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Supplemental information

Monitoring norepinephrine release *in vivo*

using next-generation $\mathsf{GRAB}_{\mathsf{NE}}$ sensors

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	lgK	GPCR	linker	cpEGFP	mutated
NE1h NE2m NE2h		METDTLLLWVLLLWVP METDTLLLWVLLLWVP METDTLLLWVLLLWVP *****************	GSTGDTSLYKKVG GSTGDTSLYKKVG GSTGDTSLYKKVG ****************	TTG <mark>GSLQPDAGNASWNG</mark> TGSLQPDAGNASWNG TGSLQPDAGNASWNG * ***********	TEAPGGGARATPYS TEAPGGGARATPYS TEAPGGGARATPYS *********
NE1h NE2m NE2h		LQVTLTLVCLAGLLMLI LQVTLTLVCLAGLLMLI LQVTLTLVCLAGLLMLI ******************	LTVFGNVLVIIAVI LTVFGNVLVIIAVI LTVFGNVLVIIAVI **************	FTSRALKAPQNLFLVSL FTSRALKAPQNLFLVSL FTSRALKAPQNLFLVSL ********	ASADILVATLVIPF ASADILVATLVIPF ASADILVATLVIPF
NE1h NE2m NE2h		2.04 SLANEVMGYWYFGKAW SLANEVMGYWYFGKAW SLANEVMGYWYFGKAW	CEIYLALDVLFCT CEIYLALDVLFCT CEIYLALDVLFCT **************	SSIVHLCAISLDRYWSI SSIVHLCAISLDRYWSI SSIVHLCAISLDRYWSI **********	34.52 TQATEYNLKRTPRR TQAKEYNLKRTPRR TQAKEYNLKRTPRR *** **********
NE1h NE2m NE2h		IKAIIITVWVISAVISI IKAIIITVWVISAVISI IKAIIITVWVISAVISI ****************	FPPLISIEKKGGGG FPPLISIEKKGGGG FPPLISIEKKGGGG ******	GGPQPAEPRCE I NDQKW GGPQPAEPRCE I NDQKW GGPQPAEPRCE I NDQKW ********	YVISSCIG <mark>S</mark> FFAPC YVISSCIGSFFAPC YVISSCIGSFFAPC *******
NE1h NE2m NE2h		LIMILVYVRIYQIAKRI LIMILVYVRIYQIAKRI LIMILVYVRIYQIAKRI *******	RTRVPPSRRGPDA\ RTRVPPSRRGPDA\ RTRVPPSRRGPDA\ *************	/AAPPGGTERRPNGLGP /AAPPGGTERRPNGLGP /AAPPGGTERRPNGLGP *********	ERSAGPGGAEAEPL ERSAGPGGAEAEPL ERSAGPGGAEAEPL *********
NE1h NE2m NE2h		PTQLNGAPGEPAPAGPI PTQLNGAPGEPAPAGPI PTQLNGAPGEPAPAGPI ******************	RDTDALDLEE <mark>GG</mark> N RDTDALDLEEGGN RDTDALDLEE <mark>GG</mark> N *********	/YIKADKQKNGIKANFH /YIKADKQKNGIKANFH /YIKADKQKNGIKANFH ********	IRHNIEDGGVQLAY IRHNIEDGGVQLAY IRHNIEDGGVQLAY ********
NE1h NE2m NE2h		HYQQNTPIGDGPVLLPI HYQQNTPIGDGPVLLPI HYQQNTPIGDGPVLLPI *******	DNHYLSVQ <mark>S</mark> KLSKI DNHYLSVQSKLSKI DNHYLSVQ <u>S</u> KLSKI **********	DPNEKRDHMVLLEFVTA DPNEKRDHMVLLEFVTA DPNEKRDHMVLLEFVTA ************************************	AGITLGMDELYKGG AGITLGMDELYKGG AGITLGMDELYKGG *****
NE1h NE2m NE2h		TGGSMVRKGEELFTGV TGGSMVRKGEELFTGV TGGSMVRKGEELFTGV ************************************	VPILVELDGDVNGH VPILVELDGDVNGH VPILVELDGDVNGH *************	IKFSVSGEGEGDATIGK IKFSVSGEGEGDATEGK IKFSVSGEGEGDATEGK	LTLKFICTTGKLPV LTLKFICTTGKLPV LTLKFICTTGKLPV ******
NE1h NE2m NE2h		PWPTLVTT <mark>LT</mark> YGVQCF3 PWPTLVTTLTYGVQCF3 PWPTLVTTLTYGVQCF3 *******	SRYPDHMKQHDFFH SRYPDHMKQHDFFH SRYPDHMKQHDFFH *******	(SAMPEGYIQERTIFFK (SAMPEGYIQERTIFFK (SAMPEGYIQERTIFFK ***********************************	DDGNYKTRAEVKFE DDGNYKTRAEVKFE DDGNYKTRAEVKFE ************************************
NE1h NE2m NE2h		GDTLVNRIELKGIDFKI GDTLVNRIELKGIDFKI GDTLVNRIELKGIDFKI ************************************	EDGNILGHKLEYN EDGNILGHKLEYN EDGNILGHKLEYN ************	IGAAARWRGRQNREKRF IGAAARWRGRQNREKRF IGAAARWRGRQNREKRF	FVLAVVIGVFVVC FFVLAVVIGVFVVC IFVLAVVIGVFVVC **********
NE1h NE2m NE2h		WFPFFFTVTLTAVGCSV WFPFFFTVTLTAVGCSV WFPFFFTVTLTAVGCSV *************	VPRTLFKFFFWFG VPRTLFKFFFWFG VPRTLFKFFFWFG *************	CONSSLNPVIYTIFNHD CONSSLNPVIYTIFNHD CONSSLNPVIYTIFNHD *******************	FRRAFKK ILCRGDR FRRAFKK ILCRGDR FRRAFKK ILCRGDR *****
NE1h NE2m NE2h		KRIVL* 663 KRIVL* 663 KRIVL* 663	5 3 3		

