Supplementary Information

Fluorescent Ca^{2+} indicators inhibit the Na,K-ATPase and disrupt energy metabolism and intracellular Ca^{2+} homeostasis

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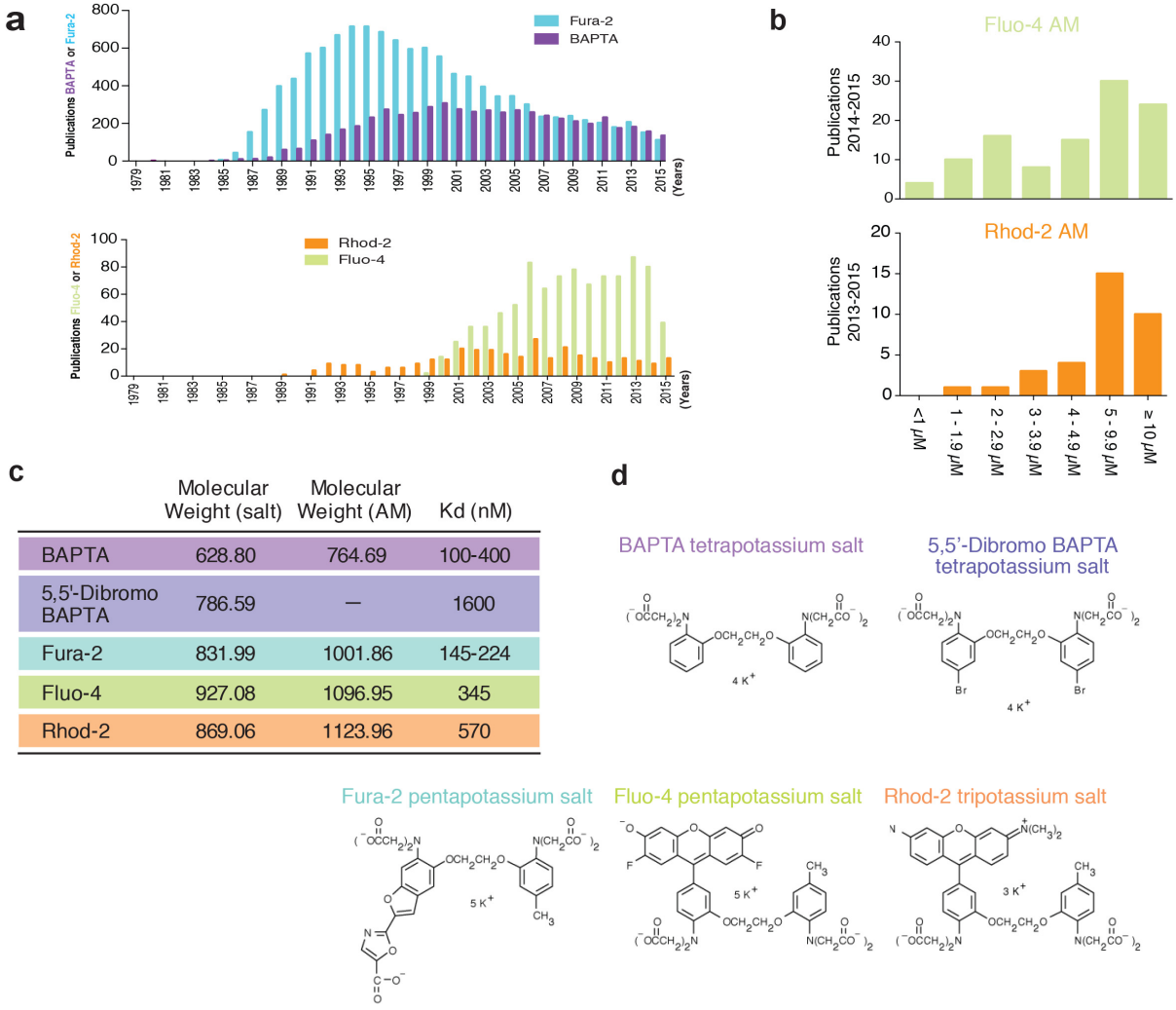
Number of Supplementary Figure and movies: 6 supplementary figures and 5 supplementary movies

