



Trans-Modulation of the Somatostatin Type 2A Receptor Trafficking by Insulin-Regulated Aminopeptidase Decreases Limbic Seizures

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Insulin-regulated aminopeptidase decreases limbic seizures

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1 **Trans-modulation of the somatostatin type 2A receptor trafficking by**
2 **Insulin-regulated aminopeptidase decreases limbic seizures**

3
4 **Running title: IRAP regulates sst2A receptor traffic and seizures**

5
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41
42

43 **Abstract**

44

45 Within the hippocampus, the major somatostatin (SRIF) receptor subtype, the sst2A receptor, is
46 localized at post-synaptic sites of the principal neurons where it modulates neuronal activity.
47 Following agonist exposure, this receptor rapidly internalizes and recycles slowly through the
48 *trans*-Golgi network. In epilepsy, a high and chronic release of somatostatin occurs which
49 provokes, in both rat and human tissue, a decrease in the density of this inhibitory receptor at
50 the cell surface. The insulin-regulated aminopeptidase (IRAP) is involved in vesicular trafficking
51 and shares common regional distribution with the sst2A receptor. In addition, IRAP ligands
52 display anticonvulsive properties. We therefore sought to assess by *in vitro* and *in vivo*
53 experiments in hippocampal rat tissue whether IRAP ligands could regulate the trafficking of the
54 sst2A receptor and, consequently, modulate limbic seizures. Using pharmacological and cell
55 biological approaches, we demonstrate that IRAP ligands accelerate the recycling of the sst2A
56 receptor that has internalized in neurons *in vitro* or *in vivo*. Most importantly, because IRAP
57 ligands increase the density of this inhibitory receptor at the plasma membrane, they also
58 potentiate the neuropeptide SRIF inhibitory effects on seizure activity. Our results further
59 demonstrate that IRAP is a therapeutic target for the treatment of limbic seizures and possibly
60 for other neurological conditions in which down-regulation of G protein-coupled receptors
61 occurs.

62

63 **Significance Statement**

64

65 The somatostatin type 2A receptor (sst2A) is localized on principal hippocampal neurons and
66 displays anticonvulsant properties. Following agonist exposure, however, this receptor rapidly
67 internalizes and recycles slowly. The insulin-regulated aminopeptidase (IRAP) is involved in
68 vesicular trafficking and shares common regional distribution with the sst2A receptor. We
69 therefore assessed by *in vitro* and *in vivo* experiments whether IRAP could regulate the
70 trafficking of this receptor. We demonstrate that IRAP ligands accelerate sst2A recycling in
71 hippocampal neurons. Because IRAP ligands increase the density of sst2A receptors at the
72 plasma membrane, they also potentiate the effects of this inhibitory receptor on seizure activity.
73 Our results further demonstrate that IRAP is a therapeutic target for the treatment of limbic
74 seizures.

75 **Introduction**

76

77 The somatostatin (SRIF) type 2A receptor (sst2A) mediates SRIF inhibitory effects on cell
78 excitability (Peineau et al., 2003; Olias et al., 2004; Bassant et al., 2005) and displays
79 anticonvulsant properties (Vezzani and Hoyer, 1999; Tallent and Qiu, 2008; Clynen et al., 2014;
80 Dobolyi et al., 2014). Like many GPCRs, sst2A receptors rapidly internalize (Dournaud et al.,
81 1998; Csaba et al., 2001; Csaba et al., 2003; Stumm et al., 2004; Csaba et al., 2012) and are
82 then specifically targeted to a perinuclear/*trans*-Golgi network (TGN) compartment, bypassing
83 the late endosomal compartment and the degradative pathway (Csaba et al., 2007; Lelouvier et
84 al., 2008). Afterwards they slowly recycle to the plasma membrane of both cell bodies and
85 dendrites. Interestingly, this particular recycling route undertaken by the sst2A receptor shares
86 common pathways with the glucose transporter 4 (GLUT4) (Leto and Saltiel, 2012). A key
87 regulator of GLUT4 storage vesicles is the insulin-regulated aminopeptidase (IRAP), a type II
88 membrane protein (Chai et al., 2004; Albiston et al., 2007; Vanderheyden, 2009; Wright and
89 Harding, 2011). In the basal state, IRAP is located principally in an intracellular compartment
90 with GLUT4, and these proteins move to the cell surface identically in response to insulin (Keller,
91 2003). In addition to its aminopeptidase activity involved in peptide hormone processing, IRAP
92 also acts as a receptor for the endogenous ligands angiotensin IV (Ang IV) and LVV-Hemorphin
93 7 (LVV-H7). IRAP ligands are on the one hand competitive inhibitors of the enzymatic activity of
94 IRAP and on the other hand regulators of its trafficking (Albiston et al., 2007). The extracellular
95 part of IRAP contains the aminopeptidase activity while its intracellular domain interacts with
96 cytosolic proteins that contribute to GLUT4 vesicles retention and translocation to the plasma
97 membrane, however the mechanisms involved in this latter process remain to be demonstrated
98 (Albiston et al., 2007).

99 Although the memory- and cognitive-enhancing effects of IRAP ligands are well
100 established (Albiston et al., 2003; Albiston et al., 2011), the role of IRAP in the central nervous

101 system is yet to be fully elucidated. Most interestingly, IRAP ligands display anticonvulsive
102 activities in different animal models (Tchekalarova et al., 2001; Tchekalarova et al., 2004;
103 Stragier et al., 2006; Loyens et al., 2011). In accordance with these central actions, IRAP was
104 found to be highly expressed in brain areas associated with cognition and epilepsy (Fernando et
105 al., 2005), regions that also displayed the highest densities of sst2A receptor immunoreactivity
106 (Dournaud et al., 1996; Schindler et al., 1997; Dournaud et al., 1998). At the cellular level, IRAP
107 immunoreactivity in neurons is associated with the *trans*-Golgi apparatus and vesicular
108 structures in the proximity of the Golgi cisternae (Fernando et al., 2007), a location remarkably
109 comparable to that of internalized sst2A receptors before recycling (Csaba et al., 2007; Lelouvier
110 et al., 2008). Because IRAP shares common distribution with the sst2A receptor and is involved
111 in vesicular trafficking, we sought to assess by *in vitro* and *in vivo* experiments whether IRAP
112 could regulate the trafficking of the sst2A receptor and, consequently, modulate its
113 anticonvulsant actions.

114

115 **Material and Methods**

116
117 *Animals.* Experimental procedures were performed using adult male Wistar rats (Charles
118 River Laboratories). All efforts were made to reduce the number of animals used and any
119 distress caused by the procedures is in accordance with the European Communities Council
120 Directive of September 22, 2010 (2010/63/UE) and complying with the guidelines of Inserm, the
121 ethics committees on animal experiments of Paris Diderot University, the Vrije Universiteit
122 Brussel and the University of Crete. Accordingly, the number of animals in our study was kept to
123 the necessary minimum.

124
125 *Antibodies.* The endogenous sst2A receptor was immunolocalized using a rabbit
126 monoclonal antibody (3582-1, Epitomics; 1:1000). This antibody is directed towards residues
127 355–369 of the human sst2A receptor. This sequence is identical in mouse, rat, and human
128 sst2A. Endogenous IRAP was detected using a mouse monoclonal antibody (9876-3E1, Cell
129 Signaling; 1:200) directed against a fusion protein corresponding to the amino terminus of rat
130 IRAP. A sheep polyclonal antibody was used to detect the *trans*-Golgi network (TGN) specific
131 integral membrane protein (TGN38) (PA1-84496, Thermo Scientific; 1:800). Secondary
132 antibodies used were Alexa Fluor 488 (A488)-conjugated donkey anti-rabbit (1:500; Life
133 Technologies, Molecular Probes) or [Cyanine 3 \(Cy3\)-conjugated donkey anti rabbit \(1:500;](#)
134 [Jackson ImmunoResearch](#)), [Cy3-conjugated donkey anti-mouse \(1:500; Jackson](#)
135 [ImmunoResearch](#)) or [A488-conjugated donkey anti-mouse \(1:500; Life Technologies, Molecular](#)
136 [Probes](#)), and Alexa Fluor 633 (A633)-conjugated donkey anti-sheep (1:500; Life Technologies,
137 Molecular Probes) to immunolocalize sst2A receptor, IRAP and TGN38, respectively. For
138 chromogenic detection of sst2A receptor and IRAP, biotinylated goat anti-rabbit and anti-mouse
139 antibodies (1:300, Vector Laboratories) were used, respectively.

