking at a mean 8.0 ± 0.44 mM (mean $\pm$ s.e.m.; n = 14 slices). The surge

258 was relatively slow, peaking only 59.9 ± 10.4s after the end of the period of illumination. We

259 also observed a drop in $[K^+]_{extra}$, that persisted throughout the entire period of illumination.

260 Brain slices from wild-type mice showed no change in $[K^+]_{extra}$ during periods of equivalent

261 illumination (n = 3 slices; Figure 1A).

262 We first investigated the nature of the drop in $[K^+]_{extra}$ during the period of illumination.

263 We performed these experiments in the presence of TTX, to rule out any possible contribution

264 arising from altered levels of neuronal firing during activation of Halorhodopsin. In the

265 presence of TTX alone, the $[K^+]_{extra}$ dropped to 1.71 ± 0.08 mM (ΔK_{extra} = ~-1.8mM from the

266 baseline level of 3.5mM), compared to 2.8 ± 0.09 mM in TEA (ΔK_{extra} = ~-0.7mM), and $2.85 \pm$

267 0.11 mM when Ba^{2+} was added (ΔK_{extra} = ~-0.65mM; Figure 2). Glial K^+ channels are relatively

268 insensitive to TEA, so the fact that the addition of Ba^{2+} ions was not significantly different from

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269 the effect of TEA alone suggests that most of the drop in $[K^+]_{extra}$ occurs via a hyperpolarization-

270 driven redistribution of K^+ into neurons. This is consistent with the pattern of opsin expression

271 in these animals, driven under the Emx1 promoter, which in adults, appears limited to the

272 pyramidal population (Gorski et al., 2002).   Whole cell patch clamp recordings of

273 Halorhodopsin-expressing pyramidal neurons recorded light-induced hyperpolarization by

274 24.5 ± 1.5 mV (mean $\pm$ sem; range 10 - 34 mV, 23 cells, from 6 mice) from resting membrane

275 potential (-62.1 $\pm$ 1.5 mV; during intense illumination E_m = -84.9 $\pm$ 2.5 mV (range = -64 to -

276 104mV; Figure 3A)).

277 **Non-specific inhibitory effects of Halorhodopsin activation**

278   We further reasoned that the drop in the extracellular $[K^+]$ may have indirect effects also

279 upon non-expressing cells, because the reduced $[K^+]_{extra}$ would be expected to lower their E_m.

280 To examine this possibility, we prepared brain slices in which Halorhodopsin was expressed in

281 the pyramidal population, but made targeted patch-clamp recordings of non-expressing cells

282 (Figure 3): non-pyramidal neurons (interneurons (n = 15), indicated by firing patterns and the

283 lack of a prominent Halorhodopsin response, and also glia (n = 2), indicated by non-firing

284 response to current injection. We found that during these periods of illumination, there was

285 also a small, but significant hyperpolarization of non-expressing cells of 2.0 $\pm$ 0.3 mV (n = 17

286 cells, comprising 2 glia (hyperpolarized by 2.0 and 3.6mV respectively) and 15 interneurons (E_m

287 = -62.4 $\pm$ 1.0 mV), from 6 mice; range of hyperpolarizing effect = 0 to 3.6mV) which was both

288 significantly different from the null response (p = 6.9 x 10^{-6}, Student's t-test), and also

289 significantly smaller than the pyramidal response (p = 7.2 x 10^{-14}; Student's t-test).   No change

290 was seen during illumination in wild-type brain slices, which lack Halorhodopsin.   A possible

291 explanation for the small hyperpolarising effect in the non-pyramidal population is that there

292 was a low level of "breakthrough" expression of Halorhodopsin in these cells. If that were the

293 explanation, though, the kinetics of the change in E_m would be comparable to that seen in

294 pyramidal cells, since this is dictated primarily at the molecular level, rather than the level of

295 expression. Notably, the time-course of the hyperpolarization of "non-expressing" cells was

