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Optical control of neuronal activity using a light operated GIRK-channel opener (LOGO)[†]

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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G-protein coupled inwardly rectifying potassium channels (GIRKs) are ubiquitously expressed throughout the human body and are an integral part of inhibitory signal transduction pathways. Upon binding of G_{βγ} subunits released from G-protein coupled receptors (GPCRs), GIRK channels open and reduce the activity of excitable cells via hyperpolarization. As such, they play a role in cardiac output, the coordination of movement and cognition. Due to their involvement in a multitude of pathways, the precision control of GIRK channels is an important endeavour. Here, we describe the development of the photoswitchable agonist **LOGO** (the Light Operated GIRK-channel Opener), which activates GIRK channels in the dark and is rapidly deactivated upon exposure to long wavelength UV irradiation. **LOGO** is the first K⁺ channel opener and selectively targets channels that contain the GIRK1 subunit. It can be used to optically silence action potential firing in dissociated hippocampal neurons and **LOGO** exhibits activity *in vivo*, controlling the motility of zebrafish larvae in a light dependent fashion. We envisage that **LOGO** will be a valuable research tool to dissect the function of GIRK channels from other GPCR dependent signalling pathways.

Introduction

G-protein coupled inwardly rectifying potassium (GIRK) channels are an integral link between metabotropic and ionotropic pathways in neurons. They constitute a subclass of inwardly rectifying potassium channels that are natively activated by G-protein coupled receptors (GPCRs) through interactions with G_{βγ} subunits.¹ One of the most important effects of GIRK activation and subsequent channel opening is the hyperpolarization of cell membranes, which immediately results in a reduction of activity in excitable cells. Therefore, neurotransmitters that target both ion channels and GPCRs, including glutamate, γ-aminobutyric acid, acetylcholine, dopamine and serotonin, can have inhibitory effects. As a consequence, GIRK channels play crucial roles in cognition, nociception, coordination, energy homeostasis and cardiac output.² Therefore, it is unsurprising that the malfunction of GIRK channels is associated with several neurological and cardiological disorders.³

GIRK channels are comprised of homo- or heterotetramers formed by the four subunits GIRK1-4.⁴ Although the expression patterns of GIRK subunits differs throughout the body, the prototypical GIRK channels of the central nervous system (CNS) are GIRK1/2 heteromers and the isoforms found in the cardiovascular system are GIRK1/4 channels.⁵ Recent breakthroughs in structural biology have identified the molecular basis for GIRK channel function, such as inward rectification and polyamine block, as well as intracellular activation by sodium ions and lipids.⁶

It has been demonstrated that GIRK channels are inhibited by a variety of drugs, as well as polyamines and barium ions.⁷ However, most of these compounds are not selective for GIRK channels.^{8,9} GIRK channels can also be activated by small molecules including volatile anesthetics,¹⁰ ethanol¹¹ and the natural product naringin.¹² Again these compounds either exhibit poor selectivity, poor potency or a combination of the two. However, the landscape of GIRK channel research was recently transformed by two small molecules, **ML297** and **VU0259369** (Fig. 1a).¹³ These compounds were shown to be the first potent and selective activators of GIRK channels bearing the GIRK1 subunit. Subtle changes to the molecular structure of **ML297** resulted in the creation of potent GIRK1 channel inhibitors, an example of which is compound **1** (Fig. 1a).¹⁴

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[†] Electronic Supplementary Information (ESI) available: Experimental procedures and characterisation data. See DOI: 10.1039/x0xx00000x

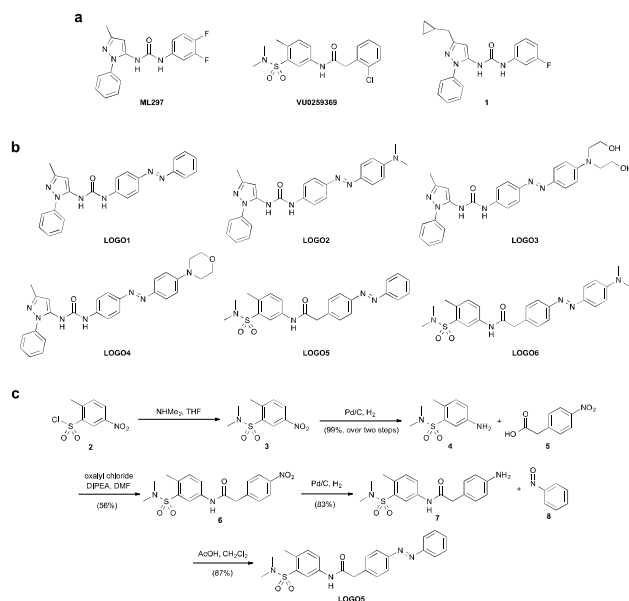


Fig. 1 Design of the photoswitchable GIRK channel agonists **LOGO1-6** using an 'azo-extension' strategy. a) Chemical structures of the GIRK channel agonists **ML297** and **VU0259369**, and the GIRK channel inhibitor **1**. b) Chemical structures of the photoswitchable GIRK agonists **LOGO1-6**. c) Synthesis of **LOGO5** using a Mills reaction.

