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A fluorescent sensor for the real-time measurement of extracellular oxytocin dynamics in the brain

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18   **ABSTRACT**

19   Oxytocin (OT), a hypothalamic neuropeptide that acts as a neuromodulator in the brain,

20   orchestrates a variety of animal behaviors. However, the relationship between brain OT

21   dynamics and complex animal behaviors remains largely elusive, partly because of the lack of

22   a suitable technique for its real-time recording *in vivo*. Here, we describe MTRIA$_{OT}$, a G

23   protein-coupled receptor-based green fluorescent OT sensor with a large dynamic range,

24   optimal affinity, ligand specificity to OT orthologs, minimal effects on downstream signaling,

25   and long-term fluorescence stability. By combining viral gene delivery and fiber photometry-

26   mediated fluorescence measurements, we demonstrated the utility of MTRIA$_{OT}$ for real-time

27   detection of brain OT dynamics in living mice. Importantly, MTRIA$_{OT}$-mediated

28   measurements revealed "OT oscillation," a hitherto unknown rhythmic change in OT levels in

29   the brain. MTRIA$_{OT}$ will allow the analysis of OT dynamics in a wide variety of physiological

30   and pathological processes.

31

INTRODUCTION

Oxytocin (OT) is a neuropeptide that was initially discovered in the early 20th century as a component of pituitary extracts that induces powerful uterine contractions[1,2]. It has since attracted attention as a key regulator of many physiological processes. In addition to its well-characterized roles as a peripheral hormone that promotes childbirth and lactation, OT is also released as a neuromodulator within the brain, thus participating in the regulation of diverse physiological functions such as sensory processing, feeding control, social cognition, and emotion[3]. To exert these central effects, OT is primarily supplied from neurons in the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus. This OT acts on target brain regions including the limbic regions, sensory cortex, and brainstem, where the OT receptor (OTR), a G protein-coupled receptor (GPCR), is highly expressed[4-6]. The release of OT in the brain is likely evoked by external inputs such as sensory cues, food intake, and social rewards[7-10]; however, it can also be controlled in an autoregulatory manner, potentially by the signaling associated with biological clocks[11-13]. It has been reported that the impairment of OT signaling in the brain may underlie the cognitive and emotional dysfunction associated with neurodevelopmental disorders (e.g., autism spectrum disorders and schizophrenia) and brain aging[14,15]. Nevertheless, despite its importance in both health and disease, the relationship between animal behaviors and the temporal distributions of OT in the brain remains largely elusive.

In addition to its important roles as an endogenous ligand, OT has emerged as a potential therapeutic agent for psychiatric disorders based on a finding that exogenous OT administration enhances positive emotions in humans[16]. Furthermore, OT administered peripherally, such as via intranasal or intravenous routes, was originally believed to reach the brain and exert therapeutic effects. However, the potency of exogenously applied OT on central disorders is currently controversial[17-20]. One of the biggest questions is whether OT

57 administered from a peripheral route can efficiently reach the brain through the nose–brain

58 pathway and/or the blood–brain barrier; this has not yet been well addressed[17].

59   In this context, techniques that allow the detection of brain OT dynamics are urgently

60 needed. However, the currently available methods have critical limitations. Although the

61 levels of OT in the brain have classically been inferred from levels in peripheral body fluids,

62 such as plasma, saliva, and urine, peripheral OT levels do not always reflect central OT levels,

63 mainly because of the impermeability of the blood–brain barrier to OT[21]. For the direct

64 measurement of brain OT levels, biochemical analyses of sampled cerebrospinal fluid or

65 dialysate collected through microdialysis probes have been the most prevailing

66 approach[12,22,23]. More recently, a reporter gene-based assay (iTango) has been performed for

67 the detection of OT-stimulated cells in the brain[24]. However, the slow sampling rate of these

68 techniques (usually from several tens of minutes to several hours) is unsuitable for tracking

69 brain OT dynamics in real time.

70   Optical measurements using genetically encoded fluorescent sensors are a potential

71 technique for the *in vivo* detection of signaling molecules, and have good sensitivity, high

72 specificity, and excellent spatiotemporal resolution[25]. However, the utility of optical

73 measurements hinges on the successful development of sensitive fluorescent sensors for

74 specific ligands. Recently, fluorescent sensors for neurotransmitters and neuromodulators

75 such as dopamine, acetylcholine, norepinephrine, and adenosine have been engineered as

76 chimera proteins, consisting of a GPCR with a fluorescent protein (FP) replacing the amino

77 acids in its third intracellular loop (IL3)[26-30]. These sensors show robust fluorescence

78 responses upon agonist binding, likely because of optimal coupling between the

79 conformational change of the IL3 and the environmental change of the FP chromophore.

80 Because the ligands of GPCRs cover a large proportion of extracellular signaling molecules[31],

81 GPCRs are expected to be used as the ligand-binding scaffolds in the development of

82   fluorescent sensors for various ligands.

83   Here, we developed a new green fluorescent OT sensor named MTRIA$_{OT}$, which is

84   composed of a medaka OTR and a circularly permutated green FP (cpGFP)-based fluorescent

85   module named MTRIA (_M_odular fluorescence unit fused with _TR_ansmembrane region-to-

86   _I_ntr_A_cellular loop linkers). We demonstrated that MTRIA$_{OT}$ can be used to analyze changes in

87   brain OT levels following exogenous OT application in anesthetized mice. Moreover,

88   MTRIA$_{OT}$ was able to reveal the existence of oscillatory OT dynamics in a cortical region of

89   freely behaving adult mice, the patterns of which were altered by anesthesia, food deprivation,

90   and aging. Finally, we also demonstrated the utility of MTRIA, the fluorescent module of

91   MTRIA$_{OT}$, for the efficient development of a variety of GPCR-based fluorescent sensors.

92   Together, our findings indicate that MTRIA$_{OT}$ offers opportunities for the detection of OT

93   dynamics in the living brain, and potentially expands the repertoire of GPCR-based

94   fluorescent sensors for extracellular ligands.

95

96   **RESULTS**

97   **Development of an ultrasensitive fluorescent OT sensor**

98   To develop a GPCR-based fluorescent sensor for extracellular OT, we screened for an optimal

99   OTR that showed good targeting to the plasma membrane (PM). Because most species have

100   only a single subtype of OTR, we examined the PM localization of six OTRs derived from

101   different groups of vertebrates (human, mouse, chicken, snake, frog, and medaka) in human

102   embryonic kidney 293T (HEK293T) cells. We chose the medaka OTR (meOTR) as the

103   scaffold for the development of the new fluorescent sensor because its localization had the

104   best correlation with that of a PM marker (Fig. 1a−c and Extended Data Fig. 1a).

105   To engineer a fluorescent OT sensor from meOTR, we determined the optimal

106   insertion site of cpGFP in the IL3 of meOTR (Extended Data Fig. 1b). Through the

107   examination of 21 potential mutants expressed in HEK293T cells, a fusion protein in which

108   cpGFP was inserted into the region between K240 and I268 of meOTR (K240−I268) showed

109   the best performance. This fusion protein had robust fluorescence, good PM targeting, and a

110   slight fluorescence response ($\Delta F/F_0 \sim 0.3$) to 100 nM OT (Extended Data Fig. 1c−g). Thus,

111   we selected this chimera protein as the template for further engineering.

112   To improve the fluorescence response of the initial OT sensor, we screened the

113   mutant sensors expressed in HEK293T cells using the following three steps (Fig. 1d). First,

114   we optimized the linkers in the N- and C-terminal regions surrounding the cpGFP. By

115   examining the fluorescence responses of 124 mutants to 100 nM OT stimulation, we obtained

116   OT-1.0, which had an approximately 150% $\Delta F/F_0$ response (Fig. 1d1). Next, we extended the

117   mutagenesis to the neighboring regions, ranging from the transmembrane helix to the

118   intracellular loops (TM-to-loop). Based on structural information about the conformational

119   transition during GPCR activation[32,33], we introduced mutations at sites from position 5.62 in

120   the fifth transmembrane helix (S230 in meOTR) to position 6.36 in the sixth transmembrane

121 helix (M278 in meOTR) (Extended Data Fig. 2a−c). We examined 55 variants, of which five

122 (S230C, F231Y, Q236R, L240G, and T274R in meOTR) showed increased fluorescence

123 responses upon 100 nM OT stimulation. We then developed an improved sensor, OT-2.0,

124 which contained all five of these mutations. OT-2.0 had about a three-fold brighter basal

125 fluorescence and larger fluorescence response ($\sim$390% $\Delta F/F_0$; Fig. 1d2). Finally, we further

126 developed OT-2.0 by introducing a mutation within the cpGFP. Based on previous knowledge

127 of cpGFP mutagenesis[29], we screened 34 variants, of which three (S57T, S96M, and N202H

128 in cpGFP; Extended Data Fig. 2b−c) had an increased rate of fluorescence change. We then

129 constructed OT-3.0, a variant that contained all three of these mutations (Fig. 1d3). OT-3.0

130 had an approximately 720% $\Delta F/F_0$ fluorescence response upon stimulation with 100 nM OT,

131 which was suppressed by the OTR antagonist L-368,899 (L-36) (Fig. 1e; top and middle). We

132 also generated an OT-insensitive sensor (OT-3.0 mut) that contained the Y206A mutation,

133 which abolished its ligand-binding capacity[32] (Fig. 1e; bottom). We characterized the dose-

134 dependent fluorescence responses of OT-1.0−3.0 in HEK293T cells (Fig. 1f), and the results

135 suggested that our screening had successfully improved the dynamic range of fluorescence

136 responses (F_{max}) with little change to the half maximal effective concentration (EC_{50}) values.

137 Herein, we designated the fluorescent module of OT-3.0 as MTRIA (Extended Data Fig.

138 2b−c) and renamed OT-3.0 as MTRIA$_{OT}$.

139   We then further characterized the ligand specificity of MTRIA$_{OT}$ expressed in

140 HEK293T cells. Compared with its sensitivity to OT, the sensor was similarly sensitive to

141 isotocin (an OT analog in fish), was much less sensitive to vasopressin orthologs (vasopressin

142 and vasotocin) and inotocin (an OT/vasopressin ortholog in insects), and was insensitive to

143 nematocin (an OT/vasopressin ortholog in nematodes) (Extended Data Fig. 3a−b). We also

144 examined the basic properties of MTRIA$_{OT}$, such as its coupling capacity with downstream

145 effectors, long term-fluorescence stability, and kinetics. We used a Ca^{2+} imaging experiment

146 and a split-luciferase complementation assay to assess the coupling of $MTRIA_{OT}$ with $G_{\alpha q}$

147 protein and β-arrestin signaling pathways, which are the downstream effectors of the wild

148 type-meOTR. $MTRIA_{OT}$ had no detectable effects on either of these effectors, whereas the

149 wild type-meOTR was able to couple with them (Extended Data Fig. 3c−d). Furthermore,

150 internalization of $MTRIA_{OT}$ from PM was not detected when $MTRIA_{OT}$-expressing

151 HEK293T cells were chronically exposed to 100 nM OT (Extended Data Fig. 3e). Finally, a

152 kinetic analysis of $MTRIA_{OT}$ was conducted using a local puff of OT followed by L-36,

153 which yielded on- and off-rates of approximately 12 s and 5 min, respectively (Extended Data

154 Fig. 3f). Taken together, these results suggest that our $MTRIA_{OT}$ sensor is equipped with

155 enough sensitivity, specificity, and stability to accurately detect extracellular OT dynamics.