Supplementary Fig. 1

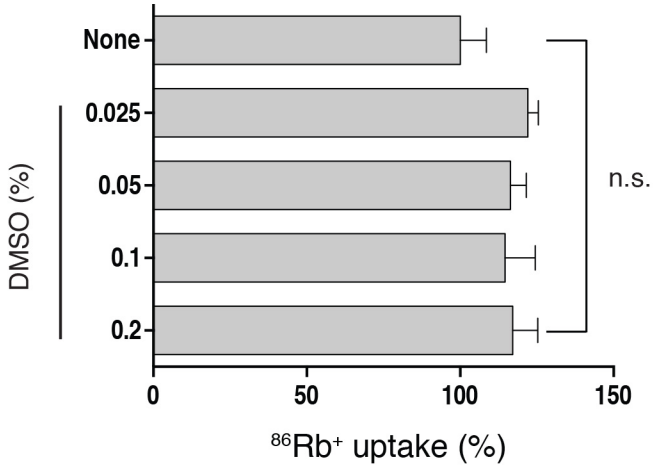


c

	Molecular Weight (salt)	Molecular Weight (AM)	Kd (nM)
BAPTA	628.80	764.69	100-400
5,5'-Dibromo BAPTA	786.59	—	1600
Fura-2	831.99	1001.86	145-224
Fluo-4	927.08	1096.95	345
Rhod-2	869.06	1123.96	570

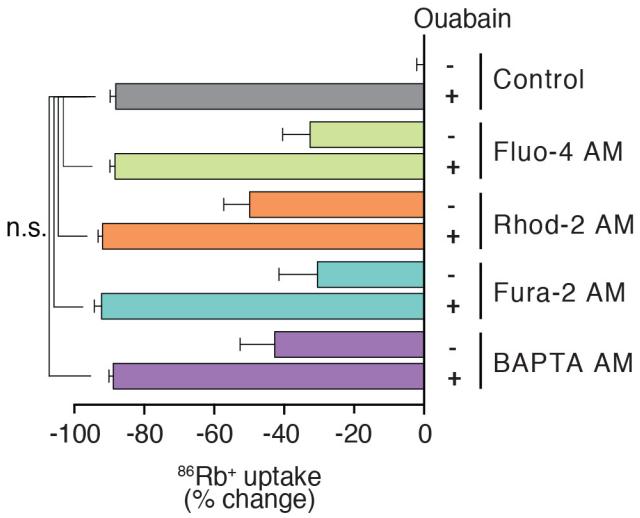
Supplementary Fig.1. Structures and characters of Ca^{2+} indicator derivatives used for published papers listed in PubMed
(**a**) The numbers of publications using BAPTA, Fura-2, Fluo-4 or Rhod-2 since 1980. (**b**) Concentrations of Ca^{2+} indicators dyes used in the papers published in 2014-2015 (Fluo-4 AM) and 2013-2015 (Rhod-2 AM). (**c**) Comparison of molecular weight and Ca^{2+} affinity of Ca^{2+} indicator derivatives. (**d**) Structures of Ca^{2+} indicator dyes. See also Supplementary Note 1.

Supplementary Fig. 2



Supplementary Fig.2. Dimethyl sulfoxide has no effect on $^{86}Rb^{+}$ uptake by astrocytes
Varying concentrations of dimethyl sulfoxide (DMSO, 0-0.2%) were loaded and $^{86}Rb^{+}$ uptake was assessed in cultured mouse astrocytes. Application of DMSO did not significantly alter astrocyte $^{86}Rb^{+}$ uptake (n=5-6 wells, p=0.35, one-way ANOVA followed by Tukey-Kramer test). Displayed are means ± S.E.M.