140
141 *Stereotaxic injection of octreotide.* Rats under isoflurane anesthesia were mounted on a
142 stereotaxic frame and injected into the dorsal hippocampus (coordinates: 2.8 mm posterior; 1.4
143 mm lateral; 3.2 mm ventral from Bregma) with octreotide (OCT; [gift from Novartis Pharma AG](#))
144 as previously described (Csaba et al., 2007). After 3h of survival, rats were deeply anesthetized
145 with sodium pentobarbital (60 mg/kg i.p.) and perfused through the ascending aorta with 400 ml
146 of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Brains were cryoprotected,
147 frozen in liquid isopentane at -45°C and sectioned in the coronal plane at a thickness of 30 µm
148 as previously described (Csaba et al., 2007).

149
150 *Immunohistochemistry.* Brain sections at the level of the dorsal hippocampus were
151 selected for chromogenic and immunofluorescent labeling. For chromogenic labeling,
152 endogenous peroxidase activity was first quenched by 0.3% H₂O₂ in 0.01 M phosphate-buffered
153 saline, pH 7.4 (PBS) for 30 min at room temperature (RT), washed in PBS, incubated in PBS
154 with 5% normal goat serum (NGS; Sigma-Aldrich) and 0.3% Triton X-100 for 30 min at RT,
155 incubated with primary antibodies diluted in PBS with 1% NGS and 0.3% Triton X-100 overnight
156 at RT, rinsed in PBS, incubated in biotinylated secondary antibodies diluted in PBS with 3%
157 NGS and 0.3% Triton X-100 for 1 h at RT, washed in PBS, and incubated in avidin-biotinylated
158 horseradish peroxidase complex (ABC; Vector Laboratories) diluted in PBS for 90 min at RT.
159 Peroxidase activity was revealed with 0.05% of 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) in
160 0.05 M Tris buffer, pH 7.6 (TB), in the presence of hydrogen peroxide (0.0048%). The reaction
161 was stopped by several washes in TB. Sections were mounted on gelatin-coated slides,
162 dehydrated in graded ethanols, delipidated in xylene and coverslipped with Permount (Fisher
163 Scientific) for light microscopic observation. For immunofluorescent labeling, sections were
164 preincubated in PBS with 5% normal donkey serum (NDS, Sigma-Aldrich) and 0.3% Triton X-
165 100 for 30 min at RT, incubated in primary antibodies diluted in PBS with 1% NDS and 0.3%

166 Triton X-100 overnight at RT, rinsed in PBS, and incubated in fluorescent secondary antibodies
167 diluted in PBS with 3% NDS and 0.3% Triton X-100 for 1 h at RT. Finally, sections were rinsed in
168 PBS, mounted on glass slides and coverslipped with Fluoromount (Southern Biotech) for
169 confocal microscopic analysis.

170
171 *Hippocampal cell cultures.* Hippocampi were dissected from 18-day-old embryo Wistar
172 rat brains and dissociated in HBSS with 0.25% trypsin and 0.1% DNase 1. Hippocampal
173 neurons were plated on glass coverslips previously coated with gelatin and poly-L-lysine.
174 Neurons were grown in Neurobasal medium (Invitrogen) supplemented with B27 and glutamine
175 (Invitrogen) and maintained in an incubator at 37°C in a 95% air/5% CO₂ humidified atmosphere.
176 All experiments were carried out at 12-13 days *in vitro*.

177
178 *In vitro pharmacological treatment.* For time-course experiments of sst2A receptor
179 internalization, neurons were treated with 1 μM of the sst2A receptor agonist octreotide (OCT) in
180 Neurobasal medium for 5, 10, 15 and 20 min at 37°C. For time-course experiments of sst2A
181 receptor recycling, neurons were pretreated with 1 μM Ang IV (or PBS as control) in Neurobasal
182 medium for 60 min at 37°C, followed by addition of 1 μM OCT or SRIF (Sigma-Aldrich) for 20
183 min. After rinsing, neurons were treated with 1 μM Ang IV (or PBS as control) in Neurobasal
184 medium for 15, 30, 45, 60, 120 and 180 min at 37°C. Additional control conditions included
185 neurons pretreated with 1 μM Ang IV (or PBS) without subsequent OCT or SRIF treatment
186 (Control w/o OCT; Control w/o agonist). At the end of incubation times, neurons were fixed with
187 4% paraformaldehyde supplemented with 4% sucrose in PB for 20 min at RT.

188
189 *shRNA lentiviral transduction.* Rat IRAP expression was inhibited in cultured
190 hippocampal cells using SMART vector 2.0 PGK-TurboGFP shRNA particles according to

191 manufacturer's protocols (Thermo Scientific, Dharmacon). Three predesigned individual IRAP-
192 gene specific shRNA lentiviral particles were obtained from Dharmacon and provided as ready-
193 to-use lentiviral particles (SH06-093146-01-10, SH06-093146-02-10 and SH06-093146-03-10).
194 The shRNA target sequences were as follows: IRAP LV-shRNA1: GATTGCTACAAACGGGAAA,
195 IRAP LV-shRNA2: AAGTTGGAGCAAGAACCGA and IRAP LV-shRNA3:
196 GATTAGTGACCAGAGTACA. SMART vector 2.0 Non-targeting PGK-Turbo GFP particles (S06-
197 0050000-01) was used as control (NT LV-shRNA; TGGTTTACATGTTGTGTGA). Hippocampal
198 cells were transduced immediately after dissociation for 1 h at 37°C (at a multiplicity of infection-
199 moi-of 10) and used for sst2A receptor recycling experiments at 12-13 days *in vitro*. Based on
200 TurboGFP expression, neuronal efficiency of transduction was 38.3% for the IRAP LV-shRNA1,
201 32.3% for the IRAP LV-shRNA2, 49.1% for the IRAP LV-shRNA3 and 45.0% for the NT LV-
202 shRNA.

203
204 *Immunocytochemistry.* Cells were washed in PBS and incubated 30 min in PBS with 5%
205 NDS and 0.1% Triton X-100. Primary antibodies were diluted in PBS with 1% NDS and 0.1%
206 Triton X-100 and incubated overnight at 4°C. Neurons were washed in PBS and subsequently
207 incubated with secondary antibodies diluted in PBS with 1% NDS and 0.1 % Triton X-100 for 1 h
208 at RT. Finally, coverslips were washed in PBS and mounted with Fluoromount (Southern
209 Biotech) for confocal microscopic analysis.

210
211 *Confocal microscopy.* Immunofluorescent sections were analyzed using a Leica TCS
212 SP8 confocal scanning system (Leica Microsystems) equipped with 488-nm Ar, 561-nm DPSS
213 and 633-nm HeNe lasers or a Zeiss LSM 5 Exciter confocal scanning system (Carl Zeiss) with
214 488-nm Ar, 543-nm HeNe and 633-nm HeNe lasers. Eight-bit digital images were collected from
215 a single optical plane using a 63x HC PL APO CS2 oil-immersion Leica objective (numerical
216 aperture 1.40) or a 63x Plan-Apochromat oil-immersion Zeiss lens (numerical aperture 1.4). For

217 each optical section, double- or triple-fluorescence images were acquired in sequential mode to
218 avoid potential contamination by linkage specific fluorescence emission cross-talk. Settings for
219 laser intensity, beam expander, pinhole (1 Airy unit), range property of emission window,
220 electronic zoom, gain and offset of photomultiplier, field format, scanning speed were
221 optimized initially and held constant throughout the study so that all sections were digitized
222 under the same conditions.

223
224 *Quantitative analysis of immunofluorescence.* To assess the extent of IRAP knockdown,
225 images of IRAP immunofluorescence from neuronal cell bodies were collected from a single
226 optical plane using a 63x Plan-Apochromat oil-immersion Zeiss lens (numerical aperture 1.4).
227 Images were segmented using identical threshold values, and integrated signal density was
228 measured using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of
229 Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2014.). This parameter,
230 corresponding to the intensity of the immunofluorescence staining, was measured in an average
231 of 30 neurons/group from 3 independent experiments. Mean values for the 4 experimental
232 groups (IRAP LV-shRNA1-3 constructs and NT LV-shRNA control) were calculated and
233 statistically analyzed.

234 To quantify sst2A receptor immunoreactivity at the plasma membrane, images of sst2A
235 receptor and TGN38 immunofluorescence from neuronal cell bodies were acquired from a single
236 optical plane using a 63x Plan-Apochromat oil-immersion Zeiss lens (numerical aperture 1.4).
237 The z-axis level was selected where the TGN38-labeled *trans*-Golgi network was clearly
238 identified and the sst2A receptor labeling at the plasma membrane was identified as a line-like
239 ring in the green channel around the TGN38 labeling of the red channel (Fig. 6a). A 1 μm wide
240 band containing the line-like sst2A receptor labeling was defined and the average pixel intensity,
241 corresponding to the intensity of the immunofluorescent staining, was measured within the
242 selection using the ImageJ software in an average of 30 neurons/group from 3 independent

243 experiments. Three sets of experiments were analyzed. One set contained 14 groups of the
244 time-course experiments of sst2A receptor recycling (Control w/o OCT; 15, 30, 45, 60, 120 and
245 180 min after OCT washout with or without Ang IV). The second set contained 6 groups (Control
246 w/o agonist; 45 min after OCT washout and 45 min after SRIF washout with or without Ang IV).
247 The third set contained 4 groups of the lentiviral shRNA experiments (IRAP LV-shRNA1-3
248 constructs and NT LV-shRNA control; 45 min after OCT washout). Mean values for the
249 experimental groups were calculated and statistically analyzed.