156

157 **Detection of an OT increase in the brain after exogenous OT administration**

158 Having validated the basic properties of $MTRIA_{OT}$ in HEK293T cells, we next tested whether

159 our OT sensor was applicable to experiments in the brains of living mice. Using an adeno-

160 associated virus (AAV), we expressed $MTRIA_{OT}$ in the anterior olfactory nucleus (AON), a

161 major target site of OT in the brain with a very high OTR expression level[6,7]. We then

162 conducted fiber photometry recordings through an implanted cannula that was placed above

163 the injection site (Fig. 2a and Extended Data Fig. 4a−b). First, we examined the responses of

164 $MTRIA_{OT}$ after the intracerebroventricular infusion of OT in anesthetized mice. We prepared

165 a range of solutions that contained different amounts of OT (0, 0.002, 0.02, 0.2, 2, or 20 μg).

166 When these solutions were serially applied through a stainless cannula implanted into the

167 lateral ventricle (Fig. 2a), significant fluorescence increases were observed upon stimulation

168 with OT at doses of 0.2 μg and above (Fig. 2b−c). This result was consistent with previous

169 findings, that intracerebroventricular infusion of a comparative dose of OT is required to

170 trigger OT-dependent animal behaviors[34-36]. This finding indicates that $MTRIA_{OT}$ is capable

171   of real-time detection of extracellular OT in living brains.

172   In the past decade, whether the peripheral administration of OT can alter brain OT

173   levels has been controversial[17]. Because it has been reported that the peripheral administration

174   of less than 20 µg of OT is sufficient to affect animal behaviors[37,38], we next evaluated

175   whether OT levels in the brain increased after the application of exogenous OT from two

176   distinct peripheral administration routes: intranasal or intraperitoneal. Neither intranasal nor

177   intraperitoneal administration of high concentrations of OT induced significant fluorescence

178   responses of MTRIA$_{OT}$ in anesthetized mice (Fig. 2d−e). Taken together with the finding that

179   MTRIA$_{OT}$ even had robust responses to the intracerebroventricular administration of OT

180   solution diluted at 1:100 (Fig. 2b−c), out data suggest that the blood–brain barrier

181   permeability to OT is less than 1%.

182

183   **OT oscillation in the brains of freely behaving mice**

184   Having shown that MTRIA$_{OT}$ is functional in the mouse brain, we next examined whether

185   MTRIA$_{OT}$ can be used to assess endogenous OT dynamics in the brains of adult mice. We

186   virally expressed MTRIA$_{OT}$ in the AON and measured the fluorescence responses using a

187   fiber photometry technique (Fig. 3a). Surprisingly, MTRIA$_{OT}$-mediated fluorescence

188   measurements revealed that transient OT signals were repeated at approximately 2-hour

189   intervals when mice were freely behaving in a cage with food and water supplied *ad libitum*

190   (Fig. 3b−d). We named this ultradian OT rhythm "OT oscillation." OT oscillations were

191   absent in simultaneously recorded reference signals (405 nm of MTRIA$_{OT}$; Fig. 3b; left

192   bottom) and in signals recorded using control sensors (470 nm and 405 nm of MTRIA$_{OT}$-mut;

193   Fig. 3b; right), excluding the involvement of artifacts derived from movements and/or

194   autofluorescence. The expression of tetanus toxin light chain, which prevents vesicular

195   transmitter release[39], in OT-expressing neurons in the PVN suppressed the increase in OT

196 compared with the control (Fig. 3e−h), confirming that OT oscillation is dependent on OT

197 release from oxytocinergic neurons in PVN.

198 We next explored what might affect the patterns of OT oscillation. Because the

199 sleep–wake cycle is a key regulator of biological rhythm, we evaluated OT oscillation in mice

200 when sleep was induced by anesthesia. When an anesthetic mixture of dexmedetomidine,

201 butorphanol, and midazolam was intraperitoneally administered, the fluorescence signal of

202 MTRIA$_{OT}$ fell to a level below the baseline (Fig. 4a, b), suggesting that brain OT levels are

203 suppressed by anesthesia.

204 Given that the feeding process is reportedly associated with OT release[40], we

205 examined the impact of fasting stress on brain OT dynamics. Interestingly, after about half a

206 day of food deprivation, the patterns of OT oscillation gradually became disturbed; the

207 oscillatory signals undershot to a level below the initial baseline (Fig. 4c, d). We named this

208 phenomenon "OT turbulence" (Fig. 4c; dark blue-shaded region). After refeeding, this OT

209 turbulence halted and normal OT oscillation quickly recovered (Fig. 4c, d). This result

210 suggests that fasting stress is likely to be encoded as OT turbulence in the brain.

211 Because aging is associated with a decline in the neuroendocrine system[15,41], we next

212 analyzed the differences in AON OT dynamics between adult (~2 months old) and old-aged

213 (~2.5 years old) mice. The fiber photometry-mediated fluorescence measurements revealed

214 the occurrence of oscillatory OT responses in both groups, but the frequency of OT transients

215 became much slower in the old-aged animals (~24-hour intervals) compared with those in

216 adult animals (~2-hour intervals), although there were no significant changes in amplitudes

217 (Fig. 4e, f). These results suggest that an altered frequency of OT oscillation may underlie

218 aging-associated declines in brain function.

219

220 **Development of fluorescent sensors for various ligands by conjugating MTRIA to other**

GPCRs

Because MTRIA was able to successfully produce a highly sensitive optical readout of OT-dependent OTRs, we finally examined whether MTRIA was also able to detect ligand binding-induced conformational changes of other GPCRs (Fig. 5a). We cloned 184 receptors for 46 ligands derived from either the human, mouse, zebrafish, or medaka, and conjugated MTRIA to a region ranging from position 5.62 of TM5 to position 6.36 of TM6 in the receptors (Extended Data Fig. 2a). To our surprise, almost 30% of the engineered sensors (54/184 proteins) showed a marked fluorescence increase ($> 50\%$ $\Delta F/F_0$) upon stimulation with high concentrations of their specific ligand (Fig. 5b). We listed the 24 sensors that showed the largest fluorescence response among the sensors sharing the same ligand (Fig. 5c, Extended Data Fig. 5) and named them MTRIA sensors (e.g., a sensor for dopamine was called MTRIA$_{DA}$). These results demonstrate the utility of MTRIA for the efficient development of new GPCR-based fluorescent sensors. This simple and efficient approach, MTRIA system, will contribute to accelerating the engineering of various GPCR-based sensors.

238 **DISCUSSION**

239 In this study, we described the development and application of a new fluorescent sensor for

240 OT, MTRIA$_{OT}$, which displayed excellent properties for measuring extracellular OT; it had a

241 large dynamic range (~720% $\Delta F/F_0$), an optimal affinity to OT (EC$_{50}$ of ~20 nM), ligand

242 specificity to OT orthologs, minimal effects on cellular signaling, and long-term fluorescence

243 stability. We then demonstrated the applications of MTRIA$_{OT}$ for measuring exogenously

244 applied and endogenous OT dynamics in the living brain. We also described the utility of the

245 fluorescent module of MTRIA$_{OT}$ (i.e., MTRIA) for the efficient development of functional

246 GPCR-based fluorescent sensors for various ligands. Our new sensor will be valuable for

247 analyzing the dynamics of extracellular signaling *in vivo*.

248 Although the impacts of exogenous OT administration on mood and emotions are

249 expected to lead to new treatments for psychiatric disorders, direct evidence for an increase in

250 brain OT levels following peripheral OT administration remains limited. Here, we

251 demonstrated that MTRIA$_{OT}$-mediated OT recording is useful for detecting changes in brain

252 OT levels following exogenous OT administration. In the present study, neither intranasal nor

253 intraperitoneal administration of a relatively large amount (20 µg) of OT resulted in

254 significant changes in the fluorescence signal of MTRIA$_{OT}$ in the brain of adult mice. In

255 contrast, intracerebroventricular administration of OT at concentrations as low as 0.2 µg

256 triggered a robust increase in brain OT levels (Fig. 2), supporting the notion that the efficacy

257 of OT transport through the blood–brain barrier is very low[42]. As has been mentioned

258 elsewhere[17,43], invasive measurements of OT using acutely implanted probes may lead to the

259 leakage of OT from injured blood vessels into the brain parenchyma following peripheral OT

260 administration. Given that peripheral OTRs are distributed in various tissues including the

261 heart, adrenal medulla, adipose tissue, and peripheral neurons[3], the actions of exogenously

262 applied OT on peripheral OTRs may have an impact on animal behaviors.

263   Accumulating evidence suggests that rhythmic hormonal signaling, with frequencies

264   ranging from a few hours (ultradian) or around a day (circadian) to more than a day

265   (infradian), plays an important role in the regulation of a range of biological processes,

266   including body homeostasis (e.g., sleep, feeding, and metabolism), mood, and cognitive

267   performance[44-46]. Although circadian changes in OT levels in the cerebrospinal fluids of

268   primates[12,47] and rodents[48] have been proposed, it has not yet been explored whether ultradian

269   regulation of OT levels exist; this is presumably because of the limited sensitivity and slow

270   sampling rates of previous techniques. Taking advantage of the faster time resolution of our

271   time-lapse fluorescence recordings, we revealed that OT levels in the AON exhibit ultradian

272   rhythms with approximately 2-hour intervals in freely behaving mice. However, we cannot

273   exclude the existence of much faster OT transients, which may be masked by the slow

274   kinetics of MTRIA$_{OT}$ in fluorescence decay ($\tau_{off} > 10$ min) upon agonist dissociation. Our

275   findings lead to a question: how is OT oscillation regulated? It should be noted that PVN

276   neurons in slice cultures show spontaneous synchronized oscillations in Ca^{2+} activity at

277   intervals of a few hours[49], implicating that ultradian OT rhythms may be controlled by a Ca^{2+}-

278   dependent biological clock. There may also be rhythmic changes in other neuromodulators

279   that show synchronization with OT oscillation. It is of particular interest that ultradian

280   rhythms of glucocorticoid secretion, which is thought to be coupled with OT levels[50], is

281   important for good sleep quality and cognitive function in healthy humans[44]. Thus, future

282   efforts are needed to clarify the regulatory mechanisms and physiological significance of OT

283   oscillation.

