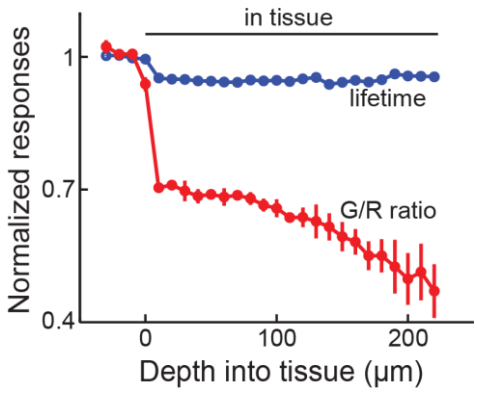

Figure S1. Related to Figure 1. Comparison of fluorescence lifetime measurements and two-color ratiometric measurements in brain tissue.

Cultured hippocampal slices were incubated in 5 μM fluorescein in ACSF. The fluorescence at various depths outside (negative) and inside (positive) of the slice tissue was imaged simultaneously using 2pFLIM imaging of the green emission channel and conventional two-photon imaging of both the green and red channels. Fluorescein's emission bleeds through into the red channel and can be reliably detected in both channels. Inside the tissue, while lifetime measurements remained stable, the green/red (G/R) ratio continued to change as the depth increased. This was because red photons (longer wavelength) were scattered less compared to green photons in this light scattering brain tissue. n = 3 slices.

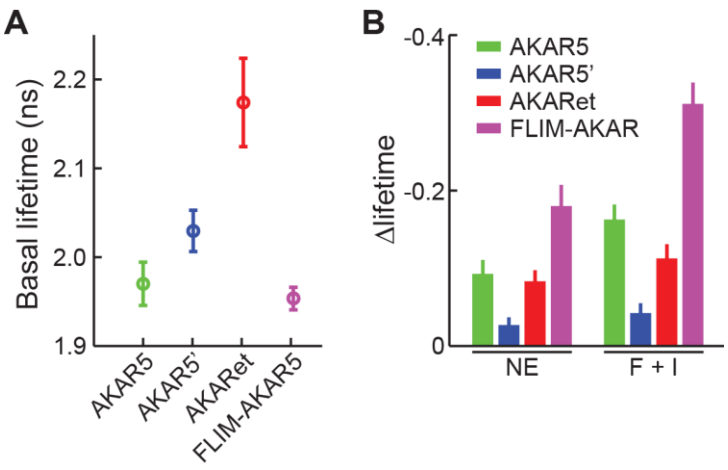

Figure S2. Related to Figure 1. Currently available AKAR sensors exhibit different basal lifetimes and stimulated lifetime changes.

Basal lifetimes (A) and the lifetime changes (Δlifetime) (B) stimulated by 1 μM norepinephrine (NE), or by 25 μM forskolin/50 μM IBMX (F + I) of the indicated AKAR sensors.