250

251 *Binding of IRAP ligands to sst2A receptor in stably transfected CHO-K1 cells.* Chinese
252 Hamster Ovary (CHO-K1) cells stably transfected with the sst2A receptor were plated in 24-well
253 plates (Becton Dickinson) and cultured until confluence in Dulbecco's modified essential medium
254 (DMEM) supplemented with L-glutamine (2 mM), 2% (v/v) of a stock solution containing 5000
255 IU/ml penicillin and 5000 µg/ml streptomycin (Invitrogen), 1% (v/v) of a stock solution
256 containing non-essential amino acids, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum
257 (Invitrogen). Before the experiment, cells were washed twice with 500 µl per well of Krebs buffer
258 (20 mM, CaCl₂, 2.7 mM KCl, 2.1 mM MgCl₂, 137 mM NaCl and 20 mM HEPES, pH 7.4) at RT.
259 Incubations were carried out for 45 min at 37°C in a final volume of 500 µl per well consisting of
260 400 µl Krebs buffer, 50 µl of increasing concentrations of Ang IV or LVV-H7 (10⁻⁹ M – 10⁻⁵ M)
261 and 50 µl ¹²⁵I-labeled SRIF (0.5 nM). At the end of the incubation period, the plates were placed
262 on ice and washed three times with ice-cold PBS. For whole-cell binding (i.e. without distinction
263 between surface-binding and intracellular accumulation of the radioligand) cells were then
264 solubilized by addition of 300 µl NaOH (1 M). After 60 min treatment at RT, solutes were
265 transferred in scintillation vials. Three ml of scintillation liquid (Optiphase Hisafe, Perkin Elmer)
266 was added and the remaining radioactivity was measured in a γ-counter (Perkin Elmer). The
267 experiments were repeated four times and results are shown in a compilation graph as the
268 percentage of ¹²⁵I-labeled SRIF binding to sst2A receptors, in the presence of Ang IV or LVV-H7.

269
270 *Focal pilocarpine-induced seizures in rats.*
271 *Surgery.* Adult male Wistar rats (275-300 g) were anaesthetized with a mixture of
272 ketamine and diazepam (start dose: 90:4.5 mg/kg), placed on a stereotaxic frame and implanted
273 with a microdialysis guide cannula (CMA/12, CMA Microdialysis/AB) above the left (or right)
274 hippocampus at flat skull coordinates: A -5.6 mm, L \pm 4.6 mm, V -4.6 mm relative to Bregma. For
275 experiments with intrahippocampal electroencephalographic (EEG) recording, the guide cannula
276 was customized by gluing an insulated electrode wire to the guide cannula and exposing it over
277 a length of 0.5 mm beyond the tip of the guide cannula. The guide was secured to the skull with
278 dental cement. For experiments with intrahippocampal EEG recordings, rats were s.c. implanted
279 with a sterilized radiotelemetric transmitter (F20-EET, Data Sciences International) which was
280 then subcutaneously tunneled to the intrahippocampal measuring electrode and the reference
281 electrode which was attached to the skull with a stainless steel screw implanted 1 mm anterior of
282 λ . Postoperative analgesia was provided by s.c. ketoprofen (3mg/kg). A concentric
283 microdialysis probe (3 mm length, CMA/12 Elite, CMA Microdialysis/AB) was inserted into the
284 guide cannula. Following transfer of the animals to the microdialysis cage, the microdialysis
285 probe was perfused with a modified Ringer's solution (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl₂)
286 at a flow rate of 2 μ l/min using a CMA infusion pump (CMA120, CMA Microdialysis/AB). Rats
287 had free access to food and water. Confirmation of unilateral probe placement was established
288 at the end of each experiment on 50 μ m brain slices prepared on a cryostat (Reichert-Jung
289 1206, Cambridge Instruments).

290 *Microdialysis and seizure severity assessment.* All microdialysis experiments were
291 performed on the day following surgery. A microdialysis flow rate of 2 μ l/min was maintained
292 throughout the pilocarpine experiments. Seizures were evoked by adding 10 mM pilocarpine to
293 the perfusion liquid during two 20 min sampling periods. Animals were rated on a seizure
294 severity scale based on Racine's scale which was adopted to include behavioral changes

295 observed in the focal pilocarpine model: (0) normal non-epileptic activity; (1) stereotypical mouth
296 and facial movements, wet dog shakes; (2) head nodding, tremor, staring; (3) forelimb clonus,
297 forelimb extension; (4) rearing, tonic-clonic activity; (5) falling (Meurs et al. 2008). The total
298 seizure severity score (TSSS) was the sum of the highest seizure severity score (SSS) observed
299 in each of the seven collection periods following the start of pilocarpine administration, and was
300 used as a measure of seizure severity. Additional experiments were performed in rats implanted
301 with an intrahippocampal EEG recording electrode and radiotelemetric transmitter. These rats
302 were placed in a microdialysis bowl equipped with an EEG monitoring unit with radiotelemetric
303 receiver (PhysioTel RPC-1, Data Sciences International) coupled to a Notocord-hem Evolution
304 acquisition system (Notocord). The intrahippocampal EEG was sampled at 100 Hz during
305 microdialysis sampling and the total EEG seizure duration and latency until the first EEG seizure
306 was determined. The observed behavioral seizures were correlated to EEG seizures by linear
307 regression analysis. Protocols differed between experimental groups as detailed below.

308 *Effect of intra-hippocampal Ang IV and LVV-H7 in the focal pilocarpine model.* Following
309 the collection of 8 baseline samples 0 μM , 1 μM or 10 μM Ang IV or LVV-H7 were perfused
310 through the microdialysis probe from the 9th sample collection period onwards. Pilocarpine was
311 co-perfused at a concentration of 10 mM during the 13th and 14th sample collection periods.
312 Hippocampal dialysate samples were subsequently collected for an additional 5 collection
313 periods.

314 *Involvement of the sst2A receptor in the anticonvulsive effects of Ang IV and LVV-H7.*
315 The involvement of the sst2A receptor in the observed effects of Ang IV and LVV-H7 was
316 investigated in experiments with collection of 6 baseline samples, followed by perfusion of the
317 microdialysis probe with 0.1 μM cyanamid 154806 (Sigma Aldrich) or 0.1 μM BIM-23627 (kindly
318 provided by Dr. Michael D. Culler, Ipsen Pharma) from the 7th sample collection period onwards.
319 From the 9th sample collection period onwards, 0 μM , 10 μM and 15 μM Ang IV or 10 μM LVV-
320 H7 were added to the perfusion liquid. Pilocarpine was co-perfused at a concentration of 10 mM

321 during the 13th and 14th sample collection periods. Hippocampal dialysate samples were
322 collected for an additional 5 collection periods.

323 *Selectivity of the effects of Ang IV and LVV-H7 in the focal pilocarpine model.* The
324 involvement of the AT1 receptor in the observed effects of Ang IV and the involvement of the μ/κ
325 opioid receptors in the anticonvulsant effects of LVV-H7 was investigated in experiments
326 according to exactly the same microdialysis protocol as described above for the sst2A receptors.
327 AT1 receptor antagonist candesartan and μ/κ opioid receptor antagonist naltrexone were used
328 at 0.1 μM concentration.

329 *Potentiation of the anticonvulsive effect of SRIF by Ang IV in the focal pilocarpine model.*
330 The effect of Ang IV on the anticonvulsive effect of SRIF was further investigated according to
331 the previously described microdialysis protocol. Ang IV and SRIF were used in a subthreshold
332 concentration that did not affect pilocarpine-induced seizures as such. Essentially we used 1 μM
333 for Ang IV and 0.1 μM for SRIF. Ang IV and SRIF were perfused through the microdialysis
334 probe, either alone or in combination, from the 9th sample collection period onwards. Pilocarpine
335 was co-perfused at a concentration of 10 mM during the 13th and 14th sample collection periods.
336 Hippocampal dialysate samples were subsequently collected for an additional 5 collection
337 periods. Two rats treated with the combination of Ang IV and SRIF did not show EEG seizure
338 activity and were excluded for analysis of EEG seizure latency.