284   Overall, our MTRIA$_{OT}$-mediated measurements suggest the importance of temporal

285   information about OT dynamics in the brain. Moreover, our analyses indicate that the patterns

286   of ultradian OT oscillation can be affected by anesthesia, food deprivation, and aging. Thus, it

287   is important to carefully consider the experimental conditions and the state of subjects when

288   interpreting OT measurement data in the brain. The context- and subject-dependent

289   inconsistencies regarding the effects of OT in human clinical trials[20] may be related to such

290   parameters. The use of MTRIA$_{OT}$ will allow us to extend our knowledge of brain OT

291   dynamics and their associations with complex behaviors.

292

293 **METHODS**

294 **DNA constructions**

295 DNA encoding GPCRs, a red fluorescent Ca^{2+} sensor jRGECO1a[51], and PM-targeted

296 mScarlet[52] ($mSca_{mem}$) were amplified by polymerase chain reaction (PCR), and were then

297 subcloned into the pCIS vector, which is a CAG promoter-driven expression vector[53]. The

298 template DNA for GPCRs was prepared using the following four approaches. First, cDNA

299 libraries of a mouse (*Mus musculus*) brain, zebrafish (*Danio rerio*) brain, and medaka

300 (*Oryzias latipes*) brain were prepared using the RNeasy Kit (Qiagen, Hilden, Germany) and

301 QuantiTect Reverse Transcription Kit (Qiagen). Second, genomic DNAs of human (*Homo*

302 *sapiens*) and mouse were isolated from HEK293T cells and a mouse brain, respectively.

303 Third, gene fragments of OTRs derived from chicken (*Gallus gallus*), snake (*Notechis*

304 *scutatus*), and frog (*Xenopus laevis*) were synthesized by Integrated DNA Technologies

305 (Coralville, IA, USA). Finally, for cloning of human dopamine receptor DRD1, human

306 muscarinic receptor CHRM3, and human adrenergic receptor ADRA2A, plasmids encoding

307 dLight1.1, GACh2.0, and GRAB$_{NE}$1m were used as the templates, respectively[27-29]. GPCRs

308 used in Fig. 5 and the primer pairs for their cloning were listed in Extended Data Table 1. To

309 assess the localization of OTRs in HEK293T cells, a human influenza hemagglutinin (HA)-

310 tag was added to the N-terminal regions of the OTRs using PCR. For the construction of the

311 green-fluorescent transmitter sensors, cpGFPs derived from GCaMP6s were inserted into the

312 IL3 regions of GPCRs using either overlap extension PCR[54] or the Gibson assembly

313 protocol[55]. For mutant sensor screening, site-directed mutagenesis was performed using

314 primers containing mutated nucleotide(s). In particular, randomized site-directed mutagenesis

315 was achieved using primers containing randomized codons (NNN). For the construction of

316 plasmid DNA in the β-arrestin recruitment assay, fragments of NanoLuc—SmBit and LgBit—

317 were amplified from pNL1.1 (Promega, Madison, WI, USA). They were then fused with the

318   C-terminus of OTR and the N-terminus of rat β-arrestin[56], followed by cloning into the pCIS

319   vector. For the construction of transfer plasmid DNA for AAVs, MTRIA$_{OT}$ and MTRIA$_{OT}$-mut

320   were cloned into the pAAV Syn woodchuck hepatitis virus post-transcriptional regulatory

321   element (WPRE) vector, which is a plasmid driven by a human synapsin promoter. In

322   addition, mScarlet (mSca) and tetanus toxin light chain-P2A-mSca were cloned into the

323   pAAV Oxt WPRE vector, which is a plasmid driven by a mouse OT promoter.

324

325   **Cell culture**

326   HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (FUJIFILM-Wako,

327   Osaka, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific,

328   Waltham, MA, USA), 100 U/ mL penicillin, and 100 µg/mL streptomycin at 37°C under a

329   humidified atmosphere of 5% CO_2 and 95% air. For the fluorescence microscopy

330   observations, cells were transfected with 1 µg of plasmid using 2 µg of Polyethylenimine Max

331   (PEI MAX) transfection reagent (Polysciences, Warrington, PA, USA), and were seeded onto

332   either glass bottom dishes or glass bottom chambers (Matsunami, Osaka, Japan) coated with

333   collagen (Nitta Gelatin, Osaka, Japan). After a 3-hour incubation, the medium was changed,

334   and the cells were further cultured for 24–36 hours before the imaging experiments.

335

336   **AAV production**

337   pHelper, XR8, and a transfer plasmid were simultaneously transfected into HEK293T cells

338   using PEI MAX transfection reagent. After overnight incubation, the medium was changed.

339   Supernatant was collected at 48- and 96-hour time points, after medium exchange. Viral

340   particles were purified using a polyethylene glycol-mediated precipitation method[57]. The

341   purified viral particles were then concentrated using an ultrafiltration membrane unit (Amicon

342   Ultra; EMD Millipore, Burlington, MA, USA). Virus titers were determined by quantitative

343 PCR using a pair of primers for the WPRE sequence (forward: actgtgtttgctgacgcaac, reverse:

344 agcgaaagtcccggaaag). Viral vectors with titers $> 10^{12}$ gc/mL were used for the fiber

345 photometry measurements.

346

347 **Immunostaining of transfected cells**

348 For the immunostaining analysis of the cellular localization of OTRs, HEK293T cells that had

349 been co-transfected with an HA-tagged OTR and $mSca_{mem}$ (10:1 ratio) were fixed in 4% (w/v)

350 paraformaldehyde-containing phosphate-buffered saline (PBS; FUJIFILM-Wako) for 10 min

351 at room temperature. Next, cells were permeabilized and blocked for 30 min at room

352 temperature in blocking solution (PBS containing 0.2% TritonX-100 [PBST] and 5% normal

353 goat serum [FUJIFILM Wako]), and were then incubated with anti-HA antibody (rabbit;

354 MBL, Aichi, Japan) for 30 min at room temperature. After washing with PBST, samples were

355 incubated with Alexa 488-conjugated anti-rabbit IgG antibody (goat; Jackson

356 ImmunoResearch, West Grove, PA, USA) for 30 min at room temperature. After three washes

357 with PBST, the stained cells in PBS underwent microscopic analysis.

358

359 **Sensor screening and evaluation by fluorescence microscopy**

360 The fluorescence images in Figures 1 and 5 and Extended Data Figures 1, 3, 4, and 5 were

361 obtained using Dragonfly 301, a spinning-disk confocal microscope system (Andor

362 Technology, Belfast, UK) equipped with a Nikon Eclipse Ti2 (Nikon, Tokyo, Japan), lasers

363 (405, 488, 561, and 637 nm), emission filters (450 ± 25 nm for the 405 nm laser, 525 ± 25 nm

364 for the 488 nm laser, 600 ± 25 nm for the 561 nm laser, and 700 ± 37.5 nm for the 637 nm

365 laser), and an electron multiplication charge-coupled device (EMCCD) camera (iXon Life

366 888; Andor Technology). Either a dry objective lens (CFI PLAN APO 20×, NA 0.75; Nikon)

367 or a water-immersion lens (CFI Plan Apo IR 60×C WI, NA 1.27; Nikon) was used to

368   illuminate the lasers. For the live-imaging experiments, data were acquired every 5 s. To

369   analyze the cellular localization of fluorescent sensors, z-stack images were acquired at 0.5

370   μm steps. For the screening of fluorescent sensors, transfected cells seeded onto glass-

371   bottomed chamber slides were soaked in 200 μL artificial extracellular solution (ECS; 150

372   mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 5.6 mM glucose; pH

373   7.4). They were then stimulated with 200 μL drug-containing ECS, in which the drug

374   concentration was twice the final concentration, administered through a pipette. To analyze

375   the ligand dose–response of the fluorescent sensors, time-constant measurements, Ca^{2+}

376   imaging, and the evaluation of long-term signal stability were performed. The transfected

377   cells were seeded onto a collagen-coated glass-bottomed dish and continuously perfused with

378   ECS. They were then rapidly stimulated with drug-containing solution using a custom-made

379   perfusion system equipped with solenoid valves.

380

381   **β-Arrestin recruitment assay**

382   The recruitment of β-arrestin to meOTR and MTRIA$_{OT}$ was assessed using the split-luciferase

383   complementation assay. Either meOTR-SmBit or MTRIA$_{OT}$-SmBit was co-transfected with

384   LgBit-arrestin in HEK293T cells that were seeded onto an opaque 96-well plastic plate using

385   PEI MAX reagent. Twenty-four hours after transfection, the cells were washed with Hanks'

386   balanced salt solution (HBSS), and stimulated with HBSS containing 100 nM OT. After the

387   addition of a luciferase substrate (ONE-Glo™ Luciferase Assay System, Promega),

388   luminescence was measured using a plate reader (Infinite 200 Pro F Plex; Tecan, Männedorf,

389   Switzerland).

390

391   **Reagents**

392   OT, angiotensin (Agt), [Arg8]-vasopressin (Avp), bradykinin (Bdk), cholecystokinin (Cck),

393 corticotropin-releasing hormone (Crh), endothelin-1 (ET-1), galanin (Gal), ghrelin (Ghr), α-

394 melanocyte stimulating hormone (Msh), melanin-concentrating hormone (Mch), neuromedin

395 B (Nmb), neuromedin U (NmU), neuropeptide Y (Npy), neurotensin (Nts), met-enkephalin

396 (Enk), nociceptin (Ncp), orexin B (Ox), prolactin-releasing hormone (Prlh), parathormone

397 (Pth), somatostatin (Sst), substance P (SP), neurokinin B (Nkb), and urotensin (Uts) were

398 purchased from Peptide Institute (Osaka, Japan). L-368,899 hydrochloride, neuropeptide ff

399 (Npff), sphingosine-1-phosphate (S1P), uridine 5′-diphosphate disodium salt (UDP), and

400 anandamide (Ana) were purchased from Tocris Bioscience (Bristol, United Kingdom).

401 Isotocin, C3a anaphylatoxin (C3a), and C5a anaphylatoxin (C5a) were purchased from

402 Bachem (Bubendorf, Switzerland). Vasotocin and thrombin receptor agonist (Thr) were

403 purchased from Anygen (Gwangju, Korea), and inotocin was purchased from Phoenix

404 Pharmaceuticals (Mannheim, Germany). Histamine disodium salt (His) and melatonin (Mtn)

405 were purchased from FUJIFILM-Wako. Leukotriene B4 (Ltb), leukotriene E4 (Lte), 1-oleoyl

406 lysophosphatidic acid (LPA), platelet-activating factor C-16 (PAF), and prostagrandin F2a

407 (Pgf) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Prostagrandin D2

408 (Pgd), prostagrandin E2 (Pge), prostagrandin I2 sodium salt (Pgi), 5-hydroxytryptamine

409 hydrochloride (5-HT), dopamine hydrochloride (DA), acetylcholine chloride (ACh), and

410 adenosine 5′-triphosphate disodium salt (ATP) were purchased from Nacalai (Kyoto, Japan).