With the first small molecular agonists of GIRK channels at our disposal, we sought to develop photoswitchable versions that can endow light control to GIRK channels.¹⁵ This approach, termed photopharmacology, is able to confer exquisite spatiotemporal control onto biological systems using the unrivalled precision of light and has previously been used to optically control ion channels,¹⁶ GPCRs,¹⁷ enzymes¹⁸ and antibiotics.¹⁹

Herein, we present **LOGO** (the Light Operated GIRK-channel Opener), an azobenzene photoswitch based on **VU0259369** that endows GIRK channels with remarkable photocontrol using UV and blue light. **LOGO** is the first example of a photochromic K^+ channel opener, as opposed to blocker,²⁰ and the first photoswitch to selectively target GIRK channels. We envisage that **LOGO** will be a valuable tool for GIRK channel research, allowing the dissection of GIRK signaling from other GPCR dependent pathways. This will further our understanding of GIRK channel function and facilitate their development as a therapeutic target.

Results and Discussion

Using the compounds **ML297** and **VU0259369** as a basis for our photoswitch design, we employed an 'azo-extension' strategy to furnish our family of **LOGOs** (Fig. 1b).²¹ In the **ML297** series, we prepared **LOGO1-4** bearing different electron-donating groups in the 4'-position of the azobenzene. In a similar fashion, **LOGO5** and **LOGO6** were derived from the compound **VU0259369**. The representative synthesis of

LOGO5 started from sulfonyl chloride **2**. Nucleophilic substitution with dimethylamine, followed by reduction of the aromatic nitro group, furnished sulfonamide **4** (Fig. 1c). An amide coupling reaction between **4** and 4-nitrophenylacetic acid (**5**) afforded nitro amide **6**, which was then reduced to the aniline **7**. Mills reaction of aniline **7** with nitrosobenzene (**8**) in the presence of acetic acid then afforded **LOGO5**. The synthesis of **LOGO1-4** and **LOGO6** proceeded in a similar fashion and is discussed in the supporting information (Fig. S1).

With our small library of **LOGOs** in hand, we set about determining their photoswitching properties using UV-Vis spectroscopy. From these experiments, we found the optimum wavelengths for the photoswitching of each of the compounds (Fig. S2). This information was then applied to our initial compound evaluation using patch clamp electrophysiology in HEK293T cells expressing GIRK1/2 channels (Fig. 2a). Beginning with **LOGO1**, we found that it could efficiently open GIRK1/2 channels as its *trans*-isomer. However, upon isomerisation to its *cis*-isomer using UV light (390 nm), only a small decrease of the current in the patch clamp experiment was observed. Based on these results, we postulated that **LOGO2-4** would endow the GIRK channels with more light dependent current because of the increased steric interactions between the ligand-binding site of the GIRK channel and the substituents in the 4'-position of the azobenzene.