339
340 *Effects of IRAP ligands on rat hippocampal SRIF.* Stereotactic surgery for bilateral probe
341 implantation in left and right hippocampus was performed as described above for the unilateral
342 intra-hippocampal probe implantation. Microdialysis probes (3 mm length, CMA/12 Elite, CMA
343 Microdialysis/AB) were continuously perfused with artificial cerebrospinal fluid (aCSF, 125 mM
344 NaCl, 2.5 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgCl_2 , 3.5 mM NaH_2PO_4 , 0.2 mM ascorbic acid,
345 0.025% bovine serum albumin, pH 7.4) at a flow rate of 1.0 $\mu\text{l}/\text{min}$ using a microdialysis pump

346 (CMA120, CMA Microdialysis/AB) following transfer of the animals to a microdialysis cage with
347 free access to food and water. The day following surgery, samples from the two bilateral probes
348 were collected every 30 min and pooled yielding a total volume of 60 μ l. At the beginning of each
349 experiment, samples were collected under baseline conditions, corresponding to perfusion of the
350 probe with aCSF alone. Then 10 μ M Ang IV or 10 μ M LVV-H7 was added to the perfusion liquid
351 for the remainder of the experiment. SRIF dialysate content was determined using a
352 radioimmuno assay (RIA) with [125 I]-Tyr 11 -Somatostatin (IM 161, Amersham) and a SRIF
353 antibody (N 1611, Amersham) raised against cyclic SRIF. A standard curve was prepared using
354 [125 I]-Tyr 11 -somatostatin (6000 cpm, 2200 Ci/mmol), the antibody (final dilution 1/3000) and
355 synthetic cyclic SRIF in a 0.8 ml solution containing 50 mM sodium phosphate, 10 mM EDTA
356 and 0.3% bovine serum albumin (pH 7.4). The radioactivity was counted with a γ -counter.
357 Results were read from the standard curve and the minimal detectable dose was 39.3 pg/100 μ l.
358 Confirmation of bilateral probe placement was again established at the end of each experiment
359 on 50 μ m brain slices prepared on a cryostat (Reichert-Jung 1206, Cambridge Instruments).

360

361 *Electron microscopy.* For electron microscopic detection of the sst2A receptor in
362 pilocarpine-treated rats in the presence or absence of Ang IV, a similar microdialysis protocol as
363 described above under “*Effect of intra-hippocampal Ang IV and LVV-H7 in the focal pilocarpine*
364 *model* “ was used. However, after the 15th collection period (time point where maximal seizures
365 are detected), rats were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.) and
366 perfused through the ascending aorta with 100 ml of saline followed by 400 ml of ice-cold 4%
367 paraformaldehyde and 0.05% glutaraldehyde in PB. Brains were post-fixed overnight in 4%
368 paraformaldehyde at 4°C. Coronal sections were cut on a vibratome at 70 μ m and collected in
369 PBS. Sections within 1 mm of the microdialysis probe were selected and equilibrated in 25%

370 sucrose and 10% glycerol in 0.05 M PB, frozen rapidly in isopentane cooled in liquid nitrogen,
371 and thawed in PBS at RT.

372 For pre-embedding immunocytochemistry, sections were preincubated for 30 min in 5%
373 NGS in PBS. Sections were then incubated overnight at RT in rabbit anti-sst2A receptor
374 antibody (1:1000) diluted in PBS containing 1% NGS. After washing in PBS, sections were
375 incubated for 120 min in Nanogold®-conjugated goat anti rabbit IgG (1:100; Nanoprobes) diluted
376 in PBS containing 2% of bovine serum albumin-c and 0.2% of cold water fish gelatin. Sections
377 were washed in PBS and post-fixed in 1% glutaraldehyde in PBS for 10 min. After washings in
378 PBS and 0.1 M sodium acetate buffer, pH 7.0, sst2A receptor immunogold labeling was
379 intensified using a silver enhancement kit (HQ Silver; Nanoprobes) for 5-10 min in the dark at
380 RT. After washings in acetate buffer and in PB, sections were post-fixed in 1% osmium tetroxide
381 in PB for 10 min at RT. After washings in PB, they were dehydrated in an ascending series of
382 ethanol, which included 1% uranyl acetate in 70% ethanol. They were then treated with
383 propylene oxide twice for 10 min, equilibrated overnight in Durcupan ACM (Fluka), mounted on
384 glass slides and cured at 60°C for 48 h. Areas of interest were cut out from the slide and glued
385 to blank cylinders of resin. Blocks were cut in semithin sections (1 µm) and then in ultrathin
386 sections on a Reichert Ultracut S microtome. Ultrathin sections were collected on pioloform-
387 coated single-slot grids. Sections were stained with lead citrate and examined with a Philips
388 CM120 electron microscope equipped with Morada Soft Imaging System (Olympus Soft Imaging
389 Solutions). On the ultrathin sections, only areas at the surface of the vibratome sections were
390 analyzed. The acquired images were equally adjusted for brightness and contrast, and
391 composite illustrations were built in Adobe Photoshop CS3 (Adobe Systems).

392
393 *Quantification of electron microscopic immunocytochemistry.* The subcellular distribution
394 of sst2A receptor in CA1 pyramidal cell dendrites was analyzed using the Measure command
395 and Cell counter plugin of ImageJ in 3 control and 3 Ang IV-treated rats. An average of 10

396 dendrites per animal was analyzed. The length of the plasma membrane and the caliber of
397 dendrites (expressed in cross-sectional surface; μm^2) were measured. The sst2A receptor
398 immunoparticles were identified and counted in association with the plasma membrane and in
399 the intracellular compartment. Results were expressed as: (1) the percentage of
400 immunoparticles associated with the plasma membrane and the intracellular compartment (%);
401 (2) density of immunoparticles per membrane length (μm) or cytoplasmic surface (μm^2).

402
403 *Data analysis.* Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad
404 Prism Software Inc.). Statistical tests applied to the data are indicated in the legends of the
405 figures. Differences were considered to be significant at $p < 0.05$. All data in text and figures are
406 presented as mean \pm S.E.M.

407

408 **Results**

409
410 **Sst2A receptor-expressing neuronal cells colocalize with IRAP in the rat**
411 **hippocampal formation**

412 The sst2A receptor and IRAP display similar regional expression patterns (Fig. 1a,c).
413 Whereas IRAP immunoreactivity was localized in the principal cells of CA1-3 and dentate gyrus
414 (Fig. 1c,d), sst2A receptor immunoreactivity is distributed in both dendritic fields and principal
415 cells of the hippocampus (Fig. 1a,d inset) (Csaba et al., 2001; Csaba et al., 2007). *In vivo*
416 internalization of the receptor through intra-hippocampal octreotide (OCT) injections revealed
417 sst2A receptor-expressing cell bodies (Fig. 1b,e), as previously reported (Csaba et al., 2001;
418 Csaba et al., 2007). Double-labeling experiments demonstrated that all hippocampal sst2A
419 receptor-positive neurons displayed IRAP immunoreactivity (Fig. 1e-g).

420
421 ***Internalized sst2A receptors are targeted to IRAP-positive vesicles in neurons***

422 IRAP and sst2A receptor sub-cellular distribution was then studied in cultured rat
423 hippocampal neurons to analyze whether both proteins could colocalize during receptor
424 internalization and recycling. In non-treated control cells (Fig. 2), a predominant plasma
425 membrane distribution of the endogenous sst2A receptor was detected at the surface of
426 perikarya and dendrites, confirming our former results (Csaba et al., 2001; Csaba et al., 2007;
427 Lelouvier et al., 2008). In accordance with earlier studies (Fernando et al., 2005; Fernando et al.,
428 2007), endogenous IRAP immunoreactivity was mainly confined to intracytoplasmic granules in
429 the perikarya and was also detected in proximal dendrites (Fig. 2). Finally, in the neuronal cell
430 body, IRAP-positive vesicles were located in a perinuclear compartment and overlapped with the
431 *trans*-Golgi marker TGN38, as previously reported (Fernando et al., 2007; Fernando et al., 2008)
432 (Fig. 2). In agreement with these latter studies, a faint IRAP signal was also apparent at the

433 plasma membrane. Therefore, little colocalization between sst2A receptor and IRAP was
434 observed in control conditions.