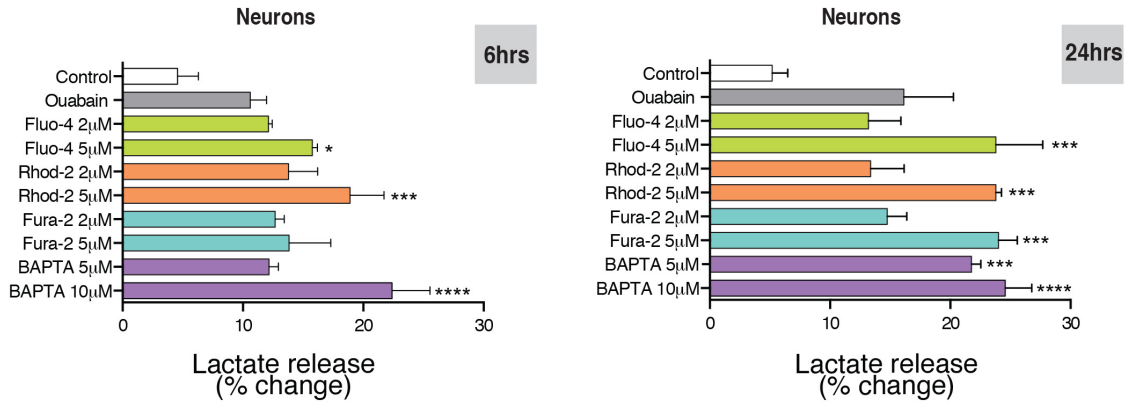
Supplementary Fig. 3



Supplementary Fig.3. Ca^{2+} indicators have no effect on ouabain-insensitive $^{86}Rb^{+}$ uptake
Cultured mouse astrocytes were loaded with Fluo-4 AM (10 μM), Rhod-2 AM (10 μM), Fura-2 AM (8 μM) and BAPTA AM (40 μM) with or without ouabain (1 mM), and $^{86}Rb^{+}$ uptake was examined. Co-application of Ca^{2+} indicators and ouabain did not show further suppression in $^{86}Rb^{+}$ uptake compared to single application of ouabain (n=4-15 wells, one-way ANOVA followed by Tukey-Kramer test). Displayed are means ± S.E.M.

Supplementary Fig.4

a



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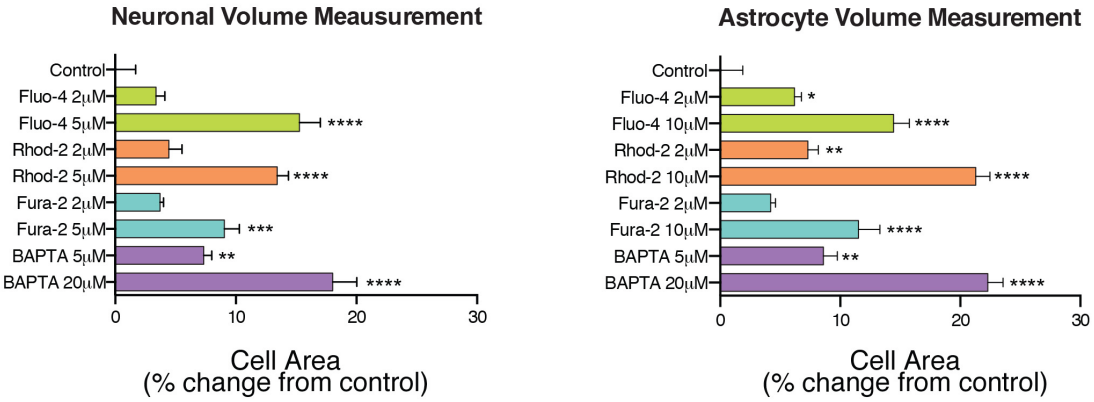
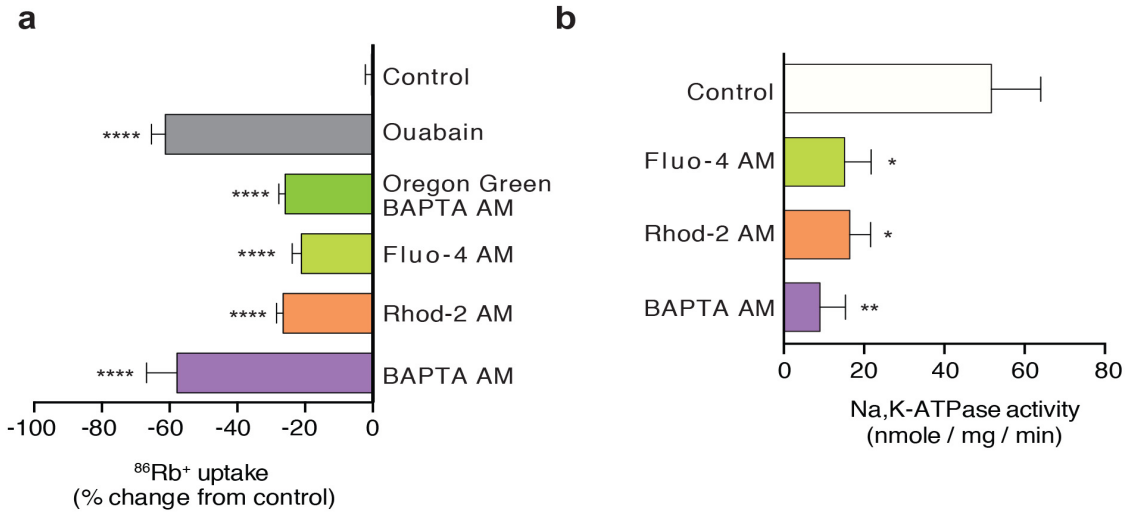