296 very slow, reaching its peak level at 1061 $\pm$ 150ms (n = 13 interneurons - discounting the two

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297 cells which showed no discernible shift in E_m; the two glia reached peak effect at 800 and
298 870ms respectively) after the start of illumination, in marked contrast to a rapid peak
299 hyperpolarization achieved in pyramidal cells, which occurred at just 47.6 ± 5.6ms (n = 23)
300 which was highly significantly faster than in non-expressing cells (p = 3.2 x 10^{-10}, Student's t-
301 test). The slow evolving hyperpolarizing effect in the non-expressing cells had a similar time
302 course to the drop in $[K^+]_{extra}$, and so we concluded that this was indeed due to indirect effects,
303 and not due to break-through expression in cells without the Emx1 promoter.

304 We reasoned that the drop in $[K^+]_{extra}$ may also influence neuronal firing in non-expressing
305 neurons. To test this, we measured the input-output function, in fast-spiking interneurons
306 (FSIs), in response to step changes in somatically injected current, in baseline conditions and
307 during a sustained period of pyramidal-Halorhodopsin activation (n = 9 cells, 6 mice; Figure 4).
308 Consistently, we found that firing rates was reduced, in these non-expressing cells, when the
309 tissue was illuminated, consistent with our results regarding the drop in $[K^+]_{extra}$ (Figure 2) and
310 also of E_m in non-expressing cells (Figure 3). This was the case if the effect was quantified either
311 by the total number of spikes in the 1s current injection (Figure 4Bi) or as the maximal
312 instantaneous firing rate (reciprocal of the shortest interspike interval) (Figure 4Bii). For each
313 cell, we fitted a sigmoidal function (Figure 4Ci) to the input-output functions for both conditions
314 (light vs no light; see Methods for fitting details). In every case, the half-maximal current was
315 increased during periods of illumination to activate Halorhodopsin in the pyramidal population
316 (Figure 4Cii,iii; 9/9 cells; mean increase = 12.7 $\pm$ 3.3 %; significantly different from null
317 response, p = 0.0049). Across the 9 cells, there was no significant change in either the maximal
318 firing rate (p = 0.20), or the slope of the fit (p = 0.37), in the two conditions (light vs no light).

319 **A large rebound surge in $[K^+]_{extra}$ follows Halorhodopsin activation**
320 We next examined the nature of the large rebound surge in peak $[K^+]_{extra}$, following
321 Halorhodopsin activation (range 2.0 – 7.1 mM above baseline, 3.5 mM). One component of the
322 rebound surge will be extrusion of K^+ ions that moved into the neurons during the period of
323 illumination. Notably, however, in a set of experiments with 90s illumination, when the
324 illumination-induced lowering of $[K^+]_{extra}$ reaches a steady state, there was no correlation

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325 between the amplitude of the illumination-induced drop in $[K^+]_{extra}$, and the rebound surge in

326 $[K^+]_{extra}$ (R^2 = 0.006, p = 0.79).

327   A second explanation is that the rebound [K+]extra surge was caused by rebound spiking

328 following a period of hyperpolarization. However, the large post-Halorhodopsin surge persisted

329 in the presence of the Na+ channel blocker, tetrodotoxin (TTX), indicating that rebound spiking

330 was not the source of the rebound peak in [K+]extra. In contrast, the peak was substantially

331 reduced, although not abolished, by blocking the K+-Cl- co-transporter, KCC2, using VU0463271

332 (p=0.0037, Kruskal-Wallis test with a Dunn's multiple comparisons test). The post-illumination

333 peak in [K+]extra was also reduced by the K+ channel blocker, TEA (Figures 4C,D; p = 0.0064,

334 Kruskal-Wallis test with a Dunn's multiple comparisons test). The time to the peak of the

335 rebound in [K+]extra was delayed by blocking KCC2 (Figure 4C, p = 0.0014, two-tailed paired t-

336 test) , but not by blocking K+ channels (Figure 4D, p = 0.61, two-tailed paired t-test).