435 After 5-10 min of agonist stimulation with 1 μ M OCT, endogenous sst2A receptors were
436 no longer present at the cell surface, and bright fluorescent accumulations appeared in the
437 cytoplasm of perikarya and dendrites (Fig. 2). [The same redistribution of sst2A immunoreactivity](#)
438 [was observed after 1 \$\mu\$ M SRIF treatment \(data not shown\), as previously reported \(Lelouvier et](#)
439 [al., 2008\)](#). Very interestingly, extensive colocalization between sst2A receptor and IRAP
440 immunoreactivity was detected both in perikarya and proximal dendrites between 5 and 20 min
441 following the beginning of the OCT treatment, as shown by the yellow pseudocolor signal
442 present in the overlaid images (Figs. 2, 3). This indicates that internalized receptors are
443 massively targeted to IRAP-positive intracellular compartments in hippocampal neurons. This
444 colocalization gradually decreased in time as the receptor recycled back to the plasma
445 membrane (Fig. 4).

446

447 ***IRAP ligand accelerates sst2A receptor recycling in hippocampal neurons***

448 Next, we performed kinetic experiments to analyze whether IRAP ligand Ang IV could
449 impact on the trafficking and recycling of endogenous sst2A receptor following agonist-induced
450 internalization (Fig. 5). In hippocampal neurons treated with OCT but in the absence of Ang IV,
451 reappearance of sst2A receptors at the plasma membrane of the cell body was first evident 30
452 min after OCT washout. Three hours after OCT stimulation, receptor distribution and density
453 were similar to control conditions, i.e. receptors were localized at the plasma membrane of both
454 the cell body and dendrites.

455 In hippocampal neurons treated with OCT but in the presence of Ang IV, we observed a
456 shift of the reappearance of the sst2A receptors at the plasma membrane ([Figs. 5, 6](#)). At 30, 45
457 and 60 min after OCT washout, the amount of membrane-associated receptors was significantly
458 higher in Ang IV-treated neurons (OCT + Ang IV) than in respective controls (OCT + PBS) (30

459 min: +69%, 45 min: +82%, 60 min: +64%; Fig. 6b). No difference was observed in the recycling
460 kinetics of the sst2A receptor following SRIF- or OCT-induced internalization. Notably, Ang IV
461 treatment resulted in a faster sst2A receptor recycling with both agonists (Fig. 6b). Interestingly,
462 within the Ang IV-treated group, we observed that 45 and 60 min after OCT washout the density
463 of receptors at the plasma membrane was significantly higher than in cells not treated with OCT
464 (Ang IV without OCT) (45 min: +33%, $p < 0.001$; 60 min: +18%, $p < 0.01$; Dunnett's Multiple
465 Comparison Test; Fig. 6b). In cells treated with OCT in the absence of Ang IV, this phenomenon
466 was also apparent but only 120 min after OCT washout (+16% vs. PBS without OCT, $p < 0.001$,
467 Dunnett's Multiple Comparison Test; Fig. 6b). These data suggest that massive pools of sst2A
468 receptors are first targeted from the perinuclear recycling compartment to the plasma membrane
469 of the cell body, before diffusing to the dendritic fields along the membranes, and this process is
470 accelerated in Ang IV-treated neurons. Together, these results suggest that the IRAP ligand has
471 shortened the recycling time of internalized sst2A receptors to the plasma membrane.

472

473 ***IRAP ligands do not bind the sst2A receptor***

474 To analyze whether the Ang IV effects on sst2A receptor recycling could be due to
475 putative cross-reaction between the IRAP ligands and the sst2A receptor, cultured hippocampal
476 neurons were treated with 1 μ M Ang IV or LVV-H7 and fixed 20 to 180 min thereafter for
477 immunoreactive detection of the endogenous sst2A receptor. Neither Ang IV nor LVV-H7 did
478 change the distribution of sst2A receptor immunoreactivity (data not shown). The possible direct
479 interaction between IRAP ligands and the sst2A receptor was also investigated in CHO-K1 cells
480 stably transfected with the sst2A receptor incubated with increasing concentrations of Ang IV
481 and LVV-H7. The total percentage of 125 I-labeled SRIF binding to sst2A receptor did not change
482 throughout the whole experiment, in the presence of either Ang IV or LVV-H7 (Fig. 7). Together,
483 these results indicate that direct interactions between IRAP ligands and sst2A receptors are
484 unlikely.

485
486 **IRAP knockdown also accelerates sst2A receptor recycling in hippocampal**
487 **neurons**

488 To confirm the specificity of IRAP effects on sst2A receptor trafficking, we next aimed to
489 silence IRAP expression in hippocampal neurons through lentiviral-mediated RNA interference.
490 The protein expression level of IRAP was analyzed by semi-quantitative laser scanning confocal
491 microscopy in individual transduced neurons (Fig. 8a,b). Data revealed a significant inhibition of
492 IRAP expression by 3 separate IRAP-targeting shRNAs as compared to control shRNA (IRAP
493 LV-shRNA1: -76%, IRAP LV-shRNA2: -70%, IRAP shRNA3: -60%; Fig. 8b). This selective
494 down-regulation of IRAP had no effect on the targeting of the sst2A receptor to the plasma
495 membrane or on the capacity of the receptor to internalize after OCT stimulation. Importantly,
496 when we analyzed the recycling of the receptor at the time point 45 min after OCT washout (Fig.
497 9a,b; chosen on the basis of pharmacological experiments for which the difference between Ang
498 IV-treated and respective control neurons was the highest), we found that the density of the
499 receptor at the plasma membrane was significantly higher in neurons transduced with IRAP-
500 targeting shRNAs than in neurons transduced with control shRNA (IRAP LV-shRNA1: +56%,
501 IRAP LV-shRNA2: +62%, IRAP shRNA3: +53%, Fig. 9b). These results suggest that reduction
502 of IRAP expression has shortened the time of recycling of internalized receptors to the plasma
503 membrane and that IRAP is therefore a negative regulator of sst2A receptor recycling.

504
505 ***Intra-hippocampal Ang IV or LVV-H7 administration is anticonvulsive in the rat***
506 ***focal pilocarpine model***

507 To further analyze whether a faster recovery of internalized sst2A receptors at the
508 plasma membrane in response to IRAP ligands could be relevant *in vivo*, we next studied the
509 effects of intra-hippocampal administration of Ang IV and LVV-H7 in a rat model of focal
510 seizures. Indeed, an increase in SRIF immunoreactivity and secretion is known to occur in

511 epileptic hippocampal tissues (Tallent and Qiu, 2008) and several studies argue for a
512 concomitant internalization of the sst2A receptors (Csaba et al., 2004; Csaba et al., 2005).

513 Intra-hippocampal perfusion of 10 mM pilocarpine via a microdialysis probe induced a
514 range of behavioral changes including wet dog shakes, typically beginning within 20 min
515 following the start of pilocarpine administration. These changes progressively evolved into bouts
516 of staring, stereotypical mouth and facial movements, forelimb clonus, and rearing. The
517 behavioral changes correlated with electrocorticographic seizures, as previously demonstrated
518 (Meurs et al., 2008). Pilocarpine-treated animals received intra-hippocampal co-perfusions of 1
519 or 10 μ M of the IRAP ligands Ang IV or LVV-H7 through a microdialysis probe. The maximum
520 seizure severity score (SSS) (Fig. 10a,b; Bin 12) of animals perfused with 1 μ M Ang IV or 1 μ M
521 LVV-H7 was not significantly different from the maximum SSS of animals in the pilocarpine
522 control group. Similarly, the mean total seizure severity score (TSSS) of animals treated with 1
523 μ M Ang IV or 1 μ M LVV-H7 were not significantly different from the mean TSSS observed in the
524 pilocarpine control group. By contrast, the maximum SSS (Fig. 10a,b; Bin 12) for animals treated
525 with 10 μ M Ang IV or 10 μ M LVV-H7 were significantly lower compared to the SSS in animals of
526 the pilocarpine control group (Fig. 10a,b). In animals treated with 10 μ M Ang IV or 10 μ M LVV-
527 H7, only minor seizure-related behavioral changes were observed, such as wet dog shakes and
528 less commonly staring. Correspondingly, the mean TSSS of animals treated with 10 μ M Ang IV
529 or 10 μ M LVV-H7 were significantly lower compared to the mean TSSS observed in the
530 pilocarpine control group (Fig. 10a,b). Taken together, these results demonstrate that IRAP
531 ligands, at a microdialysis probe perfusion concentration of 10 μ M, display anticonvulsant
532 effects.

