Optogenetic ion pumps differ with respect to the secondary pattern of K⁺ redistribution

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Abstract

We recently reported that strong activation of the optogenetic chloride pump,

Halorhodopsin leads to a secondary redistribution of K^+ ions into the cell, through tonically

open, "leak" K^+ channels. Here we show that this effect is not unique to halorhodopsin, but is

also seen with activation of another electrogenic ion pump, archaerhodopsin. The two opsins

differ however in the size of the rebound rise in extracellular potassium, $[K^+]_o$, after the end

of activation, which is far larger with halorhodopsin than for archaerhodopsin activation.

Multiple linear regression modelling indicates that most of the variance in the post-

illumination surge in $[K^+]_o$ was explained by the type of opsin, and almost nothing by the size

of the preceding, illumination-induced drop in $[K^+]_o$. These data provide additional support

for the hypothesis that intense chloride-loading of cells, as occurs naturally following intense

bursts of GABAergic synaptic bombardment, or artificially following halorhodopsin activation,

is followed by extrusion of both Cl^- and K^+ coupled together. We discuss this with respect to

the pattern of $[K^+]_o$ rise that occurs at the onset of seizure-like events.

Introduction

The development of optogenetics techniques and tools have presented new ways of manipulating neuronal excitability (Deisseroth, 2015), using either light activated ion channels (Boyden et al., 2005) or electrogenic ion pumps (Chow et al., 2010; Zhang et al., 2007). The real power of these remarkable tools is that they may be targeted very precisely to specific subsets of cells, or even to subcellular domains, allowing experimental control to be delivered at these sites. The induced ion movements, however, do alter the internal and external milieu, meaning that there may be unintended secondary effects both at and away from the site of expression. These, though, may be put to good use for exploring neuronal physiology and pathology, so it is worth documenting these effects.

Halorhodopsin was the first optogenetic protein to be used for inhibiting neurons, using light to drive chloride into neurons to hyperpolarize them (Zhang et al., 2007). We recently showed that this light-activated chloride movement has several interesting secondary effects (Parrish et al., 2023). Firstly, the hyperpolarization of membrane potential alters the balance between the electrical and chemical gradients for other ions; since the open "leak" channels at rest are mainly K^+ channels, there follows a redistribution of K^+ ions into expressing cells, causing the extracellular K^+ concentration, $[K^+]_o$, to drop. Consequently, there is a negative shift in membrane potential in other non-expressing neurons, with a measurable drop in their excitability (an "off-target" inhibition that may be important to recognize when interpreting optogenetic experiments). Most surprising, though, was that activation of halorhodopsin triggered cortical spreading depolarization (CSD) events that occurred even during periods of ongoing illumination, when most neurons were hyperpolarized and $[K^+]_o$ was below baseline levels. Interestingly, on the trials where CSD were not induced and once illumination ended, there was then a large rebound surge in $[K^+]_o$.

Archaerhodopsin, another commonly used inhibitory optogenetic ion pump, delivers hyperpolarization of neurons in a different way, by driving protons out of the cell (Chow et al., 2010). As such, archaerhodopsin activation should show some of the secondary effects of halorhodopsin, but not others. It has already been shown that activation of halorhodopsin, but not archaerhodopin, induces a pronounced chloride-loading of neurons that may persist for

many seconds (Alfonsa et al., 2015; Raimondo et al., 2012). Since Cl⁻ clearance is achieved

primarily by coupling to K^+ extrusion, through the electroneutral chloride-cation cotransporter,

KCC2 (Kaila et al., 2014; Rivera et al., 1999), the degree of chloride loading will influence the

amplitude of the post-illumination surge in $[K^+]_o$. To examine these differences, we present

recordings of $[K^+]_o$ in neocortical brain slices in which archaerhodopsin is expressed widely in

the pyramidal population, to show that it shares with halorhodopsin the effect of inducing an

inward movement of K^+ ions during the period of illumination. In contrast, the post-

illumination rebound surge in $[K^+]_o$ following archaerhodopsin activation appears much smaller

than for halorhodopsin.

Methods

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

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

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

were used for experimentation.