Supplementary Fig.4. Ca^{2+} indicators also induce Lactate Release in Neurons as well as Cell Volume in neurons and astrocytes

(**a**) Effects of Ca^{2+} indicators (Fluo-4 AM, Rhod-2 AM and Fura-2 AM) or BAPTA AM on lactate release in cultured mouse neurons (n=5 wells; *p<0.05, ***p<0.001, ****p<0.0001 compared to vehicle control (0.2% DMSO). (**b**) Effects of Ca^{2+} indicators (Fluo-4 AM, Rhod-2 AM and Fura-2 AM) or BAPTA AM on volume changes in cultured mouse neurons (n= 4, **p<0.01, ***p<0.001, ****p<0.0001 compared to control group and astrocytes (n= 4 wells; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control group. Displayed are means ± S.E.M.

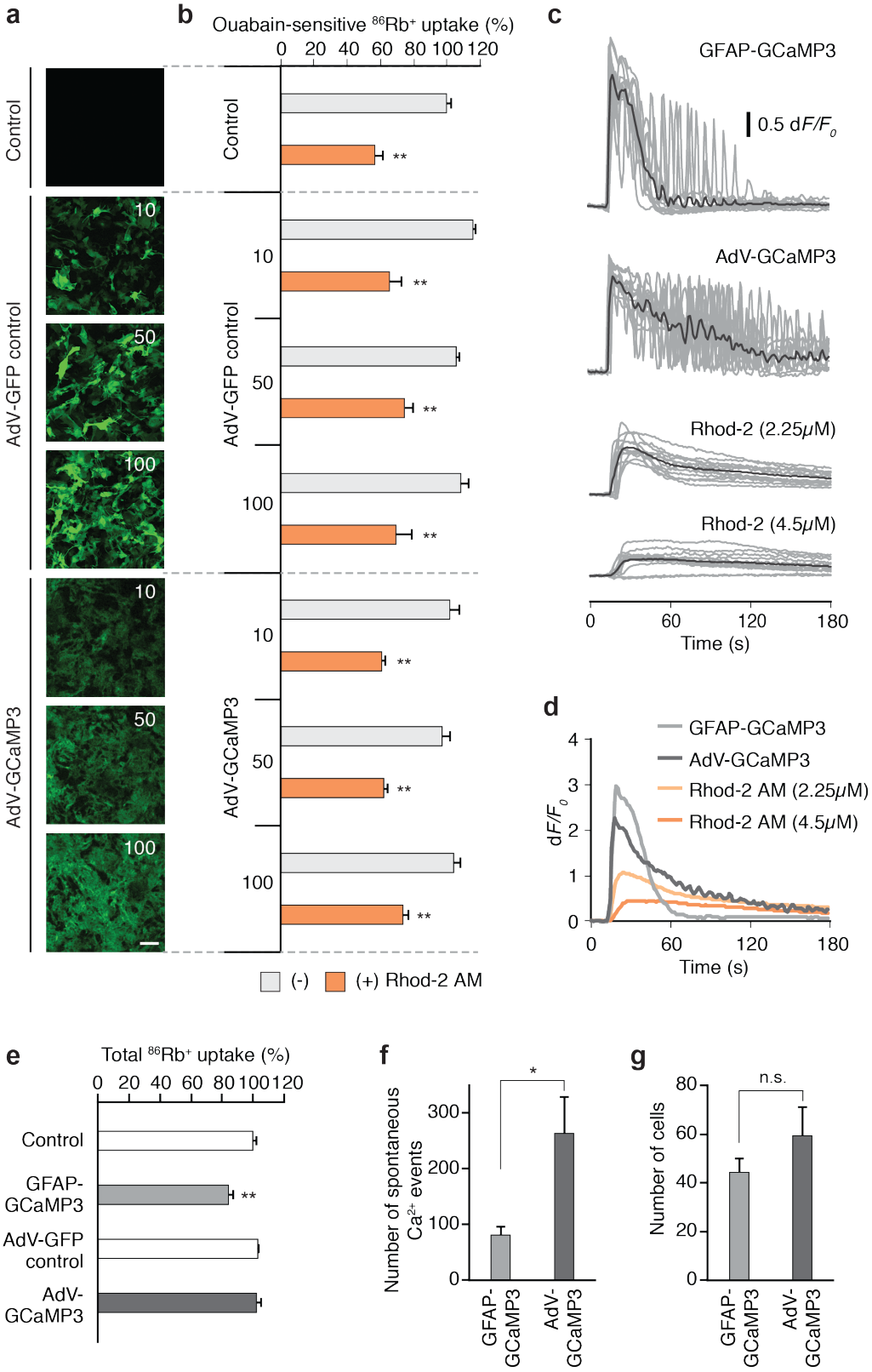
Supplementary Fig. 5



Supplementary Fig.5. Ca^{2+} indicators also inhibited Na,K-ATPase-mediated ATP hydrolysis and $^{86}Rb^{+}$ uptake in rat astrocyte cultures

(**a**) Effects of Ca^{2+} indicators (Oregon green 488 BAPTA-1 AM, 4.5 μM; Fluo-4 AM, 4.5 μM; Rhod-2 AM, 4.5 μM) and BAPTA AM (20 μM) on $^{86}Rb^{+}$ uptake in rat cultured astrocytes (n= 4-23 wells; ****p<0.01 compared to control group). (**b**) Effects of Ca^{2+} indicators (Fluo-4 AM, 4.5 μM; Rhod-2 AM, 4.5 μM) and BAPTA AM (20 μM) on ouabain-sensitive ATP hydrolysis in membrane preparations derived from rat cultured astrocytes (n= 7-8 wells; *p<0.05, **p<0.01 compared to control group). Displayed are means ± S.E.M. See also Supplementary Note 2.

Supplementary Fig. 6



Supplementary Fig.6. Quantification of spontaneous and pharmacologically evoked Ca^{2+} signals and $^{86}Rb^{+}$ uptake in GCaMP3-expressing astrocytes