533

534 ***The anticonvulsant effects of IRAP ligands are mediated by the sst2A receptor***

535 To further analyze whether the anticonvulsant properties of IRAP could be mediated by
536 the sst2A receptor, we next studied the effect of intra-hippocampal co-administration of sst2A

537 receptor antagonists and IRAP ligands in the same limbic seizure model. The sst2A receptor
538 antagonists cyanamid 154806 or BIM-23627 did not significantly affect seizure severity
539 compared to the pilocarpine control groups when perfused in the hippocampus at a
540 concentration of 0.1 μ M (Fig. 11a,b). However, seizure severity was significantly higher in
541 animals perfused with 0.1 μ M cyanamid 154806 followed by 10 μ M Ang IV (Fig. 11a) or 10 μ M
542 LVV-H7 (Fig. 11c) and 0.1 μ M BIM-23627 followed by 15 μ M Ang IV (Fig. 11b) compared to Ang
543 IV and LVV-H7 alone (Fig. 11a-c). Altogether, these experiments demonstrate that sst2A
544 receptor antagonists abolish the anticonvulsant effects of Ang IV and LVV-H7 and that the sst2A
545 receptor is thus a key factor for the anticonvulsant effects mediated by the IRAP ligands.

546
547 ***The anticonvulsant effects of IRAP ligands are independent of angiotensin AT1***
548 ***and μ /k opioid receptor activation***

549 Because Ang IV and LVV-H7 were previously characterized as low affinity angiotensin
550 AT1 (Yang et al., 2008; De Bundel et al., 2009; De Bundel et al. 2010) and opioid μ /k receptor
551 (Zhao et al., 1997; Lee et al., 2001) agonists, we therefore investigated the possible involvement
552 of these receptor subtypes in the anticonvulsant effects of Ang IV and LVV-H7. The angiotensin
553 AT1 receptor antagonist candesartan and the opioid μ /k receptor antagonist naltrexone,
554 perfused into the hippocampus at a concentration of 0.1 μ M, had no significant effect on seizure
555 severity compared to the pilocarpine control group (Fig. 12a,b). Furthermore, pre-treatment with
556 candesartan or naltrexone did not abolish the anticonvulsive effect of Ang IV and LVV-H7 as
557 compared to the pilocarpine control group (Fig. 12a,b).

558
559 ***IRAP ligands have no effect on extracellular hippocampal SRIF levels***

560 Anticonvulsant effects of IRAP ligands could be due, in addition to their effects on sst2A
561 receptor recycling, to an increase in hippocampal SRIF concentrations through inhibition of its
562 degradation, as reported in one *in vitro* study in PC12 cells (Matsumoto et al., 2001). We

563 therefore analyzed the effects of intra-hippocampal administration of anticonvulsant
564 concentrations of Ang IV or LVV-H7 on hippocampal SRIF levels. The mean baseline
565 concentrations of SRIF in hippocampal microdialysates was 0.80 ± 0.15 nM. Intra-hippocampal
566 perfusion of 10 μ M Ang IV or 10 μ M LVV-H7 had no effect on baseline SRIF levels of the
567 hippocampal microdialysates (Fig. 13). The inhibition of enzymatic SRIF degradation is therefore
568 not implicated in the mechanism of anticonvulsant action of the IRAP ligands.

569

570 ***Ang IV potentiates the anticonvulsive effect of SRIF***

571 We next investigated whether intrahippocampal administration of the IRAP ligand Ang IV could
572 potentiate the anticonvulsive effect of SRIF (Aourz et al., 2011). We found that 1 μ M Ang IV and
573 0.1 μ M SRIF had no effect on pilocarpine-induced seizures. Co-perfusion of both peptides in the
574 same concentration suppressed behavioral seizures (Fig 14a) and EEG seizures (Fig 14b-g).
575 These data clearly demonstrate that Ang IV potentiates the anticonvulsive effect of SRIF in the
576 focal pilocarpine model, potentially by increasing the availability of sst2A receptors at the
577 membrane of hippocampal neurons.

578

579 ***IRAP ligands increase the density of membrane-associated sst2A receptors in vivo*** 580 ***following evocation of a limbic seizure event***

581 To further demonstrate that Ang IV antiepileptic effects are driven by an increase of
582 membrane-associated receptors *in vivo*, as demonstrated *in vitro*, the density of sst2A receptors
583 was quantified by immunogold electron microscopy in CA1 pyramidal cell dendrites of
584 pilocarpine-injected animals treated or not with an anticonvulsive dose of Ang IV (Fig. 15a-d).
585 Whereas in the pilocarpine control group, 70% of immunoparticles were found in intracellular
586 compartments and 30% membrane-associated (Fig. 15a,c), in the animals receiving intra-
587 hippocampal perfusion of Ang IV and pilocarpine, the distribution was reversed: 30% of
588 immunoparticles were intracellular and 70% membrane-associated (Fig. 15b,c). The latter

589 distribution is close to that we have previously reported in naive animals, i.e. 20% intracellular
590 and 80% membrane-associated (Csaba et al., 2007). Quantitative analysis revealed that the
591 density of sst2A receptors at the plasma membrane was significantly higher in Ang IV-treated
592 animals as compared to controls (Fig. 15d). Conversely, the density of intracellular sst2A
593 receptor immunoparticles was significantly lower in Ang IV-treated animals as compared to
594 controls (Fig. 15d). These results suggest that, as demonstrated *in vitro*, Ang IV accelerates the
595 recycling of internalized receptors induced by SRIF release during the evoked seizure process.
596 Together with the fact that sst2A receptor antagonists prevent the anticonvulsive effects
597 mediated by Ang IV or LVV-H7, the recovery of the sst2A receptors at the plasma membrane
598 appears as the key mechanism involved in the anticonvulsive effects mediated by IRAP ligands.
599

600 **Discussion**

601

602 The life cycle of GPCRs includes biosynthesis in the endoplasmic reticulum, maturation
603 in the Golgi and transport to the cell surface followed by signal transduction at the plasma
604 membrane and often internalization. Additional signaling can also occur in early endosomes as
605 recently demonstrated (Irannejad et al., 2013). GPCR-interacting proteins have been
606 demonstrated to play a critical role along these different phases (von Zastrow and Williams,
607 2012). However, much remains to be learnt about mechanisms that regulate post-endocytic
608 sorting and recycling of GPCRs. [As documented in our study](#), the insulin-regulated
609 aminopeptidase, IRAP, appears as a new modulator of GPCR trafficking by affecting the kinetics
610 of the sst2A receptor recycling after ligand activation in neurons. *Trans*-modulation of the
611 recycling of a GPCR through ligand binding to a non-GPCR membrane protein, has, to our
612 knowledge, not yet been described. *Trans*-effects on GPCR trafficking were until now only
613 described through oligomerization of receptors (von Zastrow and Williams, 2012), which mostly
614 affect the endocytosis process. Importantly, [our study also demonstrates](#) that modulating the
615 recycling of a particular receptor can indeed impact its physiological fate and therefore present a
616 potential therapeutic value.

617 Within the hippocampus, the major SRIF receptor subtype, the sst2A receptor, is
618 localized at post-synaptic sites of the principal cells (Dournaud et al., 1996; Schindler et al.,
619 1997; Csaba et al., 2007). Although this receptor appears to be an attractive therapeutic target
620 in epilepsy, it rapidly internalizes *in vivo* before a slow recycling process (Csaba et al., 2007;
621 Lelouvier et al., 2008). This long route has been suggested to have an impact on sst2A receptor-
622 mediated signaling when, in pathophysiological conditions such as epilepsy, a high and chronic
623 release of SRIF occurs (Vezzani and Hoyer, 1999). Indeed, a decreased density of this receptor
624 subtype has been demonstrated in both rat and human epileptic dentate gyrus (Csaba et al.,
625 2004; Csaba et al., 2005). Importantly, our present data suggest that down-regulation of the

626 sst2A receptor can be counteracted and consequently can increase endogenous SRIF
627 anticonvulsant properties. We first found that internalized receptors travel massively through an
628 intra-cytoplasmic pool of IRAP-positive vesicles. IRAP has been proposed to serve as a cargo of
629 cell-specific storage vesicles and as an active player in their trafficking (Saveanu and van
630 Endert, 2012). Our findings support this notion since we demonstrate here that IRAP regulates
631 either directly or indirectly the sorting of sst2A receptors from cytoplasmic vesicles to the plasma
632 membrane. Secondly, we established that the IRAP ligand Ang IV or the knockdown of IRAP
633 expression both accelerate the recycling of sst2A receptors after their endocytosis in
634 hippocampal cells. These observations suggest a possible *trans*-modulation of sst2A receptor
635 trafficking by IRAP. We then showed that the anticonvulsive effects of both Ang IV and the
636 structurally distinct peptidergic IRAP ligand LVV-H7 were reversed by intra-hippocampal co-
637 administration of two structurally different sst2A receptor antagonists, cyanamid 154806 and
638 BIM-23627. These results unequivocally confirm the involvement of the sst2A receptor in the
639 anticonvulsive effects of the IRAP ligands. Moreover, we found that a low dose of Ang IV, which
640 does not exert anticonvulsive effects as such, potentiates the anticonvulsive action of SRIF. We
641 then asked whether Ang IV and LVV-H7 may exert their anticonvulsive effects through putative
642 inhibition of SRIF degradation via IRAP (Matsumoto et al., 2001). We have established that
643 perfusion of anticonvulsive concentrations of Ang IV or LVV-H7 into the hippocampus had no
644 effect on local SRIF levels. This suggests that IRAP is not involved in SRIF degradation in the
645 rodent brain. Other enzymes, such as endopeptidase-24.11 (Sakurada et al., 1990; Barnes et
646 al., 1995) or endopeptidase-24.15 and 24.16 (Dahms and Mentlein, 1992), may be more
647 important for SRIF degradation. Consequently, we rule out the “enzyme hypothesis” as a
648 mechanism of action in our limbic seizure model. Importantly, using immunogold electron
649 microscopy, we further demonstrate that Ang IV increased *in vivo* the availability of membrane-
650 associated sst2A receptors. Taken together, our results bring us to the conclusion that IRAP
651 ligands favor a rapid recycling and resensitization of sst2A receptors following their