337 Altogether, our data showing a large surge in [K+]extra, following a period of Halorhodopsin

338 chloride-loading, are entirely consistent with the previous report of a secondary rise in

339 [K+]extra, following chloride-loading occurring naturally caused by intense GABAergic activation

340 (Viitanen et al., 2010).

341

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343 Induction of spreading depolarization events by Halorhodopsin activation

344   While performing these investigations of Halorhodopsin-induced K^+ redistribution, we

345 observed that cortical spreading depolarization (CSD) events often occurred shortly after

346 periods of illumination (Figure 6A). This happened relatively unusually with shorter illumination

347 epochs (10-30s), but when the period of illumination were extended to 90s, CSDs occurred in

348 around 33% of trials. Notably, we were able to induce CSDs also in the presence of TTX alone (n

349 = 5 brain slices, from 4 mice), with TTX, TEA and Ba^{2+} together (to additionally block K^+

350 channels, n = 3 brain slices, from 2 mice), with TTX and KCC2 blocked using VU 0463271 (1 brain

351 slice), and with blockade of both glutamatergic and GABAergic neurotransmission in the

352 presence of TTX (1 brain slice).

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353   Using synchronous measurements of the LFP and also the $[K^+]_{extra}$, we estimated that at

354   the moment the CSD started, the median $[K^+]_{extra}$ locally was 3.1 mM (interquartile range = 2.5 –

355   3.5 mM; full range = 2.1 – 17 mM; 19 brain slices, from 8 mice; Figure 6B), and occurred shortly

356   after (1.73 $\pm$ 1.91s latency) the end of a period of illumination (typically <100s illumination).

357   Remarkably, If the illumination was maintained, then CSDs occurred in every slice (n = 6 slices,

358   from 2 mice) with a latency of 526 $\pm$ 79.6s. In contrast, CSDs were not induced by 600s

359   illumination with 565nm light either in brain slices from wild-type mice (n = 6 slices, from 1

360   mouse), or from mice expressing channelrhodopsin-eYFP (n = 12 slices, from 2 mice). In each

361   case, light was delivered at a spot, and notably, post hoc staining for DAPI, NeuN and GFAP

362   revealed no inhomogeneity of stain at that site, either in Halorhodopsin slices that experienced

363   CSDs (n = 12 slices, from 2 animals) or from the wild-type and channelrhodopsin-eYFP mice.

364   The significance of CSDs occurring during periods of Halorhodopsin activation, is that at this

365   time, the expressing population of pyramidal neurons is hyperpolarized by the direct action of

366   the opsin, and the $[K^+]_{extra}$ is below baseline.

367   Notably, spreading depolarisations could be induced using this same method, *in vivo*, in

368   anaesthetized adult mice expressing Halorhodopsin in pyramidal neurons. Illumination was

369   directed onto the cortical surface, having performed a craniotomy, and with the dura removed

370   to allow penetration of the electrode. In these experiments, the light fibre was out of the brain,

371   fractionally above its surface. The period of illumination required to induce CSDs varied from

372   animal to animal, but once we had established the requisite duration in an individual animal,

373   this same stimulation consistently triggered CSDs thereafter, having allowed at least 15 mins

374   recovery between events (n = 4 mice).

375   In a final set of *in vivo* experiments, we recorded CSD propagation in awake head-fixed

376   mice (n = 3) using multichannel graphene micro-transistor arrays (Masvidal-Codina et al., 2021),

377   thereby avoiding any confounders that may arise from anesthesia. We recorded events using a

378   4x4 graphene microarray placed upon the cortical surface. Data were collected at 16 bits,

379   allowing a wide dynamic range in DC recordings (without saturating the signal), while retaining

380   adequate voltage resolution. As such, these devices allow wide-bandwidth detection of

381   electrographic signals and are suitable for recording spreading depolarisations and higher

382 frequency activity concurrently (Figure 6Ci,ii respectively). We examined how Halo-induced

383 CSDs compared to those triggered by physical trauma, in this case, by a brief pin-prick to the

384 cortical surface. In these recordings, CSDs induced by the two different methods appeared

385 virtually identical at the individual transistors (Figure 6Ciii). We concluded therefore that even

386 though the method of induction differed, once established, the actual events were equivalent.