411 Epinephrine (Epi) was purchased from Daiichi-Sankyo (Tokyo, Japan), and norepinephrine

412 (NE) was purchased from Alfresa Pharma Corporation (Osaka, Japan). Nematocin was

413 synthesized by Cosmo Bio (Tokyo, Japan). The final concentration of the applied ligands in

414 Fig. 5b were as follows;

415 100 μM: DA, NE, ACh, His, 5-HT, ATP, UDP, and Ado; 1 μM: Enk, C3a, Ana, Lpa, Paf, Pgd,

416 Pge, Pgf, and Pgi; and 100 nM: Mtn, Agt, Avp, Bdk, Cck, Crh, ET-1, Gal, Ghr, Msh, Mch,

417 Nmb, Nmu, Npff, Npy, Nts, Ncp, Ox, Prlh, Pth, Sst, SP, Nkb, Uts, C5a, Thr, S1P, Ltb, and

418 Lte.

419

420 **Animal surgery**

421 All animal procedures were conducted in accordance with the guidelines of Kanazawa

422 University and Osaka University. C57BL/6 J or C57BL/6N female mice at either 6–8 weeks

423 postnatal (adult mice) or 2.5 years postnatal (old-age mice) were purchased from CLEA Japan

424 Inc. (Tokyo, Japan). Stereotaxic surgery was performed under anesthesia with isoflurane. The

425 depth of anesthesia was assessed using the tail pinch method. Body temperature was

426 maintained using a heating pad. One microliter of AAV suspension within a glass micropipette

427 was injected into the left medial AON (2.2 mm anteroposterior [AP] and 0.3 mm mediolateral

428 [ML] from bregma, and −4.0 mm dorsoventral [DV] from the skull surface). After the virus

429 injection, a fiber-optic cannula with a 400 μm core diameter and 0.39 NA (CFMC14L05;

430 Thorlabs, Newton, NJ, USA) was implanted just above the injection site (2.2 mm AP, 0.3 mm

431 ML, and −3.8 mm DV). For the intracerebroventricular injection experiments, a stainless

432 cannula fabricated from a 22 G needle was additionally inserted into the right lateral ventricle

433 (−0.7 mm AP, 1.5 mm ML, and −2.5 mm DV). To fix and protect the implanted cannula(s),

434 dental cement (Ketac Cem Easymix; 3M, Maplewood, MN, USA) and silicone rubber (Body

435 Double; Smooth-On, Macungie, PA, USA) were used. Carprofen (5 mg/kg; intraperitoneal), a

436 non-steroidal anti-inflammatory drug, and buprenorphine (0.1 mg/kg, intraperitoneal), an

437 opioid analgesic, were administered after the surgery. At 2 weeks or more following the AAV

438 injection, the mice were used for the fiber photometry experiments. On the measurement day,

439 the cannula was coupled to a patch cable (M79L01; Thorlabs) via an interconnector (ADAF1

440 or ADAF2; Thorlabs).

441

442 **Fiber photometry measurements**

443   The fiber photometry setup (Extended Data Fig. 4a) was constructed as described previously,

444   with a minor modification[58]. Excitation light from a light-emitting diode (LED) was directed

445   into a patch cable (M79L01) through an objective lens (CFI Plan Fluor 20× lens; Nikon), and

446   the emission light was projected onto the sensor of a scientific complementary metal-oxide

447   semiconductor (sCMOS) camera (Zyla 4.2P; Andor Technology) after passing through a

448   dichroic mirror (Di01-R405/488/561/635-25×36; Semrock, Rochester, NY, USA) and an

449   emission filter (YIF-BA510-550S; SIGMA KOKI, Tokyo, Japan). To acquire an OT-

450   dependent signal and an isosbestic signal, two different LED light sources—a 470 nm light

451   (light from M470F3 filtered with FB470-10, 4 µW at the fiber tip; Thorlabs) and a 405 nm

452   light (light from M405FP1 filtered with FB410-10, 4 µW at the fiber tip; Thorlabs)—were

453   alternatively switched. Data were acquired at 2 s intervals with an LED light exposure of 0.3

454   s. The device control and data acquisition were conducted using a custom-made LabVIEW

455   program (National Instruments, Austin, TX, USA). Data were processed using Fiji software[59],

456   and were then analyzed using a custom-made Python program. The experiments were

457   conducted within a cage (width: 270 mm, length 440 mm, and height 18.7 mm), and the

458   behaviors of mice were captured every 1 s using an overhead camera (BFS-U3-I3Y3-C; FLIR

459   Systems, Wilsonville, OR, USA). To visualize the behaviors of mice in the dark environment,

460   light from an infrared LED array (AE-LED56V2; Akizuki, Aichi, Japan) was used. Unless

461   stated otherwise, mice were fed standard pellet and water *ad libitum*. For the experiments

462   shown in Fig. 2 and Fig. 4a, mice were anesthetized by the intraperitoneal administration of a

463   mixture of 0.375 mg/kg dexmedetomidine hydrochloride, 2 mg/kg midazolam, and 2.5 mg/kg

464   butorphanol tartrate. For recovery from anesthesia, 0.75 mg/kg atipamezole hydrochloride

465   was applied intraperitoneally.

466

467   **Verification of implantation sites**

468   After the fiber photometry measurements, we confirmed the sensor expression and cannula

469   insertion sites by immunostaining. The brains of anesthetized mice were rapidly removed

470   after decapitation, and were immersed in 4% (w/v) paraformaldehyde-containing PBS

471   overnight at 4°C, followed by incubation in 20% (w/v) sucrose-containing PBS overnight at

472   4°C. After being embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo,

473   Japan), coronal sections of brains were prepared at 50 μm thickness using a cryostat

474   (CM1950; Leica Microsystems, Wetzlar, Germany). For immunostaining, the sections were

475   permeabilized in blocking solution for 30 min at room temperature, and were then incubated

476   with anti-GFP antibody (rabbit; MBL; 1:1000 dilution) overnight at 4°C. After three washes

477   with PBST, the sections were incubated with Alexa 488-conjugated anti-rabbit IgG antibody

478   (goat; Jackson ImmunoResearch; 1:2000 dilution) for 40 min at room temperature. After three

479   washes with PBST and three washes with PBS, the samples were mounted in polyvinyl

480   alcohol-based mounting medium containing 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI;

481   FUJIFILM-Wako) and 2.5% (w/v) 1,4-diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO,

482   USA) as an antifade. The stained sections were then imaged using a spinning-disk confocal

483   microscope system (Dragonfly 301). Mice with misplacement of the cannula were excluded

484   from the analysis.

485

486   **Statistical analysis**

487   All summary data are expressed as the mean ± standard error of the mean (SEM) unless stated

488   otherwise. The z-score was calculated as the average peak response divided by the standard

489   deviation of the baseline fluorescence. For the comparison of two groups, we used two-tailed

490   Student's *t*-tests. One-way analysis of variance (ANOVA) tests were performed when more

491   than two groups were compared, followed by either the Tukey–Kramer or Dunnett's *post-hoc*

492   test. Throughout the study, $P < 0.05$ was considered statistically significant. Data distribution

493   was assumed to be normal, and variance was similar between the groups that were statistically

494   compared. No statistical methods were used to predetermine the sample sizes, but our sample

495   sizes were similar to those generally used in the field[26-30].

496

AUTHOR CONTRIBUTIONS

D.I. conceived the project, performed the experiments, and analyzed the data. D.I., H.H., and M.N. discussed the results and wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

522

523   **CODE AVAILABILITY**

524   All analysis scripts are available from the corresponding author upon reasonable request.

525

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675

676

Figure 1

Figure 2

Figure 3

Figure 4

a MTRIAOT GPCRs **Screening** MTRIA

b $\Delta F/F_0$

c

Sensor	Receptor	Species	F_{max}	EC_{50} (M)
MTRIA DA	DRD1	Human	2.40	9.31×10^{-7}
MTRIA NE	Adrb2	Mouse	2.31	3.26×10^{-6}
MTRIA 5-HT	Htr1	Medaka	3.29	2.00×10^{-7}
MTRIA Mtn	Mtnr1a	Medaka	4.06	6.11×10^{-9}
MTRIA ATP	P2ry2	Mouse	1.27	3.04×10^{-6}
MTRIA Agt	Agtr2	Medaka	2.84	3.60×10^{-8}
MTRIA Avp	Avpr2	Medaka	2.46	2.00×10^{-8}
MTRIA Cck	CckrL	Medaka	2.18	3.10×10^{-8}
MTRIA Mch	Mchr1	Mouse	1.18	4.85×10^{-8}
MTRIA Nmb	Nmbr	Zebrafish	2.13	4.93×10^{-9}
MTRIA Npff	Npffr2	Mouse	1.73	7.28×10^{-8}
MTRIA Npy	Npy2r	Medaka	2.29	6.13×10^{-8}
MTRIA Nts	Ntsr1	Medaka	1.22	2.96×10^{-8}
MTRIA Enk	Oprd1a	Zebrafish	1.70	1.18×10^{-8}
MTRIA Ncp	Oprl1	Mouse	4.68	4.43×10^{-9}
MTRIA Ox	Hcrtr2	Medaka	0.95	3.95×10^{-9}
MTRIA Prlh	Prlhr2b	Zebrafish	0.72	2.75×10^{-8}
MTRIA Sst	Sstr2	Medaka	2.50	5.39×10^{-8}
MTRIA Nkb	Tac3r	Mouse	0.81	3.93×10^{-8}
MTRIA Uts	Uts2r	Zebrafish	3.47	4.24×10^{-8}
MTRIA C3a	C3ar1	Mouse	3.55	1.16×10^{-7}
MTRIA Ltb	Ltb4r	Medaka	0.46	5.60×10^{-6}
MTRIA Paf	Ptafr	Medaka	0.67	3.72×10^{-6}
MTRIA Pge	Ptger4	Mouse	1.34	1.42×10^{-6}