(**a**) Representative images of astrocytes expressing GCaMP3 or GFP. Astrocytes prepared from GCaMP3fl transgenic mice were infected with Cre recombinase-adenovirus with 10, 50 or 100 MOI to induce GCaMP3 expression (AdV-GCaMP3). As control, astrocytes infected with GFP-adenovirus were used (AdV-GFP control). Scale bar, 100 μm. (**b**) Induction of GCaMP3 expression in astrocytes had no effect on Na,K-ATPase-mediated $^{86}Rb^{+}$ uptake, whereas Rhod-2 AM loading (4 μM) consistently induced the decrease in all cell types. **p<0.01 compared to (-) Rhod-2 AM group in control culture. (**c**) Representative individual traces of Ca^{2+} transients from 12 astrocytes (grey) and averages (black) showing GFAP-GCaMP3, AdV-GCaMP3 or Rhod-2 responses to application of 5 μM ATP. In AdV-GCaMP3 astrocytes, long-lasting rhythmic oscillations in Ca^{2+} responses were observed. GFAP-GCaMP3 astrocytes exhibited shorter (~60 s) oscillations (10 out of 12 cells) in all but 2 astrocytes that exhibited comparatively longer (~120 s) oscillations. In contrast, Rhod-2 AM (2.25 and 4.5 μM) loaded cells failed to exhibit similar oscillatory patterns, instead showing only small fluctuating Ca^{2+} signals. (**d**) Representative averaged traces of Ca^{2+} transients evoked by 5 μM ATP in astrocytes measured by Rhod-2 AM or GCaMP3 (GFAP-GCaMP3 or AdV-GCaMP3, 100 MOI). GFAP-GCaMP3 and AdV-GCaMP3 showed high dF/F_0 compared to Rhod-2 (n=75 each). (**e**) Total $^{86}Rb^{+}$ uptake was slightly but significantly decreased in GFAP-GCaMP3 astrocytes. No difference in total $^{86}Rb^{+}$ uptake was observed in AdV-GFP or AdV-GCaMP3 astrocytes (100 MOI each). **p<0.01 compared to control. (**f** and **g**) Number of events (**f**) or cells (**g**) showing spontaneous Ca^{2+} rise during 5 min in GFAP-GCaMP3 or AdV-GCaMP3 expressing astrocytes. Significantly higher number of spontaneous Ca^{2+} events was observed in AdV-GCaMP3 group, but number of cells showing Ca^{2+} rise was similar in GFAP-GCaMP3 and AdV-GCaMP3 groups. *p<0.05 compared to GFAP-GCaMP3 group. Displayed are means ± S.E.M. See also Supplementary Note 3.