387 **Discussion**

388 The direct effect of illumination of Halorhodopsin is well established: that it drives an

389 inward movement of chloride ions, causing the cell to become hyperpolarized. This has been

390 used in many previous studies to inhibit neuronal activity. Here we show that there are also

391 indirect effects, but that these may have useful experimental value for probing neuronal

392 pathophysiological mechanisms. The first of these is that sustained Halorhodopsin activation

393 causes a lowering of extracellular K^+ levels. In all such cases, a steady state occurs when inward

394 ionic movement equals the outward movement. In normal baseline conditions, there is a

395 continuous small drift of K^+ ions out of the cell through leak channels, driven by the difference

396 between the membrane potential, E_m, and E_K. Since this is a steady state, it must be balanced

397 by continual inward movement of K^+ ions by other means, the most important of which is the

398 Na^+-K^+ ATPase (Larsen et al., 2014). During the illumination, hyperpolarization alters the leak

399 current driving force ($E_K - E_m$), reducing the outward movement of K^+ initially, and even

400 reversing its direction (to an inward movement) once E_m drops below E_K. The time course of

401 the K^+ redistribution is likely to have a complex relationship to the action of the Halorhodopsin

402 pump, since it is affected both by its hyperpolarizing, and also its chloride-loading, actions; the

403 latter effect will increase K^+ extrusion via KCC2, even during the period of illumination, and so

404 counter the K^+ entry previously mentioned. There may further be redistribution of K^+ through

405 the glial network that could affect how quickly the system reaches a steady state.

406 A consequence of this drop in $[K^+]_{extra}$ is a measurable reduction in excitability of non-

407 expressing neurons in the local vicinity. This consequence is important to recognize, when

408 interpreting the inhibitory consequences of Halorhodopsin, because not all the effects may be

409 attributable to the specific subpopulation of neurons expressing the opsin. Having said that, it

15

410 is also pertinent that our experiments used tissue with Halorhodopsin expressed in the

411 pyramidal population, which constitutes about 80% of the neuronal population, in murine

412 neocortex. The effect is likely to be far weaker with sparse expression patterns, and also will

413 critically depend upon the intensity of illumination.

414 We further show that following cessation of Halorhodopsin stimulation, there was a very

415 marked rebound rise in $[K^+]_{extra}$. This likely constitutes, in part, a return to the extracellular

416 space of some of the K^+ that had entered the cell. The size of the overshoot beyond resting

417 $[K^+]_{extra}$ indicates that this is further boosted by cells extruding K^+ coupled to Cl^- extrusion,

418 mediated by KCC2, as has shown in several previous publications following intense GABAergic

419 activation (Viitanen et al., 2010). The rebound $[K^+]_{extra}$ may also contribute to the excess

420 network excitability following Halorhodopsin, that has previously been noted (Alfonsa et al.,

421 2015; Raimondo et al., 2012).

422 An interesting, and unanticipated finding was the final effect, which is that protracted,

423 intense activation of Halorhodopsin proves to be a very reliable means of triggering CSDs, both

424 *in vitro* and *in vivo*. The significance of this new way of triggering CSDs is that it differs

425 fundamentally from what has been a prevailing hypotheses of CSD initiation: the critical point is

426 that instances of CSDs arising during the actual period of illumination demonstrate that they

427 can develop from a point where all the pyramidal cells are hyperpolarized and the $[K^+]_{extra}$ is

428 actually below baseline levels (i.e. < 3.5mM). CSDs are thought to underlie spreading migraine

429 auras (Lauritzen et al., 2011), and have also been described following both ischaemic and