Archaerhodopsin viral injections: C57/B6 adult mice were injected with AAV9.CAG.ArchT,

purchased from the UPenn vector core. For these adult injections, done at 8-12 weeks of age,

animals were anesthetized by ketamine–methoxamine intraperitoneal injection and placed in a

stereotaxic head holder (David Kopf Instruments). Injections were made at three to four

locations in an anterior–posterior row in one hemisphere, 1.5–2 mm lateral to the midline and

1–0.4 mm deep to the pia (0.6 µl, injected over 15 min).

Slice Preparation: We used both wild-type C57/B6 mice, and also mice expressing eNpHR3.0

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

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

eNpHR3.0/EYFP domain (Jackson Laboratory, Stock 014539; both maintained on the C57/B6

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

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

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

Mice were first decapitated, brains were removed and placed in cold cutting solution containing

(in mM): 3 MgCl2; 126 NaCl; 2.6 NaHCO3; 3.5 KCl; 1.26 NaH2PO4; 10 glucose. 400 μm

horizontal sections were made on a Leica VT1200 vibratome (Leica Microsystem, Germany).

Slices were stored at room temperature, in an interface holding chamber for 1 – 4 hours prior

to experimentation. Solutions were bubbled with carboxygen (95% O2 and 5% CO2) in artificial

cerebro-spinal fluid (aCSF) containing (in mM): 2 CaCl2; 1 MgCl2; 126 NaCl; 26 NaHCO3; 3.5 KCl;

1.26 NaH2PO4; 10 glucose.

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

recording chamber. Slices were placed in the recording chamber perfused with either aCSF,

supplemented in different experiments with different combinations of the following drugs:

1μM Tetrodotoxin (TTX) (Abcam), 10mM tetraethylammonium (TEA) (Sigma), as indicated in

the results section. Recordings were obtained using aCSF-filled ~1-3MΩ borosilicate glass

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

Extracellular potassium $[K^+]_o$ was measured using single-barrelled K^+-selective microelectrodes.

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

and the glass was exposed to vapor of dimethyl-trimethyl-silylamine (Sigma-Aldrich), baking at

200^0 C for 40 minutes. The pipettes were then backfilled with aCSF. A short column of the K^+

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

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

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

build amplifier, we calculated the $[K^+]_o$ from calibration recordings made in an open bath, using

sudden increments in $[K^+]_o$. This provided a scaling factor S, of 55-59mV, where the K^+

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

follows: $[K]_o = [K]_{o.baseline} 10^{V(t)/S}$ $[K]_{o.baseline}$ for our experiments was 3.5 mM. The temperature of

the chamber and perfusate was maintained at 33-36 ⬚C using a closed circulating heater

(FH16D, Grant instruments, Cambridge, UK). The solutions were perfused at the rate of 3

mls/min by a Watson Marlow 501U peristaltic pump (Watson-Marlow Pumps Limited, Cornwall

UK). For the collection of the HaloRhodopsin and ArchT data, the direct current local field

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

These waveform signals were digitized with a Micro 1401-3 ADC board (Cambridge Electronic

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

sampled at 10 kHz. Recordings were analysed using a custom-written code in Matlab2015b

(Mathworks, MA, USA).

Optogenetic illumination for extracellular recordings: Optogenetic illumination was delivered

for 90s continuous periods, using a Fiber-coupled LED light at 565 nm (Thorlabs, M565F3) and

driven by an LED driver (Thorlabs, LEDD1) placed in trigger mode. Light output was measured at

an average of 9 mW. The LED light was positioned just above the superficial layers of the

neocortex for the slice experiments and pointed toward the deep layers, where the

extracellular recordings were performed.

Statistics: Statistical analysis of electrophysiology was performed using GraphPad Prism

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

(MATLAB, *ranksum.m*) or multiple linear regression modelling (*fitlm.m*). Figures of

electrophysiology traces were created in Matlab2015b or 2018b (Mathworks, MA, USA).

