‡ Electronic Supplementary Information (ESI):

Supplementary Experimental section

Optogenetic modulation of real-time nanoscale dynamics of HCN channels using photoactivated adenylyl cyclases

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1. Imaging and Analysis

1.1 Ensemble photoactivation of PACs

To examine the cAMP level in Neuro-2a (N2a) cells expressing Flamindo-pUltra conjugated with bPAC, TpPAC, NgPAC1 and NgPAC3, the cells expressing these constructs were loaded in a ludin chamber (Life Imaging Services) filled with the extracellular solution (10 mM D + Glucose, 120 mM NaCl, 3 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$ and 10 mM HEPES). *In vivo* imaging was done using an inverted microscope (Olympus IX83) equipped with a 100X 1.49 NA oil objective at 37°C. Widefield fluorescence and TIRF (Total Internal Reflection Fluorescence) images of the regions of interest were acquired using an EMCCD camera (Photometrics Evolve, USA). Laser illumination and microscopy image acquisition were controlled using Metamorph 7.0 (Molecular Devices, USA). For every transfected cell, 2000 images were acquired using 560 nm laser at 50 ms exposure time. For spatial modulation of the cAMP level in cells and its detection, targeted photoactivation of PACs was done using 100% power of 405 nm (30 ms) or 488 nm (30 ms) lasers.

To study the effect of increase in cAMP on the mobility of mEos::HCN2 molecules, *in vivo* TIRF imaging of cells (2000 frames) co-expressing PAC-pUltra (bPAC, TpPAC, and NgPAC1) and mEos::HCN2 was performed using a 560 nm laser at 20 ms exposure time. The laser power of 405 nm (16 ms) was set to 100% for localized photoactivation of PACs in cells and for the photoconversion of mEos3.2 molecules from green to red fluorescent state. For targeted photoactivation, a region corresponding to the point spread function of the system was randomly selected at a distance of 2-5 µm from the periphery of the cell. Data analysis of the acquired images was performed using Metamorph 7.0 and the fluorescence intensity of the cells was quantified. The relative fluorescence intensity change (ΔF/F, fold change) was calculated as the percentile; where ΔF is defined as the change in fluorescence intensity before and after activation and F is the fluorescence intensity before activation.

Normalized average intensity values of the respective cells expressing PAC-Flamindo before and after photoactivation were fit non-linearly using a second order exponential decay in Origin 2015 (Origin Lab, Northampton, MA, USA) to calculate the fractional contribution and relaxation time for each construct. Normalized fractional contribution [(A1/(A1+A2), A2/(A1+A2)] and average lifetime [$(A1*\tau_1+A2*\tau_2)/(A1+A2)$] were calculated manually. A single order exponential decay model was used for analyzing the biophysical properties of fluorescence decay after photoactivation of cells co-expressing mEos::HCN2 and PACs.

The ratio of D_m was calculated as D_{after}/D_{before}, where D_{before} and D_{after} denote the median diffusion coefficient before and after stimulation, respectively. The relative change in the instantaneous diffusion coefficient upon stimulation (ΔD/D, average fold change) was calculated as the percentile; where ΔD is defined as the change in median diffusion coefficient (D) before and after stimulation and D is the median diffusion coefficient before stimulation.

1.2 Super-resolution by radial fluctuation (SRRF) analysis

Live N2a cells expressing mEos::HCN2 were imaged in TIRF with 488 nm. Super-resolution images were processed from 100 frames of diffraction limited images using NanoJ-SRRF[1], an image analysis module in Image J. For image reconstruction, intensity weighing and gradient smoothing were chosen. Parameters for radiality magnification were set to 5 (1 pixel = 21.4 nm), ring radius fixed to 0.5 and axes in the ring set to 6.

1.3 Photoactivation Localization Microscopy (PALM)

For photoactivation localization microscopy, cells expressing mEos::HCN2 alone and mEos::HCN2 together with PACs (referred to as mEos::HCN2+PACs) were imaged using 405 nm and 560 nm lasers, simultaneously. 405 nm laser was used for photoactivation of mEos, and the single molecules were imaged at 560 nm excitation. The laser powers for photoactivation and bleaching were balanced to have subsets of molecules separated more than the diffraction limit in each time frame. For saturation stimulation of cAMP, cells were photoactivated globally using 488 nm (100%, 30 ms) prior to PALM imaging. On the contrary, chronic stimulation of cAMP was achieved using 10% power of 405 nm (streaming) during PALM imaging for simultaneous photoactivation of PACs and photoconversion of mEos molecules. 20,000 images were acquired using Metamorph 7.0 in the streaming mode in 5 batches of 4000 frames at 20 ms exposure time. Multicolor fluorescent microbeads (100 nm, Tetraspeck, Invitrogen, USA) were used as fiduciary markers for the correction of lateral drifts and

chromatic shifts. Single molecule localization in each frame and tracking over time were performed using MetaMorph. An average of >500 trajectories per cell with a minimum trajectory length of six frames were extracted and analyzed for saturation stimulation. The median diffusion coefficient (D) and mean square displacement (MSD) was calculated from all single-molecule diffusions per cell. The average MSD and mean diffusion coefficient (D_m) were computed from the average of single-molecule diffusions of all measured cells per condition. For continuous induction of cAMP, an average of >200 trajectories per cell per stream was obtained and analyzed.

1.4 Direct stochastic optical reconstruction microscopy (dSTORM)

The immunolabeled N2a cells were imaged in a ludin chamber filled with a custom-made STORM solution containing reducing agents and oxygen scavengers. For dSTORM imaging, the ensemble fluorescence of Alexa-647 was first converted into a dark state using a 642 nm laser to get the desired density of single molecules. 20,000 frames were acquired at 20 ms exposure time. The densities of single molecules per frame were controlled using 647 nm and 405 nm lasers. Multicolor fluorescent microbeads (Tetraspeck, Invitrogen, USA) were used as fiduciary markers for the correction of lateral drifts and chromatic shifts. Single molecule localization and reconstruction of super-resolution images were performed using a custom-made plugin in MetaMorph software[2]. HCN2 protein nanoclusters were identified by adaptive thresholding of super-resolved intensity images, and the cluster properties (area, average intensity and total intensity) were calculated for the structures >0.0025 μm^2 using Integrated Morphometry Analysis module in Metamorph. Morphological features (area, average intensity, and total intensity) of each cluster were exported to calculate their respective distribution.

1.5 Statistics

GraphPad Prism 7.0 was used for statistical analysis and graphing. Normality distribution was analyzed using D'Agostino & Pearson omnibus normality test. Variability in instantaneous diffusion coefficient D_m was determined using Mann-Whitney test. Significance of difference in normalized frequency distribution, cumulative probability distribution and MSD curves between control and optogenetically/pharmacologically treated data were verified by 2-way Anova. The use of asterisks indicates statistical significance (* = $p<0.05$, ** =$p<0.01$, *** = $p<0.001$, **** = $p<0.0001$). Additional statistical details are specified in the figure legends.

References

1. M. Venkatachalapathy, V. Belapurkar, M. Jose, A. Gautier and D. Nair, *Nanoscale*, 2019, **11**, 3626-3632.
2. D. Nair, E. Hosy, J. D. Petersen, A. Constals, G. Giannone, D. Choquet and J. B. Sibarita, *J Neurosci*, 2013, **33**, 13204-13224.