430 haemorrhagic strokes (Dreier, 2011; Lemale et al., 2022), and seizures (Dreier and Reiffurth,

431 2015; Hablitz and Heinemann, 1989; Mody et al., 1987; Tamim et al., 2021). They were first

432 described by Leao (Leao, 1944, 1947), with the early realization that they could be triggered by

433 increasing $[K^+]_{extra}$, leading to the idea that a strong depolarization was the means of induction

434 and propagation. Latterly, a more nuanced view is evolving (Dreier and Reiffurth, 2015; Lemale

435 et al., 2022; Somjen, 2004), taking into account other ways in which CSDs may be triggered,

436 such as raised osmotic tension within neurons. Our main findings are consistent with this

437 hypothesis, by describing a process whereby a continuous electrogenic pump, driving first Cl^-,

438 and secondarily K^+ ions into neurons, triggers the CSD, but fall short of a conclusive proof of the

439 theory. New experimental models, however, often provide the impetus for new insights, and
440 we suggest that Halorhodopsin induction could fuel research into how CSDs arise naturally. One
441 important instance is where CSDs arise from seizure activity. Persistently intense neuronal
442 activity, as occurs during seizures, results in a large net movement of ions into cells, the
443 opening of pannexin channels in neuronal membranes (Thompson et al., 2008), allowing
444 movement of large molecules such as fluorescent dyes, and likely also allowing water to follow.
445 This, in turn, leads to cell swelling and a substantial drop in the extracellular space (Andrew and
446 MacVicar, 1994; Iwasa et al., 1980; Somjen, 2004). Indeed, the change in the tissue impedance
447 due to this redistribution can be used to characterize the spatial-temporal extent of
448 pathological discharges (Witkowska-Wrobel et al., 2021). An important research question
449 remains regarding the relationship of CSDs with seizure activity (Dreier and Reiffurth, 2015).
450 Superficially, seizure activity and Halorhodopsin activation appear rather different in terms of
451 the effects on membrane potential, but share an important feature that in both, there is a net
452 influx of ions into neurons.

453   **Figures and Table**

454   **Figure 1. Activation of Halorhodopsin in principal cell class induces strong modulation of**
455   **extracellular [K$^+$], during and after illumination.**

456   (A) Recordings of extracellular [K$^+$] in brain slices in which either Halorhodopsin (blue, top), was
457   expressed in all pyramidal cells, or in a slice with no opsin expression (black, bottom). Yellow
458   bar represents the time of illumination (90s period). Light activation of Halorhodopsin induced
459   a very marked reduction in [K$^+$]$_{extra}$, followed by an even larger, rebound increase. . Brain slices
460   without opsins showed no light induced fluctuations in [K$^+$]$_{extra}$. (B) The peak extracellular [K$^+$],
461   after the end of the period of Halorhodopsin activation (mean peak = 7.98mM).

462   **Figure 2. The main movement of K$^+$ ions from the extracellular space, induced by opsin**
463   **activation is through K$^+$ channels.**

464   (A) Recordings of extracellular [K$^+$] during a period of Halorhodopsin activation in the presence
465   of TTX and additionally TEA and Ba^{2+}, to block different K$^+$ channels. Note the far smaller
466   change in extracellular [K$^+$] when K channels were blocked.   (B) Measurements of the minimum
467   level of extracellular [K$^+$] in the different pharmacological conditions (p = 0.0021 for TTX vs TTX
468   + TEA and p = 0.0015 for TTX vs TTX / TEA vs TTX / TEA / Ba^{2+}, Kruskal-Wallis test with a Dunn's
469   multiple comparisons test).