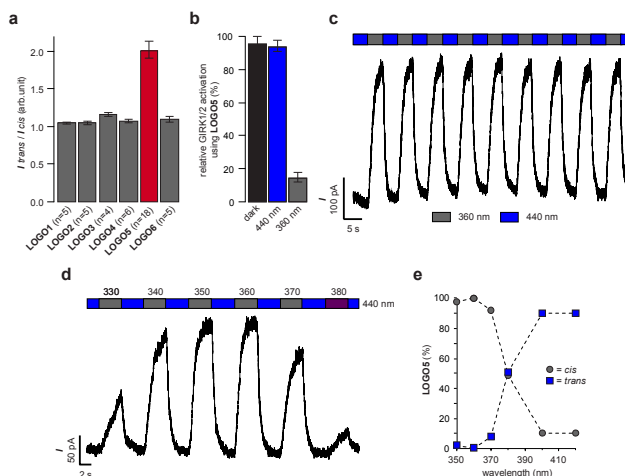


Fig. 2 Whole-cell patch clamp electrophysiology of HEK293T cells expressing GIRK1/2 channels. a) Evaluation of **LOGO1-6** ($1 \mu\text{M}$) revealed that **LOGO5** displayed the best photoswitching behaviour. b) **LOGO5** ($10 \mu\text{M}$) is almost as efficacious as **VU0259369** ($10 \mu\text{M}$) in the dark and under blue light (440 nm) illumination ($n=4$ cells). c) The photoswitching of **LOGO5** was shown to be reversible over many switching cycles in voltage-clamp mode. d) Action spectrum of **LOGO5** ($1 \mu\text{M}$) showing the accurate control of cellular currents by toggling the illumination wavelength between 440 nm and 330-380 nm. e) Photoswitching of **LOGO5** in DMSO-d_6 solution using ^1H NMR spectroscopy (see ESI for details). Values represent mean \pm SEM.

Although a small increase in the photoswitching of the GIRK channel was observed, it was still minor compared to the overall activation of the channel. We therefore turned our attention to **LOGO5** and **LOGO6**, which were derived from **VU0259369** instead of **ML297**. Pleasingly, we discovered that **LOGO5** not only activates GIRK1/2 channels, but is an excellent photoswitch (Fig. 2a). We next evaluated the red-shifted photoswitch **LOGO6**, but to our surprise the photocurrent achieved was drastically reduced compared to that of **LOGO5** (Fig. 2a). Therefore, our future investigations and biological evaluation focused on **LOGO5**. Overall, analysis of **LOGO1-6** demonstrated that potent and efficacious ligands could be easily obtained by extending portions of known agonists (Fig. 1a) to include azobenzenes. By contrast, there is a narrow 'structural window' for photoswitchable agonists, that is compounds that dramatically change their efficacy upon light-isomerisation.

Having determined that **LOGO5** is our optimal photoswitch for the control of GIRK1/2 channels, we investigated its properties in detail using patch clamp electrophysiology and NMR spectroscopy. Firstly, we established that in the dark **LOGO5** (10 μM) is almost as efficacious as **VU0259369** (10 μM). The same is true under blue light illumination, where **trans-LOGO5** also predominates (Fig. 2b). Photoisomerisation using UV light (360 nm) then showed that **cis-LOGO5** is considerably less active on GIRK1/2 channels (Fig. 2b and S3). Reversible photoactivation with **LOGO5** is very robust, with no noticeable loss of photocurrent over several switching cycles in voltage-clamp mode (Fig. 2c). Highly reproducible photoswitching of **LOGO5** was also obtained when operating in current-clamp mode (Fig. S4). Next we determined the action spectrum of **LOGO5**. When the wavelength was switched between blue light (440 nm) and different wavelengths of UV light (330–380 nm), we observed large differences in current (Fig. 2d). The minimal inward current was observed at 360 nm, whereas illumination at 330, 340, 350 and 370 nm gave more inward current. This corresponds to the photostationary states (PSS) of **LOGO5** in DMSO- d_6 solution as determined by ^1H NMR spectroscopy (Fig. 2e). Here 100% **cis-LOGO5** content was observed at 360 nm, whereas 370 and 380 nm etc. gave increasing **trans-LOGO5** content. Hence the concentration of active **trans-LOGO5** can be controlled with the colour of the light (photodosing).²² The kinetics of **LOGO5** activation were shown to be fast, with illumination at 440 nm for 1 second resulting in almost complete opening of the GIRK1/2 channel (Fig. S6).

Next, we monitored the action of **LOGO5** on GIRK1/2 channels expressed in HEK293T cells by elevating the holding potential in 10 mV steps from -100 mV to $+50$ mV. During each voltage step illumination was switched between 360/440/360 nm (Fig. 3a). Again, we found that the **cis-isomer** of **LOGO5** has very little activity at GIRK1/2 channels, whilst the **trans-isomer** of **LOGO5** shows strong GIRK1/2 activation. The amplitude of photocurrents at different holding potentials reflects the inward rectification of GIRK1/2 channels (Fig. 3b). Given the high concentration of K^+ ions (50 mM), the reversal potential was found to lie between -30 and -20 mV.