A

В

60 58 58

120 118 118

180 178 178

240 238 238

300 298 298

360 358 358

420 418 418

480 478 478

540 538 538

600 598 598

660 658 658

d sites Properties of intermediate versions of genetically encoded GPCR-based norepinephrine sensors

Version	Template	Mutation vs. template	Mutation type	Relative maximal brightness ^a	Relative response ^a	Apparent affinity index ^b
N.D.	NE1h			1.00	1.00	N.D.
NE1m	NE1h	K6.34T	GPCR	1.42	1.78	N.D.
N.D.	NE1h	K6.34A/ Y136L	GPCR/ cpEGFP	1.47	2.45	N.D.
N.D.	NE1h	K6.34A/ Y136I	GPCR/ cpEGFP	1.60	2.09	N.D.
N.D.	NE1h	K6.34A/ Y136F	GPCR/ cpEGFP	1.69	2.32	N.D.
N.D.	NE1h	L161F	cpEGFP	0.23	1.38	N.D.
N.D.	NE1h	K6.34F/ Y136E	GPCR/ cpEGFP	1.60	2.39	N.D.
NE2m	NE1h	I34.52K/ K6.34F/ Y136E	GPCR/ GPCR/ cpEGFP	1.48	2.82	0.56
N.D.	NE2m	N2.64R	GPCR	1.20	2.29	0.78
N.D.	NE2m	N2.64H	GPCR	1.09	2.27	0.72
N.D.	NE2m	T6.58I	GPCR	1.23	2.49	0.66
N.D.	NE2m	T6.58L	GPCR	1.06	2.17	0.76
N.D.	NE2m	F6.34H	GPCR	0.86	1.05	0.82
N.D.	NE2m	F6.34V	GPCR	1.10	2.05	0.18
NE2h	NE2m	F6.34I	GPCR	1.31	2.54	0.42

^a Relative data were determined by maximal brightness or response to NE1h; ^b apparent affinity index were determined by response in 10 µM divide response in 300 nM NE; N.D.: not determined;

Note: All data were collected in HEK293T cells.

Figure S1. Amino acid sequence alignments and properties of GRAB_{NE} versions and intermediates, related to Figure 1.

(A) Sequence alignments of $\text{GRAB}_{\text{NE1h}}$, $\text{GRAB}_{\text{NE2m}}$, and $\text{GRAB}_{\text{NE2h}}$. The selected mutation sites were indicated by the black box.

(B) Properties of intermediate versions of genetically encoded GPCR-based norepinephrine sensors. The beneficial mutation sites and intermediates were summarized with brightness, response and apparent affinity index.