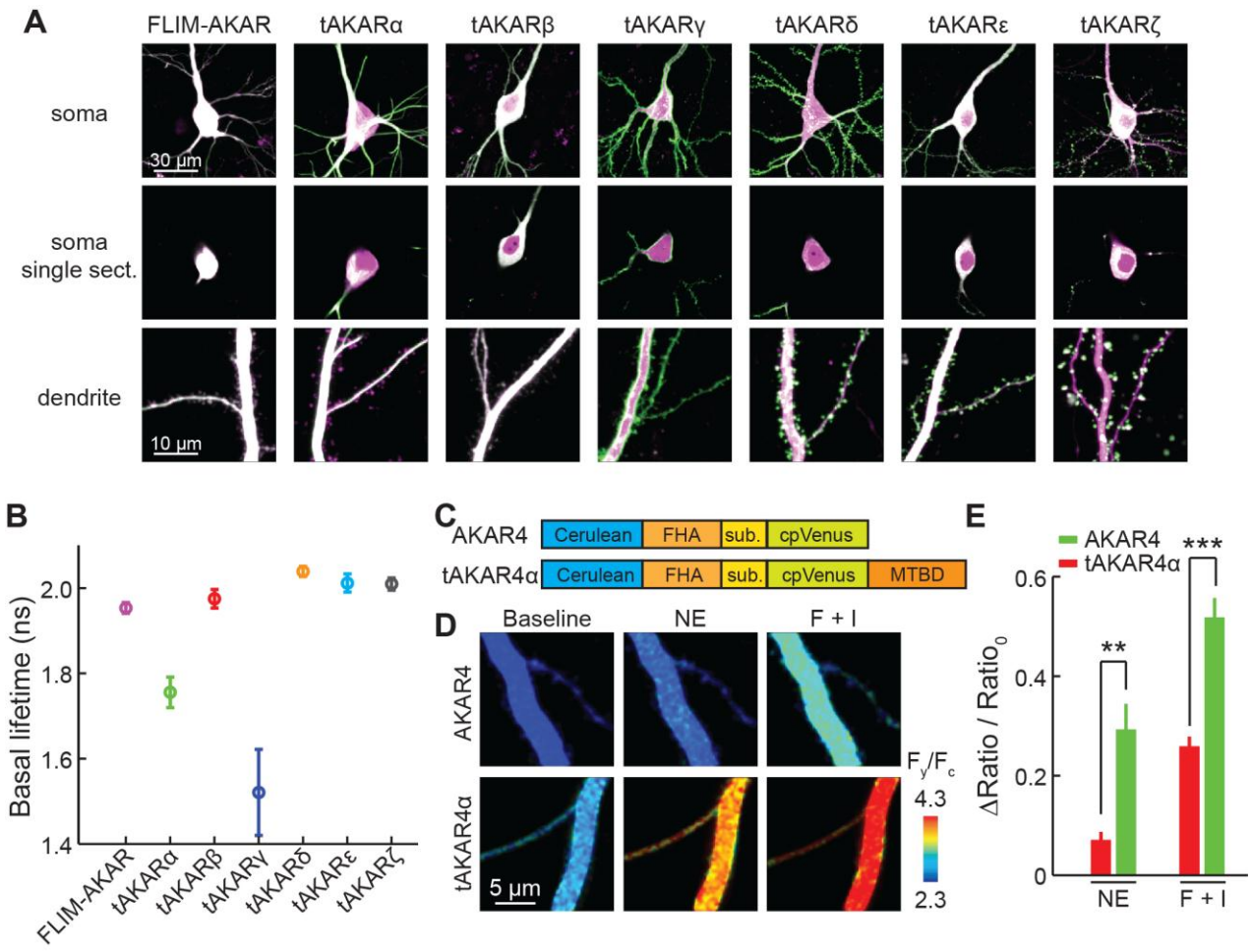

Figure S3. Related to Figure 2. Supporting data for subcellularly targeted AKARs.

(A) Representative two-photon max-projection images (upper) and corresponding single sections across the center (middle) of neuronal somas, and zoom-in images of apical dendrites (lower) of CA1 neurons in cultured hippocampal slices expressing the indicated sensors (green) and the cytosolic marker mCherry (magenta).

(B) The averaged basal lifetime at CA1 apical dendrites of the indicated AKAR sensors. n = 6 – 7 cells for all sensors.

(C) Schematic of AKAR4 and its microtubule-targeted variant tAKAR4α, showing their respective domain structures.

(D, E) Representative images (D) and collective quantifications (E) of two-photon ratiometric imaging (F_{YFP}/F_{CFP}) of AKAR4 and tAKAR4α in CA1 apical dendrites in response to 1 μM norepinephrine or 25 μM forskolin/50 μM IBMX. n = 5 dendrites for both constructs.