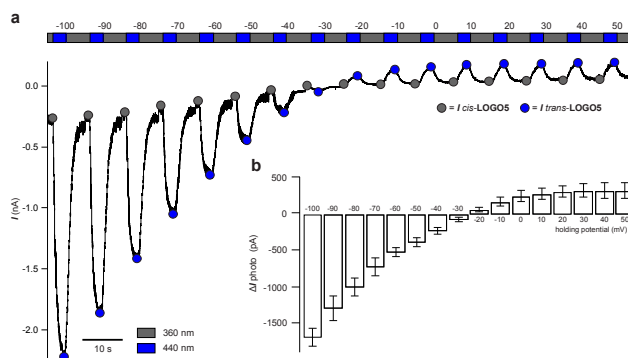


Fig. 3 Inward rectification of GIRK1/2 channels in the presence of **LOGO5** (10 μM) in voltage-clamp mode. a) The holding potential was elevated in 10 mV steps from -100 mV to $+50$ mV illuminating with 360/440/360 nm at each step to give the **cis-isomer** of **LOGO5** (360 nm) and the **trans-isomer** of **LOGO5** (440 nm). Respective peak currents are indicated as grey and blue circles. b) Averaged photocurrent amplitudes ($\Delta I = I_{\text{trans-LOGO5}} - I_{\text{cis-LOGO5}}$). Inward rectification is also translated into the photocurrents ΔI ($n=3$ cells). Values represent mean \pm SEM.

To evaluate the activity of **LOGO5** at different GIRK channel subtypes, we next employed the thallium flux assay technique (Fig. 4).²³ We found that **LOGO5** is capable of activating GIRK channels that contain the GIRK1 subunit with similar potency and efficacy (GIRK1/2: $\text{EC}_{50} = 1.2 \pm 0.09$ μM , $\%E_{\text{max}} = 95 \pm 5.0$; GIRK1/4: $\text{EC}_{50} = 1.9 \pm 0.10$ μM , $\%E_{\text{max}} = 101.5 \pm 6.4$; Table S1). However, **LOGO5** is unable to activate homodimeric GIRK2 channels, even at high concentrations.

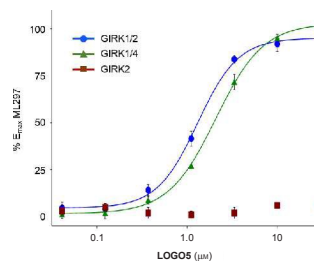


Fig. 4 Potency, efficacy and selectivity of **LOGO5**. Shown are fits to representative data obtained from testing multiple concentrations of **LOGO5** on cell lines stably expressing GIRK1/2 (blue circles), GIRK1/4 (green triangles) and GIRK2 (red squares). The measured potencies for GIRK1/2 and GIRK1/4 were 1.2 ± 0.09 μM and 1.9 ± 0.1 μM , respectively. **LOGO5** was inactive on cells that only express GIRK2. Efficacy values were normalised to the maximum activity observed using **ML297** (10 μM) on GIRK1/2-expressing cells. Error bars represent \pm SEM obtained from triplicate wells. Reported potency values represent the SEM obtained from three independent experiments.

Having demonstrated **LOGO5** on HEK293T cells, which heterologously express GIRK1/2 channels, we next wondered if this tool could be used to control excitable cells that natively express GIRK channels. To test this, we turned to dissociated rat hippocampal neurons which have been shown to express a variety of GIRK subunits.²⁴ After the application of **LOGO5** (20 μM), pyramidal neurons exhibited large membrane hyperpolarization (15.8 ± 2.5 mV, $n=7$ cells) in response to illumination with blue light (450 nm), which was reversed with UV light (360 nm) (Fig. S7a). Photoswitching of **LOGO5** in both directions was stable in the dark over tens of seconds while in current-clamp mode (Fig. 5a) indicating that constant illumination of the sample is not required. This bistability is characteristic of 'normal' azobenzenes.²⁵ In voltage-clamp mode at -60 mV, blue light (450 nm) illumination induced an outward current (50.3 ± 4.8 pA, $n=4$ cells), consistent with activation of a potassium conductance (Fig. S7b). Most importantly when at depolarised potentials, **LOGO5** was able to reversibly silence action potential firing under blue light (450 nm) illumination (Fig. 5b). Illuminating with UV light (360 nm) restored action potential firing. The photoswitching of **LOGO5** in dissociated rat hippocampal neurons was also highly reliable; photoswitching could be repeated for the entire length that a patch was maintained (~ 5 -10 minutes; Fig. S7c), indicating that **LOGO5** is a useful tool for experiments over extended periods of time.