470

471   **Figure 3. The light-induced drop in extracellular [K$^+$] indirectly affects the membrane potential**
472   **and excitability of cells that do not express Halorhodopsin.**

473   (A) Whole cell current clamp recordings of a pyramidal cell (black), a fast-spiking interneuron
474   (red) and a glial cell, made from neocortical brain slices prepared from mice which express
475   Halorhodopsin in all pyramidal cells, but not in other cell classes. In each case, intense light
476   activation was delivered diffusely to an area of several hundred microns around the cell
477   location, through the microscope objective.   Panel Ai illustrates the very large amplitude
478   hyperpolarisation in the pyramidal cells (the baseline Em for the three recordings are
479   superimposed, for ease of comparison of the light induced effect; pyramidal E$_m$ = -63 mV;
480   interneuronal E$_m$ = -70.5 mV; glial E$_m$ = -68.5mV), and the far smaller effect on the non-

18

481 expressing cell classes, while Aii shows the same plots normalised to the maximal drop, in order

482 to illustrate the difference in time course of the effects on E_m. (B) Bar charts showing the

483 difference in the amplitude (Bi, $p = 7.2 \times 10^{-14}$; Student's t-test) and the time to peak (Bii, $p = 3.2$

484 $\times 10^{-10}$; Student's t-test) of the hyperpolarisation in the Halo-expressing pyramidal cells (n = 23),

485 and the non-expressing interneurons (n = 15).

486

487 **Figure 4. Indirect suppression of firing in non-expressing, fast spiking interneurons.**

488 (A) Firing response to current injection (200pA, 1s) in a fast-spiking interneuron that did not

489 express Halorhodopsin, in control conditions (black) or while there was concurrent activation of

490 Halorhodopsin in the local pyramidal cells. (B) Input-output (IO) functions of a different fast-

491 spiking interneuron, plotted as the total number of action potentials during the 1s period of

492 current injection (Bi), or as the maximal instantaneous firing rate (reciprocal of the shortest

493 interspike interval) (Bii). In both cases, the IO function was derived without illumination (black)

494 and then with concurrent activation of Halorhodopsin in the pyramidal population (red, "light").

495 (Ci) The IO function (see methods). (Cii) Paired (light / no light) values of the half-maximal

496 current (I_{50}) for each cell, and the same data, normalised to the no-light I_{50} ($p < 10^{-6}$, paired t-

497 test).

498

499 **Figure 5. Halorhodopsin-induced K^+ redistribution involves KCC2 and voltage-activated K^+**

500 **channels.**

501 (A) Recordings of $[K^+]_{extra}$ during and after a 90s period of Halorhodopsin activation (yellow bar)

502 in the presence of the Na^+ channel blocker, TTX (blue, top), or the KCC2 blocker, VU0463271

503 (red, middle), or the blocker of voltage-gated K^+ channels, TEA (black, bottom). (B) The peak

504 $[K^+]_{extra}$, after the end of illumination, for the three different drugs ($p = 0.0037$ for TTX vs TTX +

505 VU and $p = 0.0064$ for TTX vs TTX + TEA, Kruskal-Wallis test with a Dunn's multiple comparisons

506 test).

507 (C) The time to the peak $[K^+]_{extra}$, following a 90s period of Halorhodopsin activation, with and

508 without the presence of the KCC2 blocker, VU0463271. Blockade of KCC2 causes a significant

509 delay in the time to the peak ($p = 0.0014$, Two-tailed paired t-test). All experiments were

510 performed in the presence of the Na^+ channel blocker, TTX, to exclude any contribution to the

19

511   [K$^+$] fluxes made by rebound neuronal spiking. (D) Similar measurements made with and

512   without the voltage-gated K$^+$ channel blocker, TEA, showing no significant effect.