Results

Activation of archaerhodopsin causes secondary redistribution of K^+ ions

We previously reported that activation of the optogenetic chloride pump, halorhodopsin,

induced a secondary ionic redistribution of K^+ ions into the cell during the period of activation,

followed by a surge in $[K^+]_o$ after illumination ended (Parrish et al., 2023).   The inward

movement of K^+ appears to pass through leak K^+ channels, whereas the post-illumination

outward movement included a KCC2-sensitive component, implying that it was driven in part by

the need to remove Cl^- ions that had been pumped into cells. We reasoned therefore that

these effects may be replicated in part by activation of other optogenetic pumps. To test this

prediction, we recorded the pattern of K^+ redistribution associated with activation of the proton

pump, archaerhodopsin (Figure 1). Widespread archaerhodopsin expression in the pyramidal

cell population was achieved by injecting a viral vector carrying the floxed gene into mice

expressing Cre-recombinase under the Emx1 promoter (Gorski et al., 2002).   During periods of

illumination, we recorded a highly significant drop in $[K^+]_o$ in brain slices expressing

archaerhodopsin (Figure 1), but not in brain slices from wild-type mice. This K^+ redistribution

was almost entirely eliminated by blockade of plasmalemmal K^+ channels, using TEA. In both

respects, archaerhodopsin mimicked the effect we had previously reported in halorhodopsin-

expressing brain slices. In our preparations, the amplitude of the K^+ redistribution was larger in

the halorhodopsin-expressing brain slices, which might reflect the degree of hyperpolarization

achieved.

At the end of the illumination period, we previously reported that halorhodopsin-

expressing brain slices showed a very large rebound surge in $[K^+]_o$ (7.98 $\pm$ 0.41 (mean $\pm$ s.e.m),

range 5.5 - 10.6 mM; 95% confidence interval = 7.18 - 8.78 mM). In contrast, the post-

illumination surge in archaerhodopsin-expressing slices was significantly above the 3.5mM

baseline level (4.12 $\pm$ 0.12 mM, range 3.4 - 5.1 mM; 95% confidence interval = 3.86 - 4.38 mM),

but significantly smaller than occurred with the halorhodopsin slices (ArchT vs Halorhodopsin, p

= 0.0001, two-tailed Mann-Whitey test) (Figure 2).

For both opsins, a component of the rebound surge will be extrusion of K^+ ions that

moved into the neurons during the period of illumination; since the illumination-induced

inward movement was greater in our halorhodopsin group (possibly due to stronger

expression; during Halorhodopsin activation, the range of the drop in of drop in $[K^+]_o$ = 1.2 - 2.2

mM below baseline (3.5mM); during ArchT activation, the range = 0.5 – 1.3 mM below

baseline), one possibility is that the larger rebound in that group simply reflected this

difference. One approach to test this would have been to adjust expression patterns or

illumination levels to try to match the amplitude of the illumination-induced drop for the two

different opsins, but the halorhodopsin experiments were performed first, and we were not

able to achieve the same degree of K^+ redistribution with the archaerhodopsin. Instead, we

pooled the datasets and used multilinear regression modelling of the $[K^+]_o$, with the predictive

variables being the illumination-induced drop, and the type of opsin expressed, to assess their

relative contributions to the post-illumination surge (Table 1). We found no correlation

between the amplitude of the illumination-induced drop in $[K^+]_o$, and the rebound surge in $[K^+]_o$

for either experimental group individually (Table 1; Figure 3), whereas across the entire data

set, virtually all the variance was explained by the type of opsin, and almost nothing by the size

of the preceding, illumination-induced drop in $[K^+]_o$. This is consistent with our previous data

showing that a large element of the post-illumination surge in halorhodopsin slices was

sensitive to blockade of KCC2, indicating that the surge is boosted significantly by being coupled

also to Cl^- extrusion in the halorhodopsin experiments.

A final point of difference is that in none of the archaerhodopsin experiments did we

observe cortical spreading depolarization events; in contrast, these were triggered quite readily

in halorhodopsin-expressing tissue (not illustrated, but examples are found in (Parrish et al.,

2023)).