Supplementary Movies

Supplementary Movie 1. Beating cardiomyocytes in culture prepared from mouse (2x framerate)

Supplementary Movie 2. ATP-induced Ca^{2+} response in astrocytes expressing GFAP-GCaMP3

Supplementary Movie 3. ATP-induced Ca^{2+} response in astrocytes expressing AdV-GCaMP3

Supplementary Movie 4. ATP-induced Ca^{2+} response in astrocytes loaded with Rhod-2 AM

Supplementary Movie 5. Spontaneous Ca^{2+} response in astrocytes expressing GFAP-GCaMP3

Supplementary Notes

Supplementary Note 1. Structures and characters of Ca^{2+} indicator derivatives used for published papers listed in PubMed

The initial Ca^{2+} indicator, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), was first introduced in 1979. Many different types of Ca^{2+} indicator dyes, including Fluo-4, Rhod-2 and Fura-2, have been created, and analysis of papers listed in PubMed revealed that more than 25,600 papers using Ca^{2+} indicators have been published (**Supplementary Fig. 1a**). The summary for concentrations of Ca^{2+} indicator dyes used in the recently published papers (Fluo-4 AM, 2014-2015; Rhod-2 AM, 2013-2015). Several papers that could not be obtained or are written in Chinese or Ukrainian were not included. Ca^{2+} indicator dyes have similar molecular weight and share their structures (**Supplementary Fig. 1b** and **c**). Fura-2 has high affinity for Ca^{2+} compared to Fluo-4 and Rhod-2 (**Supplementary Fig. 1b**). Despite widespread use of Ca^{2+} indicator dyes, only little attention has been paid to their side-effects. A few reports have, however, shown that Ca^{2+} indicators can alter cellular functions, such as Ca^{2+}-induced Ca^{2+} release from endoplasmic reticulum in cerebellar granule neurons and HEK-293 cells [1], field excitatory postsynaptic potentials in the brain hippocampal slice [2], K^+ channels genetically expressed in HEK293 cells [3], and K^+ currents in brain slices [4].

Supplementary Note 2. Ca^{2+} indicators inhibit Na,K-ATPase activity in rat astrocyte cultures

To broaden our findings, we also prepared astrocyte cultures from rats, which are another rodent species in which calcium indicators are widely used in basic research. As expected, $^{86}Rb^+$ uptake was suppressed by Fluo-4, Rhod-2 and BAPTA AM loading (**Supplementary Fig. 5a**). Consistently, ATP hydrolysis mediated by Na,K-ATPase was suppressed by loading of Fluo-4, Rhod-2 and BAPTA AM (**Supplementary Fig. 5b**). We also examined the effect of other Ca^{2+} indicator, Oregon green BAPTA AM, and found the comparable inhibition of $^{86}Rb^+$ uptake to other Ca^{2+} indicators (**Supplementary Fig. 5a**).

Supplementary Note 3. Quantification of spontaneous and pharmacologically evoked Ca^{2+} signals and $^{86}Rb^{+}$ uptake in GCaMP3-expressing astrocytes

Astrocytes expressing GCaMP3 (AdV-GCaMP3) or GFP (AdV-GFP control) showed no difference in Na,K-ATPase-mediated $^{86}Rb^{+}$ uptake. Conversely, Rhod-2 AM loading (4 μM) consistently induced the decrease in all cell types (**Supplementary Fig. 6a**,**b**). Analysis of Ca^{2+} transients in response to application of 5 μM ATP revealed that AdV-GCaMP3 astrocytes exhibit long-lasting pattern of rhythmic oscillations. GFAP-GCaMP3 astrocytes also exhibited both shorter (~60 s) and longer (~120 s) pattern of oscillation, whereas Rhod-2 AM (2.25 and 4.5 μM) loaded cells had no oscillation but with small, fluctuated Ca^{2+} signals (**Supplementary Fig. 6c**,**d**). For this set of experiment, we employed lower concentration of ATP (5 uM). GCaMP3 showed larger response than Rhod-2, suggesting that GCaMP3 can clearly detect intracellular Ca^{2+} changes triggered by low dose of GPCR agonists.

Supplementary References

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4 **Zhang, L. *et al.* Potentiation of a slow Ca(2+)-dependent K+ current by intracellular Ca2+ chelators in hippocampal CA1 neurons of rat brain slices. *J Neurophysiol* 74, 2225-2241 (1995).**