Figure 5

Extended Data Figure 1

a

b

MTRIA

5.62   6.36

230   240   268   278

meOTR   SFKIWQNFKLK   ...   ISKAKITTVKM

MTRIA_OT   CYKIWRNFKGK L cpGFP S V ISKAKIRTVKM

S57T

N202H

S96M

Active state (5C1M)
Inactive state (6TPK)

c

```
  1 MEIISNESEIWQFNGSWRNSSLGNGTGALNQTNPLKRNEEVAKVEVTVLA  50
 51 LVLFLALAGNLCVLLAIHTTKHSQSRMYYFMKHLSIADLVVAIFQVLPQL 100
101 IWDITFRFYGPDILCRLVKYLQVVGMFASTYMLVLMSIDRCLAICQPLHS 150
151 LHRRKDRIYVILSWLLSLIFSIPQMFIFSLREVGSAGSGVYDCWGDFVKP 200
201 WGAKAYITWISLTIYIIPVAILSICYGLICYKIWRNFKGKLNVYIKADKQ 250
251 KNGIKANFHIRHNIEDGGVQLAYHYQQNTPIGDGPVLLPDNHYLSVQTKL 300
301 SKDPNEKRDHMVLLEFVTAAGITLGMDELYKGGTGGMMVRKGEELFTGVV 350
351 PILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT 400
401 TLTYGVQCFSRYPDHMKQHDFFKSAMPEGYIQERTIFFKDDGHYKTRAEV 450
451 KFEGDTLVNRIELKGIDFKEDGNILGHKLEYNSVISKAKIRTVKMTFVIV 500
501 VAYIVCWTPFFSVQMWSAWDQAAPREAMPFIISMLLASLNSCCNPWIYMC 550
551 FAGHLFHDLRQNLLCCSTRYLKSPECRCERDFNSSHKSNSSNFAIKSTSS 600
601 SRSITQTSTT 610
```

Extended Data Figure 2

a

disulfide bond

Oxtocin	CYIQNCPLG-(amide)
Isotocin	CYISNCPIG-(amide)
Vasopressin	CYFQNCPRG-(amide)
Vasotocin	CYIQNCPRG-(amide)
Inotocin	CLITNCPRG-(amide)
Nematocin	CFLNSCPYRRY-(amide)

b

MTRIA_OT

	F_{max}	EC_{50} (nM)
OT	7.35	20.5
IT	6.93	18.9
INT	4.50	88.5
VT	3.05	192.2
AVP	2.74	207.6
NMT	—	—

$\Delta F/F_0$ vs Ligands (nM)

c

OT

meOTR + jRG1a

MTRIA_OT + jRG1a

$\Delta F/F_0$

1 min

Peak $\Delta F/F_0$

***

d

Relative Luminescence

*

NS

Oxt (−) (+) (−) (+)

meOTR-SmBit +LgBit-Arrestin

MTRIA_OT-SmBit +LgBit-Arrestin

e

MTRIA_OT

***

NS

$\Delta F/F_0$

Pre, 10 min, 30 min, 60 min, 120 min

f

OT   L-36

$\tau_{off} = 5.3 \pm 0.4$ min

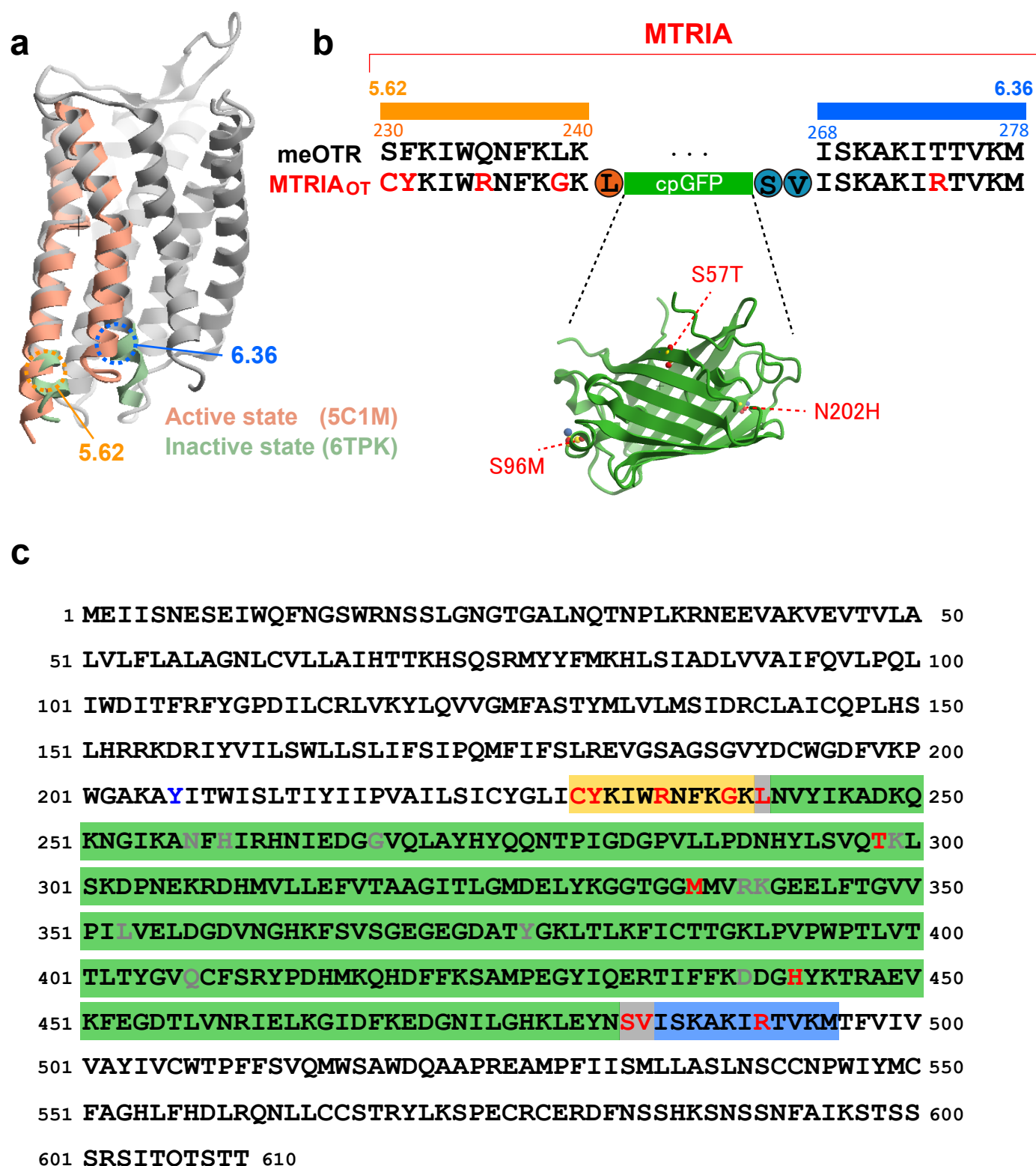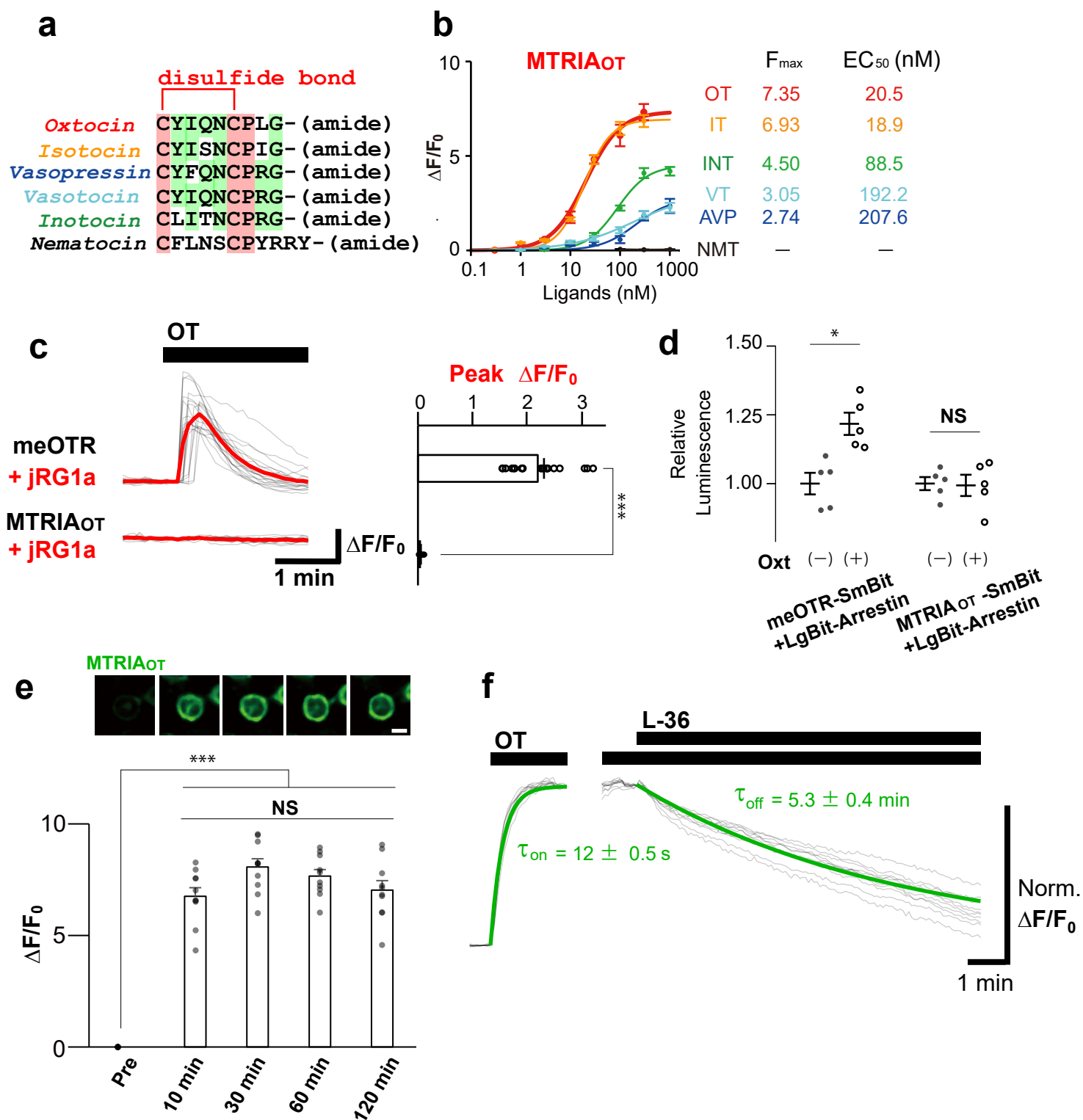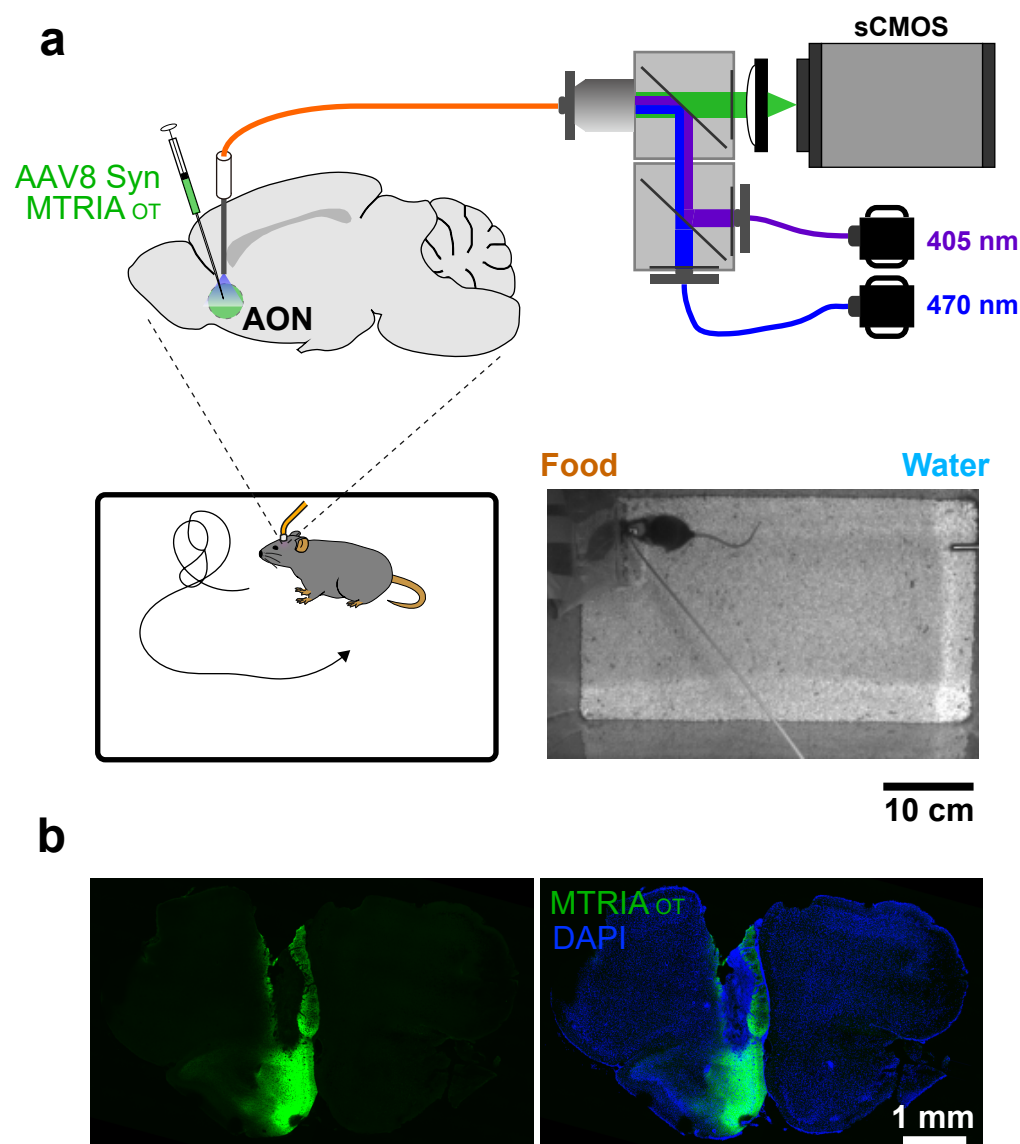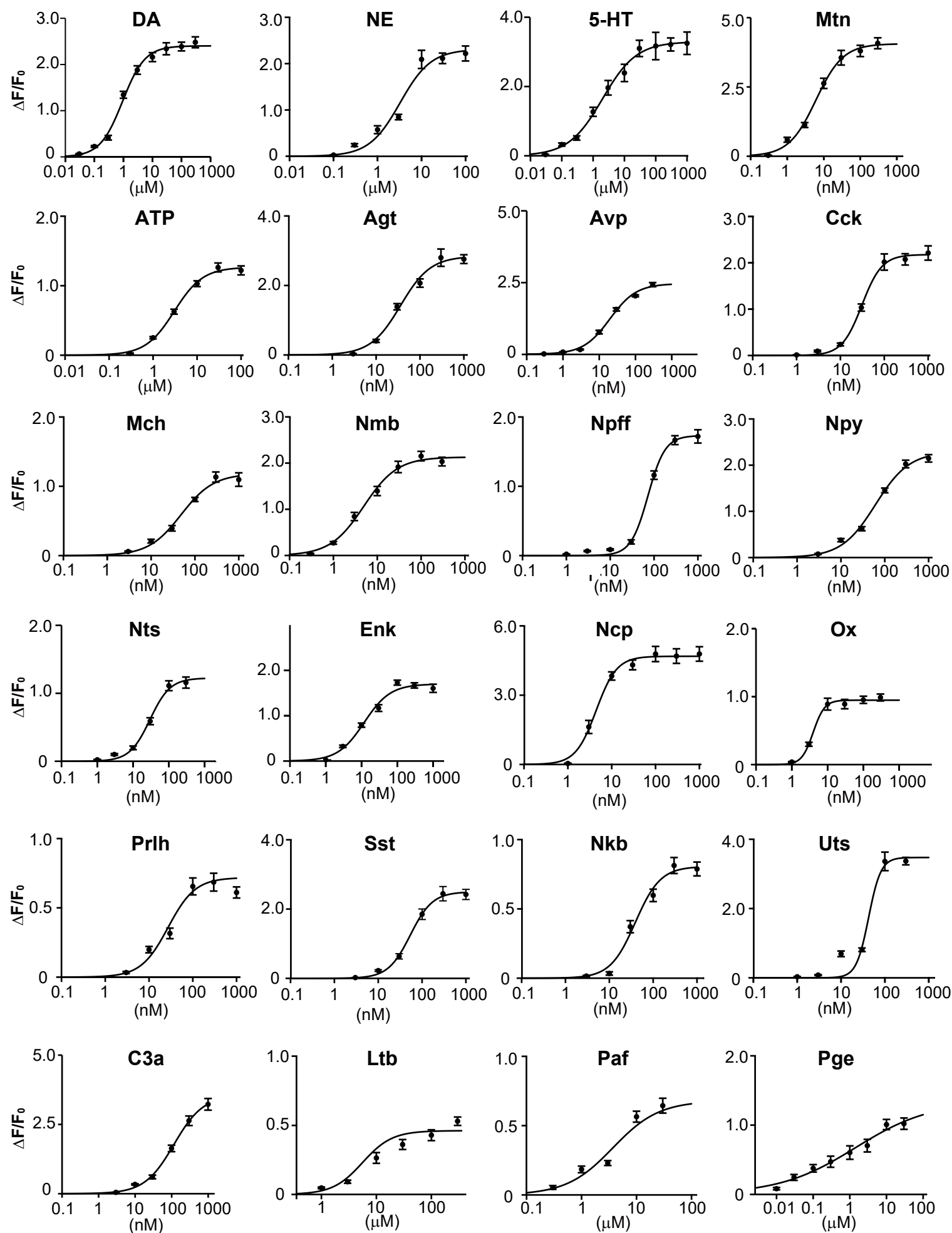$\tau_{on} = 12 \pm 0.5$ s