652 internalization and enhance endogenous SRIF inhibitory effects on seizure activity by increasing
653 the density of functional receptors at the plasma membrane. [These results are in agreement with](#)
654 [studies using the \$\beta\$ 2 adrenergic receptor \(Pippig et al., 1995; Vistein and Puthenveedu, 2013\) or](#)
655 [the Human G Protein-Coupled Receptor 17 \(Daniele et al., 2011\) demonstrating that recycled](#)
656 [receptors resensitize rapidly.](#)

657 In the hippocampal formation, SRIF interneurons and the sst2A receptor, which is highly
658 expressed in the dendritic fields of granular and pyramidal cells, are well poised to regulate
659 neuronal hyperexcitability (Johansson et al., 1984; Tallent and Qiu, 2008). Hilar SRIF inhibitory
660 interneurons and their axons that terminate on the distal dendrites of granule cells are positioned
661 to inhibit granule cells at the perforant path inputs, a major excitatory pathway to the
662 hippocampus. SRIF fibers are present in the hilus, suggesting that SRIF neurons also modulate
663 hilar neurons (Leranth et al., 1990; Lubke et al., 1998). SRIF axons are also localized throughout
664 the CA1-CA3 regions, being enriched in stratum lacunosum moleculare (Johansson et al.,
665 1984). Distinct inhibitory actions at the cellular level in rat CA1 pyramidal neurons were
666 demonstrated for SRIF. It increases two different types of K⁺ currents, the voltage-sensitive M-
667 current (Moore et al., 1988; Schweitzer et al., 1990) and a voltage-insensitive leak current
668 (Schweitzer et al., 1998). Postsynaptic hyperpolarization and reduced excitatory postsynaptic
669 currents of CA1-3 pyramidal neurons by SRIF have also been reported (Tallent and Siggins,
670 1997, 1999). Taken together, these studies and our present results emphasize the importance of
671 the hippocampal somatostatinergic network, which includes the availability of functional sst2A
672 receptors at the plasma membrane for dampening neuronal hyperexcitability.

673 Although beyond the scope of the present study, it would be of interest to decipher the
674 mechanisms by which IRAP is able to regulate the recycling of the sst2A receptor. In CHO-K1
675 and HEK 293 cells, iodinated Ang IV induced internalization of IRAP which appears to be
676 clathrin-mediated (Demaegdt et al., 2008). Ligand-induced internalization and recycling of IRAP
677 has also been suggested in neurons (Fernando et al., 2008). In hippocampal cells treated with

678 Ang IV, we did not observe a time-dependent accumulation of IRAP immunoreactivity within
679 intracytoplasmic vesicles. This suggests that intracellular stores of IRAP are constantly driven to
680 the cell surface to replace internalized IRAP, and that the IRAP exocytosis rate is higher in the
681 presence of its ligands. Because internalized sst2A receptors travel through IRAP-containing
682 vesicles, one can hypothesize that the rate of receptor exocytosis is indirectly dependent upon
683 the presence of IRAP ligands, i.e. sst2A receptors recycle faster in cells or tissue incubated with
684 Ang IV or LVV-H7. If this is the case, effects of IRAP ligands would not be specific for the sst2A
685 receptor but could also modulate other types of receptors that internalize and recycle through an
686 IRAP/TGN compartment. Most interestingly, Ang IV has been shown to reverse the kindling-
687 induced down-regulation of dopaminergic D1 and D2 receptors in the basal ganglia
688 (Tchekalarova et al., 2004), two receptors which are well known to internalize in neurons
689 (Dumartin et al., 1998; Skinbjerg et al., 2010; Lane et al., 2012). Thus IRAP ligands, in addition
690 to their potential for modulating limbic seizures, might also represent appealing therapeutic tools
691 in neurological diseases or following pharmacological treatments in which down-regulation of
692 GPCRs could occur (Bezard et al., 2001; Gainetdinov et al., 2004; Williams et al., 2013).
693 Although the above described mechanism by which IRAP ligands could impact on sst2A
694 receptor intracellular trafficking is a likely starting hypothesis, the cellular events involved could
695 be difficult to decipher. In insulin-sensitive cells, IRAP trafficking itself is far from being
696 understood, despite a large number of studies (Albiston et al., 2007). In addition, IRAP and the
697 sst2A receptor both interact through their intra-cytoplasmic domain with several proteins
698 involved in vesicle trafficking, tethering and membrane anchoring (Albiston et al., 2007; Watson
699 et al., 2008; Csaba et al., 2012). Interestingly, IRAP knockdown in adipocytes increase plasma
700 membrane GLUT4 levels due to increase of exocytosis (Jordens et al., 2010). Regarding our
701 present results showing that IRAP knockdown increases the recycling of the receptor to the
702 plasma membrane, it appears that IRAP could be a negative regulator of the exocytosis pathway

703 in different cell types. Further studies in neuronal cells are mandatory to shed lights on the
704 molecular and cellular mechanisms implicated in this process.

705 In conclusion, the present study demonstrates that two structurally different peptidergic
706 IRAP ligands protect against limbic seizures in rats. These anticonvulsive effects do not result
707 from the inhibition of the enzymatic activity of IRAP and subsequent prevention of SRIF
708 degradation. We demonstrate that IRAP, following ligand binding, *trans*-regulates the recycling
709 process of the sst2A receptor and increases the availability of this inhibitory receptor at the
710 plasma membrane. This represents a novel mechanism by which GPCR transmission can be
711 regulated and further demonstrates that IRAP is a potential therapeutic target for the treatment
712 of epilepsy. Whether IRAP could modulate the down-regulation of other types of GPCRs, thus
713 displaying additional therapeutic interests, will represent intriguing issues for future studies.

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927

928

929 **Legends**

930

931 **Figure 1. Neurons expressing the sst2A receptor also display IRAP immunoreactivity in**
932 **the rat hippocampal formation.** a) In control rats, diffuse sst2A receptor immunoreactivity is
933 observed in the strata oriens (Or) and radiatum (Rad) of CA1-3 and the molecular layer (Mol) of
934 dentate gyrus (DG). b) After intra-hippocampal injection of octreotide (OCT), sst2A receptor-
935 immunoreactive neurons are detected in the pyramidal cell layer (Py) of CA1-3 and granular
936 layer (Gr) of DG. c) IRAP-immunoreactivity is located in the principal cell layers of the
937 hippocampal formation. d) In the CA1 of control rats, double-labeling immunohistochemistry
938 reveals that IRAP is predominantly localized in pyramidal cell bodies (Py), whereas sst2A
939 receptor immunoreactivity is diffusely distributed in strata oriens (Or) and radiatum (Rad). Inset
940 shows sst2A receptor immunoreactivity in the pyramidal cells layer. e-g) In OCT-injected rats,
941 double-labeling immunohistochemistry reveals colocalization of internalized sst2A receptor and
942 IRAP in pyramidal neurons of CA1. Note that all hippocampal sst2A receptor-immunoreactive
943 neurons are IRAP-positive. Only a subpopulation of IRAP-immunoreactive neurons is sst2A
944 receptor-positive. Similar stainings were found in three different OCT-injected rats (three
945 sections per animal). Scale bars: a-c, 400 μm ; d-g, 40 μm .

946

947 **Figure 2. Kinetics of sst2A receptor internalization in primary hippocampal neurons.** In
948 control neurons, the sst2A receptor immunoreactivity is predominantly located at the surface of
949 perikarya and proximal dendrites. Conversely, IRAP immunofluorescence is mainly confined to
950 intracellular vesicles overlapping with *trans*-Golgi network marker TGN38. Little colocalization is
951 thus observed between sst2A receptor and IRAP. Treatment with the sst2A receptor agonist
952 octreotide for 5, 10 or 20 min results in a massive redistribution of sst2A receptor
953 immunoreactivity from the cell surface into intracellular vesicles, where it is colocalized with
954 IRAP (yellow pseudocolor signal on sst2A/IRAP merged images) and TGN38 (white pseudocolor

955 signal on sst2A/IRAP/TGN38 merged images). Thirty neurons/group were analyzed from 3
956 independent experiments. OCT, octreotide. Scale bars: 10 μm .