Discussion

Archaerhodopsin and halorhodopsin have both been used to inhibit neuronal activity, but

do so by driving ionic movement in opposite directions: halorhodopsin by driving Cl^- inwards,

and archaerhodopsin by driving H^+ out. We show here that this is further reflected by

differences in the pattern of secondary effects, consistent with our interpretation of these

effects reported in a previous study of halorhodopsin. The two optogenetic pumps share the

effect of hyperpolarizing neurons, and consistent with this, both show a secondary drop in $[K^+]_o$,

caused by K^+ moving into the stimulated cells through leak channels driven by the altered

electro-chemical balance for K^+ during the period of opsin activity. The rebound, post-

illumination surge in $[K^+]_o$, however, appeared much smaller for archaerhodopsin, even

allowing for the fact that the expression levels of the two opsins were not exactly equivalent

(halorhodopsin expression was achieved by cross-breeding Emx1 and floxed halorhodopsin

mouse lines; archaerhodopsin expression was obtained by injecting Emx1 mice with a viral

vector carrying the floxed archaerhodopsin gene) and so we were unable to achieve the same

degree of K^+ loading during the periods of illumination. Across the entire dataset, pooled from

both halorhodopsin- and archaerhodopins-expressing brain slices, virtually all the variance in

the peak amplitude of the rebound $[K^+]_o$ was explained by the choice of opsin, and very little by

the within-illumination drop.

The much larger rebound surge in $[K^+]_o$ with halorhodopsin is, in fact, in line with what

can be predicted from the different actions of the two opsin pumps. Halorhodopsin loads the

cell with chloride, whereas archaerhodopsin consumes protons from the abundant intracellular
buffer species. Even if halorhodopsin and archaerhodopsin current amplitudes and durations
were identical, only halorhodopsin loads the cell with parallel inward fluxes of K^+ and Cl^-,
leading to outward transport of KCl by KCC2, which is an electroneutral transporter capable of
restoring a normal intraneuronal Cl^- level rapidly after a Cl^- load. This pattern of ionic
redistribution is consistent with previous observations that the rebound $[K^+]_o$ surge, following
chloride-loading arising either naturally, following intense GABAergic activation (Viitanen et al.,
2010), or artificially from halorhodopsin activation (Parrish et al., 2023), is reduced by blockers
of KCC2.

These collected observations, including the much smaller surge following
archaerhodopsin activation, further reinforces the view that $[Cl^-]_i$ and $[K^+]_o$ are tightly coupled,
as seen with the large rise in $[K^+]_o$ that follows intense interneuronal activation or direct GABA
application (Chang et al., 2018; Viitanen et al., 2010). These observations warrant a
reconsideration of the nature of raised $[K^+]_o$ during epileptic seizures, which may comfortably
exceed 10mM at its peak (Hablitz and Heinemann, 1987; Librizzi et al., 2017; Raimondo et al.,
2015; Somjen, 2004). This has often been attributed solely to K^+ movement out of cells during
action potentials, as described originally by Hodgkin and Huxley (Hodgkin and Huxley, 1952).
Recordings of the ictal initiation period typically also show a significant rise in $[K^+]_o$ ahead of the
time when pyramidal cells are recruited, as is readily apparent in published recordings (Chizhov
et al., 2022; Librizzi et al., 2017). Notably, this early peak coincides with a period of very intense
interneuronal activity (Parrish et al., 2019; Trevelyan et al., 2006). A major contribution to this
early rise, therefore, is likely to be the extrusion of K^+ coupled to Cl^--clearance, following a
period of significant chloride-loading due to intense GABAergic bombardment. Recognising
these two different causes of K^+ extrusion – (1) the Hodgkin-Huxley model of action potential
firing and (2) coupled to the clearance of Cl^- is important to our understanding of ictal
propagation and recruitment to seizures (Graham et al., 2022; Trevelyan et al., 2022).

The most surprising secondary effect triggered by prolonged halorhodopsin activation
was the occurrence of CSDs, at times, arising from a state where the majority of neurons were
hyperpolarized to different degrees, and $[K^+]_o$ was below baseline levels (Parrish et al., 2023).

245 We did not observe such events in our archaerhodopsin preparations. Two possible

246 explanations suggest themselves for this difference, both consistent with our data: first that the

247 lack of CSDs in our archaerhodopsin experiments reflects the smaller shifts in K^+ (Figure 3), or

248 second that it reflects differences in the net ion flux during illumination. Halorhodopsin

249 activation results in both inward Cl^- and K^+ movement, generating a large net influx of

250 osmotically active particles. In contrast, archaerhodopsin drives an outward proton movement,

251 and the K^+ redistribution is much smaller. While this negative finding (no CSDs in our

252 archaerhodopsin experiments) represents a limited test of the hypothesized differences

253 between halorhodopsin and archaerhodopsin, the result is consistent with what else is known

254 about their different mechanisms of action.