Norm. $\Delta F/F_0$

1 min

Extended Data Figure 3

Extended Data Figure 4

Extended Data Figure 5

677 **FIGURE LEGENDS**

678 **Figure 1. Development of a new fluorescent OT sensor.**

679 **a**, Schematic representation of a PM-targeting OTR conjugated with an HA-tag at the N-

680 terminus. **b**, Images of HEK293T cells co-expressing an HA-tagged OTR (HA: green) and a

681 PM-targeted mScarlet (mSca$_{mem}$: red). The right traces compare the normalized fluorescence

682 intensities of HA and mSca$_{mem}$ signals on the dotted lines. **c**, Summary of the Pearson

683 correlation coefficients of fluorescence signals in **b** (n = 8, 17, 10, 10, 12, 10, and 17 cells for

684 human, mouse, chicken, snake, frog, and medaka, respectively). Statistics: one-way ANOVA

685 ($F_{5,68}$ = 2.35, P = 4.9×10^{-19}) with Bonferroni *post-hoc* test (P = 3.3×10^{-9}: human vs. medaka,

686 P = 8.9×10^{-9}: mouse vs. medaka, P = 5.4×10^{-14}: chicken vs. medaka, P = 2.4×10^{-19}: snake vs.

687 medaka, P = 2.3×10^{-8}: frog vs. medaka). **d**, Development of an ultrasensitive fluorescent OT

688 sensor over three step-screening; optimization of linker regions (**d1**), TM-to-loop regions

689 (**d2**), and cpGFP moiety (**d3**). **d1–d3**, schematics of mutagenesis (top), basal fluorescence

690 images and heatmap images depicting the responses to 100 nM OT (middle), and scatter plots

691 describing the relationship between basal brightness and the fluorescence response to 100 nM

692 OT (bottom). **e**, Traces of fluorescence responses (gray: each trace, green: average trace) of

693 either OT-3.0- or OT-3.0-mut-expressing cells upon stimulation with the indicated drugs.

694 Summary of peak $\Delta F/F_0$ (n = 10 cells per group). Statistics: one-way ANOVA ($F_{2,27}$ = 3.35, P

695 = 5.7×10^{-16}) with Bonferroni *post-hoc* test (P = 4.3×10^{-10}: top vs. middle, P = 4.3×10^{-10}: top

696 vs. bottom, P = 1: middle vs. bottom). **f**, Dose–response curves of the sensors (n = 10 cells per

697 point). The F_{max} and EC$_{50}$ values are summarized on the right. Scale bars, 10 μm (**b, d**).

698 Graphs represent the mean ± SEM (**c, e, f**). ***P < 0.001, NS: not significant (**c, e**).

699

700 **Figure 2. *In vivo* real-time measurement of brain OT dynamics following exogenous OT**

701 **administration.**

702   **a**, Schematic illustrating the fiber photometry recording of $MTRIA_{OT}$ in the AON. **b**,

703   Representative trace of the normalized fluorescence intensity of $MTRIA_{OT}$ following

704   intracerebroventricular (ICV) injection of OT at the indicated dose. **c**, Summary of

705   normalized $\Delta F/F_0$. ($n = 5$ mice). Statistics: one-way ANOVA ($F_{5,24} = 2.62$, $P = 1.4 \times 10^{-12}$)

706   with Dunnett's *post-hoc* test to compare with 0 ($P = 1$: 0.002 vs. 0, $P = 1$: 0.02 vs. 0, $P =$

707   0.0090: 0.2 vs. 0, $P = 5.4 \times 10^{-10}$: 2 vs. 0, $P = 5.2 \times 10^{-14}$: 20 vs. 0). **d**, Representative traces of

708    $\Delta F/F_0$ upon stimulation with either 20 µg OT or saline via the indicated administration routes

709   (top: ICV, middle: intranasal [IN], bottom: intraperitoneal [IP]). **e**, Summary of peak $\Delta F/F_0$.

710   ($n = 3$ mice). Statistics: one-way ANOVA ($F_{3,8} = 4.07$, $P = 3.8 \times 10^{-5}$) with Bonferroni *post-*

711   *hoc* test ($P = 0.020$: ICV+OT vs. ICV+saline, $P = 1$: IN vs. ICV+saline, $P = 1$: IP vs.