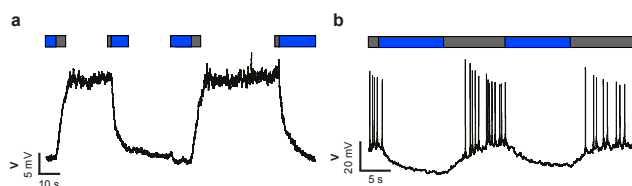


Fig. 5 Optical control of excitability via endogenous GIRK channels using **LOGO5** in rat hippocampal neurons. a) Photoswitching of **LOGO5** reversibly and repeatedly manipulated membrane potentials by 10-20 mV. Light responses were stable in the dark for tens of seconds due to the slow relaxation of the *cis*- and *trans*-isomers of **LOGO5**. b) Photoswitching of **LOGO5** results in the control of spontaneous action potential firing. (grey = 360 nm; blue = 450 nm).

With the control of native GIRK channels accomplished in rat hippocampal neurons, we investigated if **LOGO5** had any effect in living animals. We selected zebrafish larvae (*Danio rerio*) as our organism of choice as they are transparent, enabling facile light delivery and they have previously been used in conjunction with biologically active compounds containing azobenzene photoswitches.²⁶ Accordingly, zebrafish larvae 5-7 days post fertilisation were exposed to 10 second pulses of UV light (365 nm) and blue light (455 nm), in between interludes of ambient light (Fig. S8). After the first cycle of UV and blue light pulses the time that the zebrafish larvae spent swimming in the 10 seconds after the light pulse was measured to give the background swimming behaviour. The zebrafish larvae were then incubated with **LOGO5** (50 μM) for 1 hour and the

same protocol was used to determine the effect of the photochromic GIRK agonist by calculating the change in swimming time. Gratifyingly, the zebrafish larvae showed significantly different changes in swimming time in the presence of **LOGO5**, which could be modulated by alternating illumination with UV and blue light (Fig. 6). When illuminated with blue light, the zebrafish larvae exhibited reduced swimming times compared to the control experiments. After illuminating with UV light for 10 seconds, the zebrafish larvae dramatically increased the time they spent swimming. However, the zebrafish larvae also showed increased swimming time in the control experiment using only UV light. To dissect the effect of **LOGO5** from the native response to UV light, we performed additional experiments using the non-photoswitchable GIRK activator **ML297** (Fig. 6). These experiments demonstrated that there is almost no change in the swimming behaviour of the zebrafish larvae when illumination is switched between UV and blue light. This is in stark contrast to the results obtained using **LOGO5** and UV light.

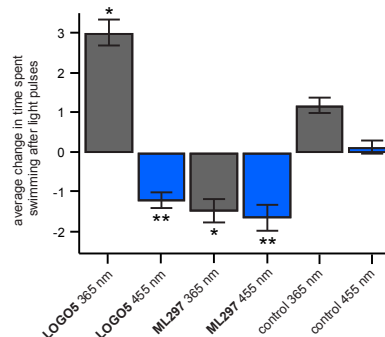


Fig. 6 Optical control of zebrafish motility in the presence of **LOGO5**. After the application of **LOGO5** (50 μM), the swimming behaviour of the zebrafish larvae was reduced under blue light illumination and increased under UV light illumination when compared to the control (1% DMSO) zebrafish. Experiments using the non-photoswitchable GIRK opener **ML297** (50 μM) showed reduction of the zebrafish motility independent of UV and blue light illumination. (**LOGO5** $n=36$ zebrafish; **ML297** $n=36$ zebrafish; control (1% DMSO) $n=72$ zebrafish) (* $P < 0.01$ versus control 365 nm; ** $P < 0.001$ versus control 455 nm). Values represent mean \pm SEM.

Conclusion

In summary, we have developed a photochromic agonist that enables the optical control of GIRK channels. Our photoswitch, **LOGO5**, is active as its *trans*-isomer, with UV light illumination converting it to its significantly less active *cis*-isomer. **LOGO5** has been shown to work with GIRK channels that bear the GIRK1 subunit and can be used to efficiently reduce neuronal excitability in dissociated hippocampal neurons in a light dependent manner. **LOGO5** can also be implemented *in vivo*, for instance in zebrafish larvae. Future work is focused on the

advanced development of LOGOS as an optical tool to control GIRK channels in higher animals.

Acknowledgements

D.M.B. gratefully acknowledges the European Commission for a Marie Skłodowska-Curie Intra-European Fellowship (PIEF-GA-2013-627990). D.T. thanks the Munich Centre for Integrated Protein Science (CIPSM) and the European Research Council (Advanced Grant 268795). D.T. and E.Y.I. thank the National Institutes of Health support of the Nanomedicine Developmental Center for the Optical Control of Biological Function (2PN2EY018241). We thank Luis de la Osa de la Rosa and Luisa Zartner for excellent technical assistance and Dr. Martin Olbrich and Cedric Hugelshofer for helpful discussions during the preparation of this manuscript.

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