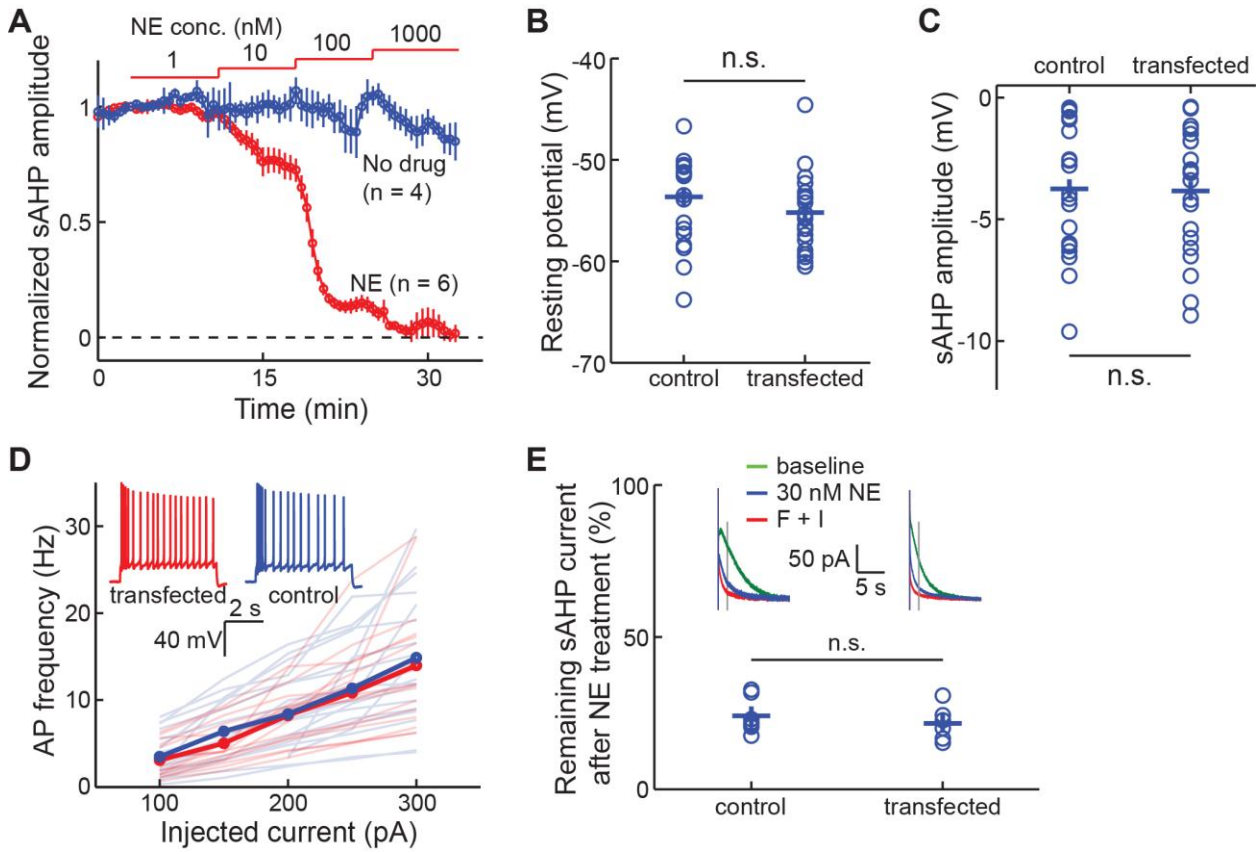

Figure S4. Related to Figure 3. The expression of tAKARα does not alter neuronal function or PKA modulation.

(A) Averaged time course of the slow afterhyperpolarization (sAHP) measurements, normalized to the baseline, with (red) or without (blue) applications of increasing concentrations of norepinephrine (NE) as indicated.

(B, C) Resting membrane potentials (B) and sAHP amplitudes (C) of neurons transfected with tAKARα and untransfected control neurons. n = 21 for all conditions.

(D) Action potential (AP) frequencies elicited by current injections of different amplitudes for neurons transfected with tAKARα and untransfected control neurons. Inset shows

representative traces in response to 200 pA current injection. n = 17 for transfected neurons and 19 for controls.

(E) Representative traces (inset) and collective results of sAHP currents being attenuated by bath application of 30 nM norepinephrine (NE). The inset also shows the maximal attenuation of sAHP currents by forskolin and IBMX (F + I). The remaining current insensitive to forskolin and IBMX has been subtracted from measurements. Gray lines indicate the measurement window. n = 6 for transfected neurons and 7 for controls.