513

514 **Figure 6. Prolonged activation of Halorhodopsin in pyramidal populations can trigger cortical**

515 **spreading depolarisations.**

516 (A) Concurrent recordings of extracellular K^+ concentration (Ai), and the local field potential

517 (Aii), during a 90s activation of Halorhodopsin in the pyramidal population, in which a CSD

518 event developed soon after the illumination period ended. (B) The $[K^+]_{extra}$ at the start of the

519 CSD for the different drug groupings (Bi), and its time of initiation (Bii) relative to the end of a

520 90s long period of Halorhodopsin activation (each data point is a single brain slice). (C) Example

521 traces of a CSD triggered *in vivo*, recorded using a graphene multi-transistor array, which

522 started while illumination was still on-going (illumination was started ~250s prior to the start of

523 the event). The top panel shows both low (Ci) and high (Cii) frequency components, to

524 illustrate how these different bandwidths can help distinguish between depolarization (the

525 voltage deflection in Ci) and depression (the reduced high frequency component in Cii). (Ciii)

526 Illustration of the propagation across multiple electrodes. The dotted line is aligned to the

527 maximal deflection of the leading electrode, to help visualize the relative delays of onset in the

528 other electrodes. A second CSD was triggered locally by a pin-prick injury, and recorded through

529 the same array. The right panel shows the overlaid electrophysiological signatures of the two

530 events, recorded through the different transistors, to illustrate the similar time course for the

531 Halo- and pinprick induced events, at each individual site (note that for the right panels, we

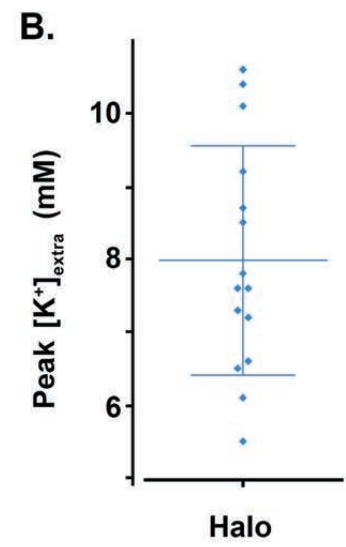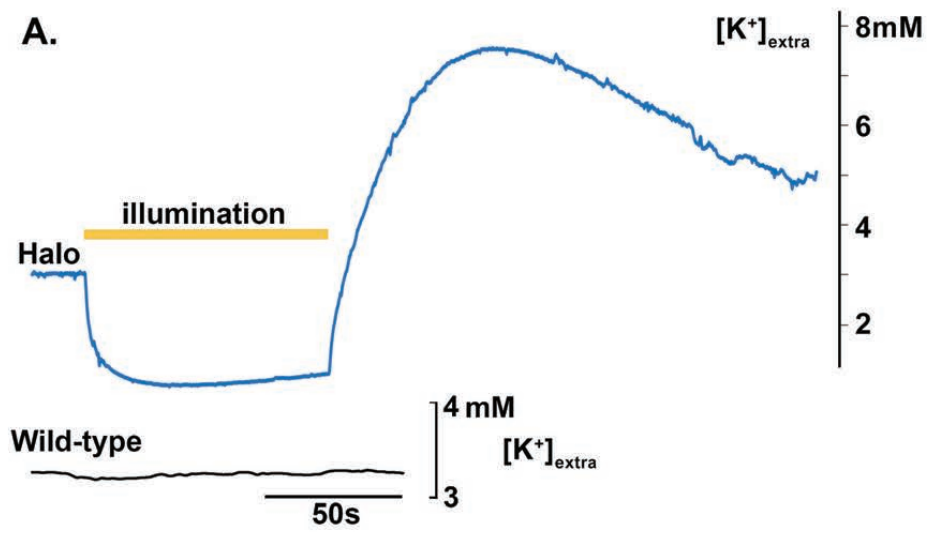532 aligned the recordings to their start; Scale bars, 50s, 50μV).