Figure and Table Legends

Figure 1. The main movement of K^+ ions from the extracellular space, induced by opsin
activation is through K^+ channels.

(A) Redistribution of K^+, assessed by recording $[K^+]_o$ during a period of halorhodopsin (black) or
of archaerhodopsin activation (red) in the presence of TTX to block neuronal firing, and
additionally TEA to block "leak" K^+ channels. (B) Pooled data of measurements of $[K^+]_o$ for all
the halorhodopsin and archaerhodopsin recordings. For both opsins, the size of the K^+
redistribution is greatly reduced by block of voltage-gated K^+ channels (Halo vs Halo + TEA, p =
0.00076; Arch vs Arch + TEA, p = 0.0279, two-tailed Mann-Whitney test).

Figure 2. The large post-illumination surge in $[K^+]_o$ following a period of halorhodopsin
activation is not replicated by archaerhodopsin activation.

(A) Recordings of extracellular $[K^+]$ in brain slices in which either Halorhodopsin (blue, top), or
Archaerhodopsin (red, middle), was expressed in all pyramidal cells, or in a slice with no opsin
expression (black, bottom). Yellow bar represents the time of illumination (90s period). Light
activation of Halorhodopsin induced a very marked reduction in $[K^+]_o$, followed by an even
larger, rebound increase. There was also a drop in $[K^+]_o$, synchronous with activation of
Archaerhodopsin, but the rebound was negligible. Brain slices without opsins showed no light
induced fluctuations in $[K^+]_o$. (B) The peak extracellular $[K^+]$, after the end of illumination, for
Archaerhodopsin (mean peak = 4.12mM, n = 11) and Halorhodopsin activation (mean peak =
7.98mM; n = 15; comparison, p = 0.0001, two-tailed Mann-Whitey test), respectively.

Figure 3. The post-illumination $[K^+]_o$ rise does not reflect the drop in $[K^+]_o$ during illumination.
A plot of the $[K^+]_o$ rise, in excess of baseline (3.5mM), following the end of illumination versus
the drop in $[K^+]o$ below baseline that occurs during illumination for 25 different brain slices, 14
expressing Halorhodopsin (red) and 11 expressing Archaerhodopsin-T (black). Halorhodopsin
activation, using the expression systems available to us, produced a larger K^+ dip during
illumination (range 1.2-2.2mM below baseline) than did activation of ArchT (0.5-1.3mM below
baseline). The dotted lines represent the correlation for the two experimental groups,

extended to the ordinate axis. The difference in the intercepts represents the effect of chloride

loading, secondary to halorhodopsin activation, producing an excess return of K^+ to the

extracellular space. Table 1 shows the R^2 values for different linear regression models, which

show that virtually all the experimental variation is explained in terms of the two experimental

groups, and almost none by the intra-illumination $[K^+]_o$ drop.

Table 1. Linear regression model analyses of Archaerhodopsin and Halorhodopsin $[K^+]_{extra}$.

Linear regression model analyses of the data presented in Figure 3, examining different models

of the post-illumination rise in $[K^+]_{extra}$ predicted by the expression of either Halorhodopsin or

ArchT, and the degree of $[K^+]_{extra}$ drop during the period of illumination. The 3rd analysis ("$[K^+]$

drop (all)") is of the dataset with ArchT and Halorhodopsin brain slices pooled together without

distinction. This distinction is made in the 4th analysis (("$[K^+]$ drop, ArchT/Halo grouped"),

whereas the 5th analysis does not include information about the $[K^+]$ drop during illumination.

Note that the adjusted R^2 actually improves without the $[K^+]$ drop information, indicating that

the post-illumination $[K^+]$ rise is explained almost solely by the difference between the opsins.

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Table 1. Models

		R^2	Adjusted R^2	p value
[K$^+$] rise vs	[K$^+$] drop (Halo)	0.006	-0.076	0.79
	[K$^+$] drop (ArchT)	0.099	-0.001	0.35
	[K$^+$] drop (all)	0.554	0.535	$< 10^{-4}$
	[K$^+$] drop, ArchT/Halo grouped	0.724	0.699	$< 10^{-6}$
	ArchT/Halo grouped	0.721	0.709	$< 10^{-7}$