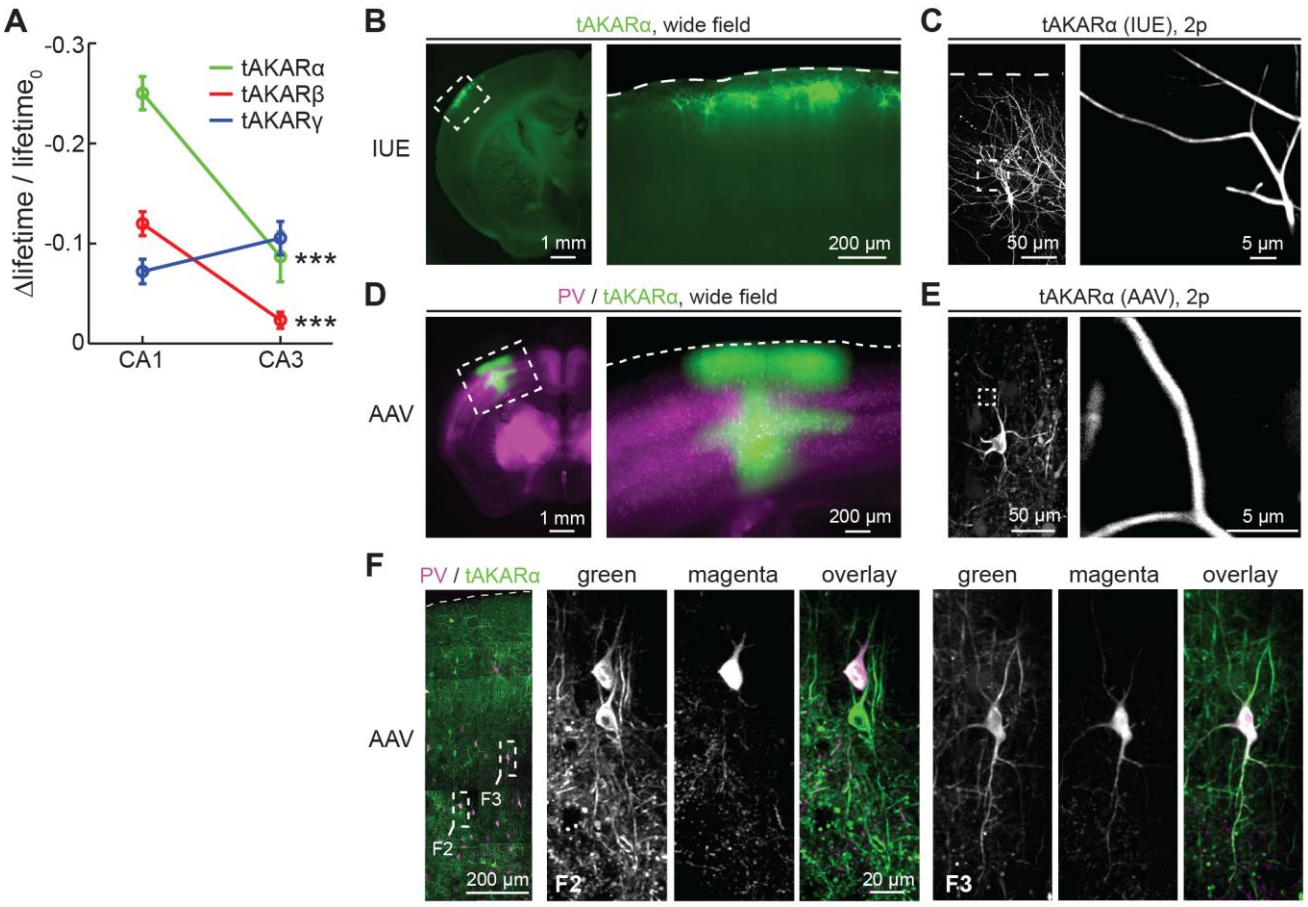

Figure S5. Related to Figure 4. Supporting data for the expression and comparison of sensors across neuronal types.

(A) The averaged amplitudes of Δlifetime/lifetime$_0$ responses of the indicated tAKARs in the apical dendrites of CA3 neurons compared to those of the corresponding sensors in CA1 neurons in cultured hippocampal slices. n = 6 – 7 for all sensors in both types of neurons.