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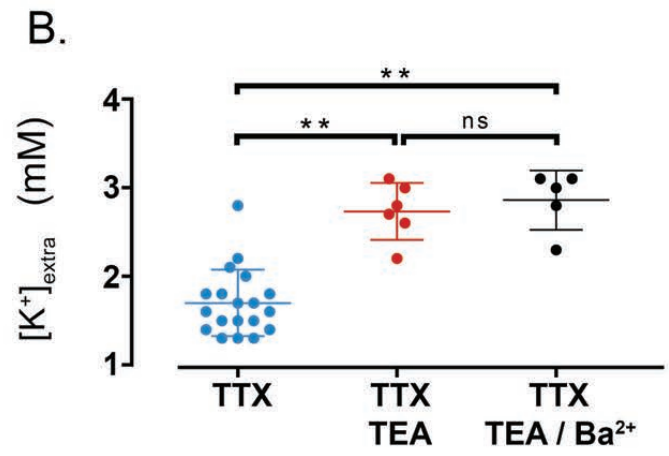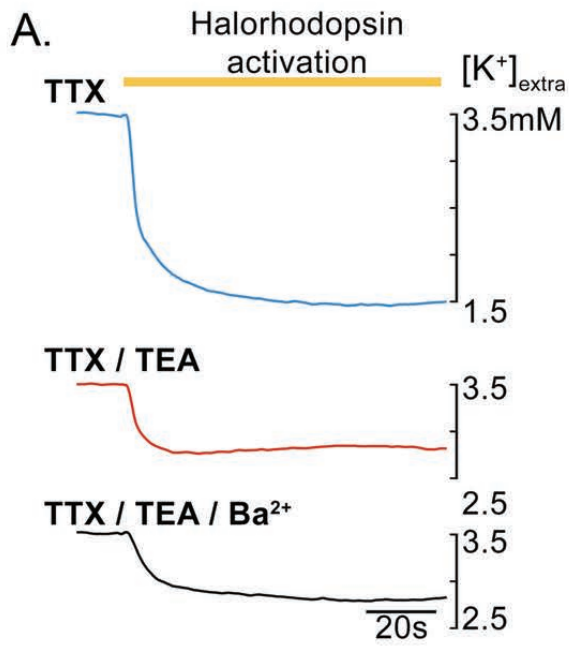572 and extracellular ions. Physiol Rev *75*, 689-723.

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624

A.

illumination

Halo

Wild-type

$[K^+]_{extra}$ 8mM

6

4

2

4 mM
$[K^+]_{extra}$
3

50s

B.

Peak $[K^+]_{extra}$ (mM)

10

8

6

Halo

A.

Halorhodopsin activation

TTX

$[K^+]_{extra}$

3.5mM

1.5

TTX / TEA

3.5

2.5

TTX / TEA / Ba^{2+}

3.5

2.5

20s

B.

$[K^+]_{extra}$ (mM)

4

3

2

1

**

**

ns

TTX

TTX
TEA

TTX
TEA / Ba^{2+}

Ai. Direct / indirect effects on Em

Halorhodopsin activation

200ms

5mV

Indirect effect
Interneuron / Glia

Direct effect
Pyramidal cells

Aii.

200ms

Bi.

p ≪ 0.001

Hyperpolarisation (mV)

20

10

0

Halo   No Halo

Bii.

p ≪ 0.001

Time to peak (s)

1

0.5

0

Halo   No Halo

A

Fast-spiking interneuron
No Halorhodopsin expression

Current injection, no light

200ms

20mV

Pyramidal Halorhodopsin activation

Current injection

Bi.

Ci

F_{max}

Firing
rate

I_{50}

Current

Bii

Cii

Ciii

Ai. *In vitro*

$[K^+]_{extra}$

mM

50

Halorhodopsin

Aii
LFP

50s

2mV

Bi. Starting $[K^+]_{extra}$

$[K^+]_o$ (mM)

20

3.5

0

▲	No drugs
▲	TTX
▲	TTX / VU
▲	TTX / TEA
▲	TTX / TEA / Ba
▲	TTX / APV / NBQX

Bii.

0   10

Time after illumination (s)

C. *In vivo* CSDs

Ci. Low frequency

1.2mm
separation

5mV
50s

Cii. High frequency

0.5mV
50s

Ciii. Ongoing
illumination

Pin-prick

Overlay

50s

50μV