Figure S2. Characterization of next-generation GRAB_{NE} sensors in cultured HEK293T cells or neurons, related to Figure 1.

Expression of GRAB_{NE} sensors, membrane-localized EGFP and RFP in cultured HEK293T cells (top) and neurons (bottom). Line plotting of GFP and RFP signals were shown in middle. Normalized colocalization ratio with membrane-target RFP were summarized in right. The scale bar is 10 µm (top) and 50 µm (bottom). Dose-dependent curves of $\text{GRAB}_{\text{NE2m}}$ and $\text{GRAB}_{\text{NE2h}}$ in response to a variety concentrations of norepinephrine (NE) and epinephrine (Epi) in HEK293T cells. The corresponding EC₅₀ values are indicated. n = 3 independent cultures each.

(C) Dose-dependent response of $GRAB_{NE2m}$ and $GRAB_{NE2h}$ to trace amines (OA: octopamine, TA: tyramine, PEA: phenethylamine) and major NE metabolites (VMA: vanillylmandelic acid, NMN: normetanephrine, MHPG: 3-methoxy-4-hydroxyphenylglycol) in HEK293T cells. n = 3 independent cultures each.

(D) Normalized changes in the fluorescence intensity of $GRAB_{NE2m}$ (top) and $GRAB_{NE2h}$ (bottom) in response to application of the indicated molecules (applied at 10 μ M), Δ F/F₀ relative to NE. NE, norepinephrine; Epi, epinephrine; ISO, isoprenaline; YO, yohimbine; ICI, ICI-118,551; ACh, acetylcholine; 5-HT, 5-

hydroxytryptamine (serotonin); Glu, glutamate; GABA, γ -aminobutyric acid; Ado, adenosine; HA, histamine. (E) Downstream G protein signaling (left) and β -arrestin signaling (right) of the native wildtype α 2 adrenergic receptor (WT- α 2AR) with or without co-expression of GRAB_{NE} sensors measured using a luciferase complementation mini-G protein assay and the Tango assay in cultured cells, respectively. n = 3 wells with $\geq 10^5$ cells.

n.s., not significant (one-way ANOVA for A and two-way ANOVA for E).



Figure S3. Characterization of next-generation GRAB_{NE} sensors *in vivo*, related to Figure 4.

(A) Transgenic mice expressing dualNECa reporters has minimal effect on animal behaviors. Quantification of behavioral parameters in the open field test (left) and elevated plus maze test (right) of transgenic mice expressing NE2m and jRGECO1a (dualNECa) driven by CaMKII α or GFAP promoter, comparing with floxed and wildtype (WT) mice. n ≥ 4 mice in each group.

(B–D) NE transients and calcium signals during NREM sleep. Typical traces with transient peaks and full width at half maximum (FWHM) were labeled in (B). NE transients and calcium signals were aligned and summarized in (C). Profiling of NE transient during NREM sleep were shown in (D). n.s., not significant (One-way ANOVA).



Figure S4. $\text{GRAB}_{\text{NE2m}}$ and $\text{GRAB}_{\text{NEmut}}$ fluorescence measured during audio stimulation, related to Figure 5.

(A) Schematic diagram depicting the delivery of AAV in P0–P1 mouse pups by injection into the transverse sinuses in P0–P1 mouse for expressing $\text{GRAB}_{\text{NEmut}}$ in neurons in the dorsal cortex. Also shown are an image of $\text{GRAB}_{\text{NEmut}}$ fluorescence and the paradigm used for audio stimulation using white noise.

(B) Representative images and time course of the change in diameter pupil, GRAB_{NE2m} (left) and GRAB_{NEmut} (right) fluorescence measured in the cortex, and the EMG recording. The shaded areas indicate the delivery of white noise.



Figure S5. Mesoscopic NE and calcium dynamics in dorsal cortex of awake mice, related to Figure 5. (A–C) Illustrations (left) of whisker stimulation and visual stimulation delivered to CaMKIIα::NECa and GFAP::NECa mice. Shown are the peak response images, representative traces, and summary of the peak responses following bilateral (A–B) or unilateral (C) stimulation of the indicated mice. black and grey lines indicate the ROIs used to analyze the representative traces. n = 3–5 animals per group. **p < 0.01, *p < 0.05, and n.s., not significant (Paired student's *t*-test).