(B, C) Wide-field (B) and two-photon (2p; panel C) images of tAKARα (green in wide-field images) expressed using *in utero* electroporation (IUE) into wildtype mice.

(D, E) Wide-field (D) and two-photon (E) images of tAKARα (green in wide-field images, expressed using AAV injection) and tdTomato (magenta in wide-field images, expressed from

the *PV-IRES-Cre;Ai9* transgenes) from mice with the *PV-IRES-Cre;Ai9* double heterozygous genotype.

(F) Two-photon images and two fields of enlargements showing that PV neurons (magenta), which also express tAKARα (green), can be readily identified.

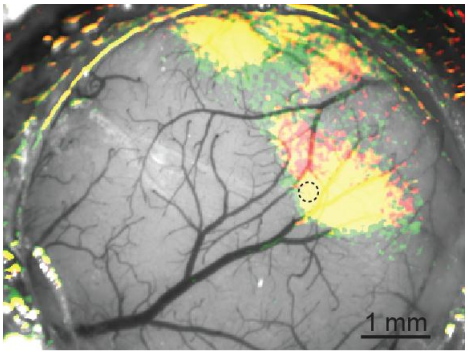

Figure S6. Related to Figure 6. Intrinsic imaging reveals the location of the barrel cortex.

Representative image of intrinsic imaging while stimulating mouse whiskers. Gray: bright-field image; green and red: intrinsic imaging signal from two independent trials; dashed circle: the 2pFLIM imaging area ($\Phi \sim 280$ μm).

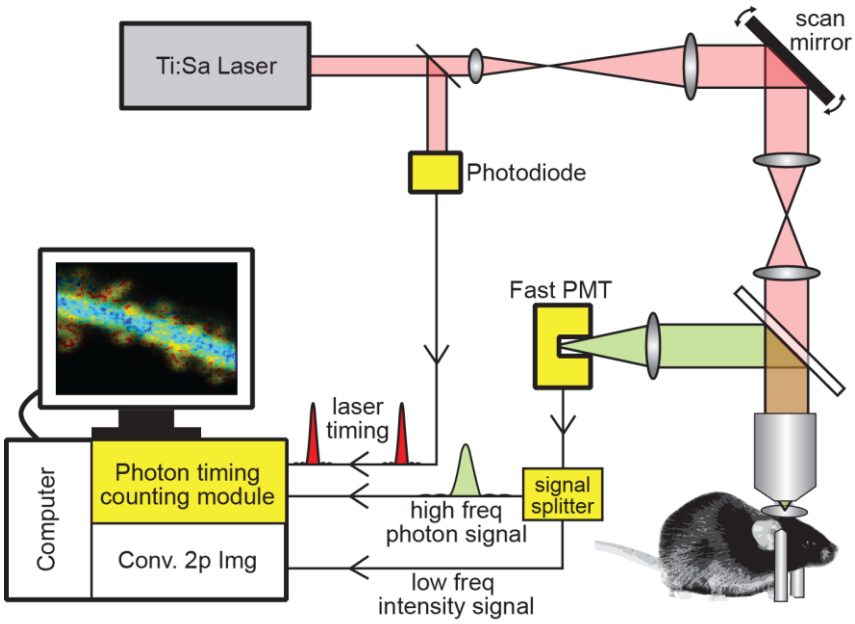

Figure S7. Related to STAR METHODS. Schematic of our *in vivo* 2pFLIM setup.

2pFLIM can be implemented onto most existing two-photon microscopes by the modular addition of the yellow highlighted hardware components: a photon timing counting module (e.g., Becker and Hickl SPC-150), a low-noise, fast photomultiplier (PMT; e.g., Hamamatsu H10769PA-40), an optional signal splitter (e.g., Becker and Hickl HPM-CON-02) that allows simultaneous 2pFLIM imaging and conventional two-photon imaging, and a photodiode (e.g., Thorlabs FDS010) that measures the laser timing. Presently, these pieces of hardware together cost approximately $40,000, depending on the exact configuration.