712   ICV+saline). Graphs represent the mean ± SEM (**c**, **e**). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$,

713   NS: not significant (**c**, **e**).

714

715   **Figure 3. *In vivo* real-time measurement of brain OT dynamics in freely behaving mice.**

716   **a**, Schematic illustrating the fiber photometry recording of $MTRIA_{OT}$ in the AON in freely

717   behaving mice. **b**, Representative traces of 470 nm-excited signals (green) and 405 nm-

718   excited signals (blue) from either $MTRIA_{OT}$- or $MTRIA_{OT}$ mut-expressing mice. **c**, Summary

719   of peak z-scores ($n = 4$ mice). Statistics: one-way ANOVA ($F_{3,12} = 3.49$, $P = 7.4 \times 10^{-5}$) with

720   Bonferroni *post-hoc* test ($P = 0.026$: 470 nm vs. 405 nm in $MTRIA_{OT}$, $P = 0.034$: 470 nm in

721    $MTRIA_{OT}$ vs. 470 nm in $MTRIA_{OT}$-mut, $P = 0.21$: 470 nm vs. 405 nm in $MTRIA_{OT}$-mut). **d**,

722   Frequency histogram showing the intervals of OT signal peaks ($n = 26$ events from four

723   mice). **e**, Schematic illustrating the experimental protocol for assessing the involvement of

724   vesicular release from PVN OT neurons in the OT signal increase in the AON. **f**,

725   Representative traces of $MTRIA_{OT}$ activities recorded from mice either expressing mSca or

726   co-expressing tetanus toxin light chain (TeLC) and mSca in the PVN. **g**, **h**, Summary of the

727   peak number every hour (**g**) and peak z-score (**h**) (n = 3 mice in mSca and n = 4 mice in

728   TeLC-P2A-mSca). Statistics: unpaired two-tailed t-test (P = 8.2×10^{-4} in **g** and P = 1.6×10^{-4} in

729   **h**). Graphs represent the mean ± SEM (**c**, **g**, **h**). ***P < 0.001, *P < 0.05, NS: not significant

730   (**c**, **g**, **h**).

731

732   **Figure 4. Alterations in OT oscillation caused by anesthesia, food-deprivation, and**

733   **aging.**

734   **a**, Representative trace of MTRIA$_{OT}$ activity showing the impact of anesthesia on OT

735   oscillation. The background of the trace is shaded to indicate the period during anesthesia

736   (dark blue) and the period after release by an antagonist (pink). **b**, Summary of the minimum

737   values of z-scores before and during anesthesia (n = 3 mice). Statistics: unpaired two-tailed t-

738   test (P = 0.018). **c**, Representative trace of MTRIA$_{OT}$ activity showing the impact of food

739   deprivation on OT oscillation. The background of the trace is shaded to indicate the period of

740   food deprivation. The period of OT turbulence is colored dark blue and the peaks of undershot

741   signal are indicated by arrowheads. **d**, Summary of the minimum z-score values before,

742   during (Food dep.), and after food deprivation (n = 3 mice). Statistics: one-way ANOVA ($F_{2,6}$

743   = 5.14, P = 0.0030) with Bonferroni *post-hoc* test (P = 0.019: before vs. Food dep., P = 0.040:

744   Food dep. vs. after, P = 1: before vs. after). **e**, Schematic illustrating the recordings and

745   representative traces of MTRIA$_{OT}$ fluorescence signals in adult (top) and old-aged (bottom)

746   mice. **f**, **g**, Summary of the peak number every hour (**f**) and peak z-score (**g**) (n = 3 mice).

747   Statistics: unpaired two-tailed t-test (P = 6.0×10^{-5} in **f** and P = 0.49 in **g**). Graphs represent

748   the mean ± SEM (**b**, **d**, **f**, **g**). ***P < 0.001, *P < 0.05, NS: not significant (**c**, **g**, **h**).

749

750   **Figure 5. Development of various transmitter sensors by conjugation with MTRIA.**

751   **a**, Schematic illustrating the development of MTRIA sensors. **b**, The $\Delta F/F_0$ values are given

752   as the mean $\pm$ SEM ($n = 5$ cells for each group). The sensors with $\Delta F/F_0 > 0.5$ are colored

753   either red or orange. The sensors with the best performance among receptors sharing the same

754   ligand are colored red. **c**, Table showing the basic properties of the MTRIA sensors: sensor

755   names, scaffold receptors, species, F_{max}, and EC$_{50}$.

756

757 **EXTENDED DATA FIGURE LEGENDS**

758 **Extend Data Figure 1. Optimization of a new fluorescent OT sensor.**

759 **a**, Representative images of HEK293T cells co-expressing an HA-tagged OTR (green) and a

760 mSca$_{mem}$ (red). The normalized fluorescence intensities on the dotted lines are shown on the

761 right. **b**, Schematic representation of cpGFP insertion into the IL3 of OTR. **c**, Sequence

762 alignment of the regions of the IL3 of human dopamine receptor D1 (hDRD1), a scaffold of a

763 dopamine sensor (dLight1), and meOTR. **d**, Representative images of HEK293T cells co-

764 expressing the indicated meOTR-cpGFP chimera (green) and mSca$_{mem}$ (red). **e**, Pearson

765 correlation coefficients comparing the meOTR-cpGFP chimeras and mSca$_{mem}$ are summarized

766 as the mean $\pm$ SEM (n = 13, 12, 12, 13, 12, 13, 13, 11, 13, 13, 13, 12, 13, and 12 cells; left to

767 right). Statistics: one-way ANOVA ($F_{13,167}$ = 1.78, P = 5.5×10^{-32}) with Bonferroni *post-hoc*

768 test (P = 5.1×10^{-9}: N236–S269, P = 7.9×10^{-14}: N236–I268, P = 1.2×10^{-13}: F237–S269, P =

769 4.1×10^{-14}: F237–I268, P = 3.1×10^{-12}: K238–S269, P = 4.9×10^{-14}: K238–I268, P = 1.6×10^{-16}:

770 L239–S269, P = 2.9×10^{-14}: L239–I268, P = 1.2×10^{-9}: K240–S269, P = 9.6×10^{-11}: T241–

771 S269, P = 5.4×10^{-15}: T241–I268, P = 2.7×10^{-16}: K242–S269, P = 2.0×10^{-16}: K242–I268,

772 compared with K240–I268). ***P < 0.001. **f**, Summary of the insertion site-dependent

773 characteristics of meOTR-cpGFP chimeras. **g,** Traces showing the fluorescence responses of

774 the K240–I268 meOTR-cpGFP chimera upon stimulation with 100 nM OT. Scale bars, 10 µm

775 (**a**, **d**).

776

777 **Extend Data Figure 2. Sequence of a new fluorescent OT sensor.**

778 **a**, Superposition of active- and inactive-GPCR structures, adapted from the Protein Data Bank

779 (PDB) archives (IDs: 5C1M and 6TPK). TM5–TM6 regions of the active and inactive states

780 are colored dark salmon and dark sea-green, respectively. **b**, Alignment of the TM5–TM6

781 region between meOTR and MTRIA$_{OT}$; the structure of cpGFP is adapted from a PDB archive

782   (ID: 3SG2). The mutations introduced in MTRIA$_{OT}$ are shown as red. **c**, Full amino acid

783   sequence of MTRIA$_{OT}$. The mutations in MTRIA$_{OT}$, the point mutation in MTRIA$_{OT}$-mut, and

784   the mutations of cpGFP that were adapted in the other fluorescent sensors are shown in red,

785   blue, and gray, respectively.

786

787   **Extend Data Figure 3. Basic properties of MTRIA$_{OT}$.**

788   **a**, Sequence alignment of OT and its orthologous neuropeptides. **b**, Dose–response curves of

789   MTRIA$_{OT}$ to OT and its orthologous neuropeptides. **c**, Assay for G protein coupling. Traces of

790   jRG1a fluorescence responses in either meOTR- or MTRIA$_{OT}$-co-expressing cells upon

791   stimulation with 100 nM OT (gray: each trace, green: average trace). Summary of the peak

792    $\Delta F/F_0$ responses are shown on the right ($n = 20$ cells). Statistics: unpaired two-tailed t-test (P

793    $= 8.1 \times 10^{-21}$). **d**, Assay for β-arrestin coupling. Traces showing the relative luminescence

794   increase induced by NanoLuc luciferase complementation upon stimulation with 100 nM OT

795   ($n = 5$ wells). Statistics: one-way ANOVA ($F_{3,16} = 3.24$, $P = 0.0009$) with Bonferroni *post-hoc*

796   test ($P = 0.029$: ($-$) vs. ($+$) in meOTR-SmBit + LgBit-arrestin, $P = 1$: ($-$) vs. ($+$) in

797   MTRIA$_{OT}$-SmBit + LgBit-arrestin). **e**, Time-course of the fluorescence intensity of MTRIA$_{OT}$

798   over 120 min following 100 nM OT stimulation. Representative images (top) and the

799   summary of $\Delta F/F_0$ responses (bottom: $n = 10$ cells). Statistics: one-way ANOVA ($F_{4, 45} =$

800   2.58, $P = 7.0 \times 10^{-22}$) with Bonferroni *post-hoc* test ($P = 9.7 \times 10^{-13}$ Pre vs. 10 min, $P = 1.9 \times 10^{-}$

801   ^{13} Pre vs. 30 min, $P = 9.3 \times 10^{-15}$ Pre vs. 60 min, $P = 2.6 \times 10^{-11}$ Pre vs. 120 min, $P = 0.24$: 10

802   min vs. 30 min, $P = 0.78$: 10 min vs. 60 min, $P = 1$: 10 min vs. 120 min, $P = 1$: 30 min vs. 60

803   min, $P = 1$: 30 min vs. 120 min, $P = 1$: 60 min vs. 120 min). **f**, Traces showing the kinetics of

804   MTRIA$_{OT}$ in the fluorescence rise (left: τ_{on}) and decay (right: τ_{off}) upon OT binding and

805   dissociation in HEK293T cells (gray: each trace, green: average trace; $n = 10$ cells). Scale

806   bars, 10 μm (**e**). Graphs represent the mean ± SEM (**b**−**d**). ***$P < 0.001$, *$P < 0.05$, NS: not

807   significant (**c**−**e**).

808

809   **Extend Data Figure 4. Fiber photometry setup and confirmation of the measurement**

810   **site.**

811   **a**, Schematic illustrating the fiber photometry measurement of a freely behaving mouse. A

812   representative image of an experimental arena captured by an overhead camera is shown in

813   the bottom right. **b**, Histology showing the expression of $MTRIA_{OT}$ (green) and the placement

814   of an implanted cannula. DAPI (blue) was used for counterstaining nuclei.

815

816   **Extend Data Figure 5. Dose–response curves of MTRIA sensors.**

817   Dose–response curves of the fluorescent sensors for 24 ligands that were examined in

818   HEK293T cells. Data are shown as the mean ± SEM ($n = 10$ cells for each data point). The

819   ligands were as follows: dopamine (DA), norepinephrine (NE), serotonin (5-HT), melatonin

820   (Mtn), adenosine 5′-triphosphate (ATP), angiotensin (Agt), arginine-vasopressin (Avp),

821   cholecystokinin (Cck), melanin-concentrating hormone (Mch), neuromedin B (Nmb),

822   neuropeptide FF (Npff), neuropeptide Y (Npy), neurotensin (Nts), enkephalin (Enk),

823   nociception (Ncp), Orexin (Ox), prolactin-releasing hormone (Prlh), somatostatin (Sst),

824   neurokinin B (Nkb), urotensin (Uts), complement component C3a (C3a), leukotriene B (Ltb),

825   platelet-activating factor (Paf), and prostagrandin E (Pge).

Extended Data Table 1: GPCRs used in Fig. 5 and the primer pairs for their cloning

Ligand	Receptor (Best performance, Responded, Not-responded in Fig. 5)	Species	Primer F	Primer R
DA	DRD1	human	aaaGAATTCaccatgaagacgatcatcgcc	aaaGGATCCtcaggttgggtgctgaccg
	Drd1	mouse	aaaCTCGAGACCatggctcctaacacttctacc	tttACTAGTtcaggttgaatgctgtccgc
	Drd2	mouse	aaaCTCGAGACCatggatccactgaacctgtcc	tttACTAGTtcagcagtgcaggatcttcatg
	Drd3	mouse	aaaCTCGAGACCatggcacctctgagccagataag	tttACTAGTtcagcaggatagaatcttgagg
	Drd1	zebrafish	aaaGAATTCACCatggcagtaaaggatttgaatg	aaaGGATCCtcagttcttgtttaggcttgg
	Drd2	zebrafish	aaaGAATTCACCatggaagtcttcacagcgtatgc	aaaGGATCCtcaacagtgcagaatcttaatgaag
	Drd3	zebrafish	aaaGAATTCACCatggcaatgtttagcagtggtg	aaaGGATCCttagcagctcaggattttaatgaagg
	Drd6	zebrafish	aaaGAATTCACCatggagagcgatggtgctg	aaaGGATCCtcaacactcctcaatctgtccc
	Drd1	medaka	AAAGAATTCACCatggatctcatgaactttacaactgtaattg	aaaGGATCCtcatttcttgtttagactggctgtc
	Drd3	medaka	aaaGAATTCACCatggcgatgtttagcagcac	aaaGGATCCtcacgtagcccagccaggtg
	Drd5	medaka	aaaGAATTCACCatggagaatccagccaagtatc	aaaGGATCCtcagtgtaatccattgggtgtg
NE	ADRA2A	human	aaaGAATTCACCatggagacagacacactcc	aaaGGATCCtcacaacacgatccgcttcc
	Adra1	mouse	aaaGAATTCACCatggtgcttctttctgaaaatgcttc	aaaGGATCCtcatggatataaagccccggg
	Adrb1	mouse	aaaGAATTCACCatgggcgcggggggcgctc	aaaGGATCCctacaccttggactccgaggag
	Adrb2	mouse	aaaGAATTCACCatggggccacacgggaac	aaaACTAGTttacagtggcgagtcatttgtac
	Adra2a	zebrafish	aaaCTCGAGACCatgatttgtggggccaatgc	tttACTAGTtcacaccactctcctcttatctctc
	Adrb1	zebrafish	aaaCTCGAGACCatgggagacgggctaccg	tttACTAGTttacagctgagactctgaatggg
	Adrb2	zebrafish	aaaGAATTCACCatgggaaacataaggtcctcaatacc	aaaACTAGTttacaacactctcatttgggctttg
	Adrb3	zebrafish	aaaCTCGAGACCatggtgatcatcatcacgattacc	tttACTAGTtcagccatcctgctcctgaag
	Adra1	medaka	aaaGAATTCACCatgagtttgagcactaacaatgtcac	aaaGGATCCtcagacccttgtcatcgtttacaatg
	Adra2a	medaka	AAAGAATTCACCatggaggacagcaaccagaccag	aaaGGATCCtcacaggtacttcctccgc
	Adrb2	medaka	aaaGGATCCACCatggcaaatgagagttctgcg	aaaGCGGCCGCtcaaaccactgaagctttatttccg
ACh	CHRM3	human	aaaGAATTCACCatggagacagacacactcc	aaaGGATCCttacaaggcctgctcgggtg
	Chrm1	mouse	aaaGAATTCACCatgaacacctcagtgcccc	aaaGGATCCttagcattggcgggagg
	Chrm4	mouse	aaaGAATTCACCatggcgaacttcacacctgtc	aaaGGATCCctacctggctgtgccgatg
	Chrm5	mouse	aaaCTCGAGACCatggaaggggagtcttatcacaatg	aaaACTAGTtcagggtagctgctgttgc
	Chrm2	zebrafish	aaaGAATTCACCatggatacaataaacttcaccttctgg	aaaACTAGTtcatcgggtggagcgaatg
	Chrm5	zebrafish	aaaGAATTCACCatgggtgtggagaaattgaccc	aaaGGATCCtcatgtgagtttgctgccc
	Chrm3	medaka	AAAGAATTCACCatgagctccaacagtacagacc	aaaGGATCCttacgttgaatctctgggaatccg
	Chrm5	medaka	aaaGAATTCACCatggaaggagaaattgcgctaaact	aaaGGATCCtcatgacatcttggagctaaccac
His	Hrh1	mouse	aaaGAATTCACCatgagccttcccaacacctc	aaaGGATCCttaggaacgaatgtgcagaattttttg
	Hrh2	mouse	aaaGAATTCACCatggagacccaatggcacgg	aaaGGATCCttacctgactgggcttccctg
5-HT	Htr1	mouse	aaaGAATTCACCatggatatgttcagtcttggccag	aaaACTAGTtcagcggcagaacttgcac
	Htr7	mouse	aaaCTCGAGACCatgatggacgttaacagcagcg	aaaACTAGTtcacagttttgtcagtgcaacagaattc
	Htr6	zebrafish	aaaGAATTCACCatgcgtgttcgctcagagg	aaaACTAGTtcaatcatctaaagtatcgacctggttg
	Htr1	medaka	aaaGAATTCACCatggattttctaacattaagcggcaac	aaaACTAGTctatggtctgtgaaatttgcattttatgatc
	Htr4	medaka	aaaGAATTCACCatgaatgtcagcgctgcagg	aaaACTAGTctacacgggcactggtctg
	Htr5	medaka	aaaGAATTCACCatgacaagcaccaacgccag	aaaACTAGTtcaccgctgccgggaaaaaag
	Htr6	medaka	aaaGAATTCACCatgtctgagccccatctgc	aaaACTAGTtcaatccagattgtccgtctgattg
Mtn	Mtnr1a	mouse	aaaGAATTCACCatgaaagggcaatgtcagcgag	gggACTAGTttaaacagagtccacctttattaagttattattg
	Mtnr1a	medaka	aaaGAATTCACCatgcttcaaaatgggtctcacc	aaaGGATCCtcagacggactccactttgac
	Mtnr1b	medaka	aaaGAATTCACCatgccggacgcaataaccctc	aaaGGATCCtcattccttgtttgtgcgatctc
	Mtnr1c	medaka	aaaGAATTCACCatgggatttggaggtgaaggatg	aaaGGATCCttatacattgatctctgctacattgttg
ATP	P2ry2	mouse	aaaGAATTCACCatggcagcagacctggaac	aaaGGATCCctatagccgaatgtccttagtctcac
ADP	P2ry1	mouse	aaaGAATTCACCatgaccgaggtgccttgg	aaaGGATCCtcacaaactcgtgtctccattctg
	P2ry12	mouse	aaaGAATTCACCatggatgtgcctggtgtcaac	aaaGGATCCtacattggggtctcttcgcttg
	P2ry1	zebrafish	aaaGAATTCACCatgacagcggagtttaataacctg	aaaGGATCCtcacatgcggttttcaccg
	P2ry12	zebrafish	aaaGAATTCACCatggagcaaacaacgcagc	aaaACTAGTtcatgtcagtgcgtttccctg
	P2ry1	medaka	aaaGAATTCACCatgaccacagacctgaacttgac	aaaACTAGTtcacagtctgcgctccc
	P2ry12	medaka	aaaGAATTCACCatggacttaaatgccactctgttc	aaaGGATCCtcaattacatgtagacatttgttggc
UDP	P2ry6	mouse	aaaGAATTCACCatggagcaggacaagatggcac	aaaGGATCCtcagactctctgcctctgcc
	P2ry6	medaka	aaaGAATTCACCatgcccccatcgtccaac	aaaGGATCCtcaggactttgacacagcgg
Ado	Adora1	mouse	aaaGAATTCACCatgccgccgtacatctcg	aaaGGATCCctagtcatcagctttctcctctgg
	Adora2a	mouse	tttAAGCTTACCatgggctcctcggtgtac	tttACTAGTtcaggaaggggcaaactctg
	Adora2b	mouse	aaaGAATTCACCatgcagctagagacgcaagac	aaaGGATCCtcataagcccagactgagagtag
	Adora3	mouse	aaaGAATTCACCatggaagccgcaacaccac	tttACTAGTtactcagtagtctgttccatgtttg
	Adora2a	zebrafish	aaaGAATTCACCatgctgaacaatgtttcgacgtg	aaaGGATCCtcaggaaacctccgtgagttc
	Adora2b	zebrafish	aaaGAATTCACCatggattcgctctacatcgcc	aaaGGATCCctatagcagagggtcaatagtcattg
	Adoa2b	medaka	aaaGAATTCACCatgagtcctgaaacggaccag	aaaGGATCCttacagcagagggtcgatggtc
Agt	Agtr2	mouse	aaaGAATTCACCatgaaggacaacttcagttttgctg	aaaGGATCCttaagacacaaaggtgtccatttctc
	Agtr2	zebrafish	aaaGAATTCACCatggaaccagactccgcc	aaaGGATCCttacgagctctgctgatccag
	Agtr2	medaka	aaaGAATTCACCatgatggcaatcccaactgac	aaaGGATCCctacgagggattagaagtctccac
Avp	Avpr1a	mouse	aaaGAATTCACCatgagtttccgcgcgaggc	aaaGGATCCtcaagtgggagacagggataaatc

	Receptor	Species	Forward	Reverse
	Avpr1b	mouse	aaaGAATTCACCatggattctgagccttcttg	aaaGGATCCctaagagatgctggtctcc
	Avpr2	mouse	aaaGAATTCACCatgatcctggtgtctaccac	aaaGGATCCctcagagaggagctggctg
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