957
958 **Figure 3. Internalized sst2A receptor colocalizes with IRAP in primary hippocampal**
959 **neurons.** The sst2A receptor immunoreactivity is confined to bright fluorescent granules in the
960 cytoplasm of octreotide-treated neurons and colocalizes with IRAP-positive vesicles (arrows) as
961 illustrated by high resolution confocal microscopy in three different neurons. Scale bars: 2 μm .

962
963 **Figure 4. Kinetics of sst2A receptor recycling in primary hippocampal neurons.** Fifteen
964 minutes after agonist washout, only intracellular sst2A receptor immunoreactivity is apparent.
965 Thirty min after OCT washout, the large majority of sst2A receptor immunofluorescence is still
966 intracellular. However, membrane-associated receptors become apparent in perikarya and
967 proximal dendrites. From 60 to 120 min after agonist washout, the surface sst2A receptor
968 labeling becomes more and more intense. In parallel, the intensity of intracellular
969 immunofluorescence gradually decreases. At 180 min after OCT washout, the distribution of
970 sst2A receptor immunoreactivity is very similar to control conditions (i.e. before agonist-induced
971 internalization of the receptor). The colocalization of sst2A receptor with IRAP (yellow
972 pseudocolor signal on sst2A/IRAP merged images) and *trans*-Golgi network marker TGN38
973 (white pseudocolor signal on sst2A/IRAP/TGN38 merged images) is important at 30 min after
974 octreotide washout and gradually decreases from 60 to 180 min. Thirty neurons/group were
975 analyzed from 3 independent experiments. OCT, octreotide. Scale bars: 10 μm .

976
977 **Figure 5. IRAP ligands accelerate sst2A receptor recycling in hippocampal neurons.** At 45
978 min after OCT washout, sst2A receptor immunoreactivity is localized in the cytoplasm,
979 overlapping with the *trans*-Golgi network marker TGN38, as well as at the cell surface in a form
980 of a fluorescent ring (arrows). Note that the surface sst2A receptor labeling is more intense in

981 the Ang IV-treated neuron than in the PBS control. OCT, octreotide; PBS, phosphate-buffered
982 saline. Scale bars: 10 μm .

983
984 **Figure 6. Analysis of sst2A receptor recycling in hippocampal neurons.** a) For
985 semiquantitative analysis, a single optical section is acquired from the neuronal cell body at a z-
986 axis level where the sst2A receptor labeling at the plasma membrane is identified as a line-like
987 ring in the green channel around the TGN38 labeling of the red channel. Scale bar: 10 μm . b)
988 Semiquantitative analysis reveals a significantly higher sst2A receptor immunofluorescence at
989 the plasma membrane 30, 45 and 60 min after OCT washout in the Ang IV-treated neurons as
990 compared to the respective PBS controls (upper panel). The sst2A receptor signal intensity is
991 higher at the plasma membrane 45 min after OCT or SRIF washout in the Ang IV-treated group
992 as compared to the respective PBS controls. Note, that no significant difference is detected
993 between respective OCT- and SRIF-treated groups (lower panel). Thirty neurons/group were
994 analyzed from 3 independent experiments. Values are expressed in relation to an arbitrary unit
995 (100%) of the control value (PBS w/o agonist). *** $p < 0.001$; ns, not significant (ANOVA followed
996 by Bonferroni's Multiple Comparison Test). Ang IV, angiotensin IV; OCT, octreotide; PBS,
997 phosphate-buffered saline.

998
999 **Figure 7. IRAP ligands do not bind to the sst2A receptor in stably transfected CHO-K1**
1000 **cells.** The specific binding of Ang IV (a) and LVV-H7 (b) to sst2A receptors in CHO-K1 cells was
1001 investigated by incubating these cells with increasing concentrations (10^{-9} M to 10^{-5} M) of IRAP
1002 ligands and 0.5 nM radiolabeled SRIF. In four independent experiments, the ^{125}I -labeled SRIF
1003 and IRAP ligands competed for binding to sst2A receptors, after which the number of counts per
1004 minute were measured. The results are shown as the percentage of ^{125}I -labeled SRIF binding to
1005 sst2A receptors. Ang IV, angiotensin IV; LVV-H7, LVV-Hemorphin 7.

1006
1007 **Figure 8. IRAP-targeting shRNAs decreases IRAP immunoreactivity in hippocampal**
1008 **neurons.** a) Using TurboGFP lentiviral particles with three IRAP-targeting shRNAs (IRAP LV-
1009 shRNA1-3) and a non-targeting control (NT LV-shRNA), neuronal transduction is demonstrated
1010 by the presence of the TurboGFP signal (green). In neurons transduced with non-targeting LV-
1011 shRNA, IRAP immunoreactivity is mainly confined to vesicles in the cell bodies and proximal
1012 dendrites (red). Note that in neurons transduced with IRAP-targeting shRNA, the intensity of
1013 IRAP immunofluorescence is low. Scale bars: 10 μ m. b) Semiquantitative analysis reveals that
1014 the intensity of IRAP immunoreactivity in the IRAP LV-shRNA1-3 groups is significantly lower as
1015 compared to the NT LV-shRNA controls. Thirty neurons/group were analyzed from 3
1016 independent experiments. *** $p < 0.001$ (ANOVA followed by Bonferroni's Multiple Comparison
1017 Test).

1018 **Figure 9. IRAP knockdown increases sst2A receptor recycling in hippocampal neurons.** a)
1019 Using TurboGFP lentiviral particles with three IRAP-targeting shRNAs (IRAP LV-shRNA1-3) and
1020 a non-targeting control (NT LV-shRNA), neuronal transduction is demonstrated by the presence
1021 of the TurboGFP signal. At 45 min after agonist washout, in neurons transduced with both non-
1022 targeting and IRAP-targeting shRNA, the sst2A receptor immunoreactivity is localized in the
1023 cytoplasm, overlapping with the *trans*-Golgi marker TGN38. Receptors are also localized at the
1024 cell surface (arrows). Note, however, that the intensity of the surface sst2A receptor labeling is
1025 higher in neurons transduced with IRAP LV-shRNAs than those with the NT LV-shRNA control.
1026 Scale bars: 10 μ m. b) Semiquantitative analysis reveals that the intensity of sst2A receptor
1027 immunoreactivity at the plasma membrane 45 min after OCT washout is significantly higher in
1028 the IRAP LV-shRNA1-3 groups as compared to the NT LV-shRNA control. Thirty neurons/group
1029 were analyzed from 3 independent experiments. Values are expressed in relation to an arbitrary
1030 unit (100%) of the control value (NT LV-shRNA). *** $p < 0.001$ (ANOVA followed by Bonferroni's
1031 Multiple Comparison Test). OCT, octreotide.

1032
1033 **Figure 10. IRAP ligands display anticonvulsive properties in the rat focal pilocarpine**
1034 **model.** Time profiles of seizure severity scores (SSS) are shown with total seizure severity
1035 score (TSSS) in the inserts. Both Ang IV (a) and LVV-H7 (b) treatments at 10 μ M concentration
1036 result in a significantly lower SSS and TSSS. **p < 0.01; ***p < 0.001 vs. pilocarpine control
1037 group (SSS: two-way ANOVA followed by Dunnett's Multiple Comparison Test; TSSS: ANOVA
1038 followed by Bonferroni's Multiple Comparison Test). Ang IV, angiotensin IV; LVV-H7, LVV-
1039 Hemorphin 7.

1040
1041 **Figure 11. Antagonists of the sst2A receptor prevent the anticonvulsive effect of Ang IV**
1042 **and LVV-H7 in the rat focal pilocarpine model.** The sst2A receptor antagonist cyanamid
1043 154806 (a and c) or BIM-23627 (b) reverse the effect of Ang IV (a, b) and LVV-H7 (c) on total
1044 seizure severity score (TSSS). *p < 0.05; **p < 0.01 between groups (ANOVA followed by
1045 Bonferroni's Multiple Comparison Test). Ang IV, angiotensin IV; LVV-H7, LVV-Hemorphin 7.

1046
1047 **Figure 12. Angiotensin AT1 (a) and μ/κ (b) receptors are not implicated in the**
1048 **anticonvulsive effect of Ang IV and LVV-H7 in the focal pilocarpine model.** AT1 receptor
1049 antagonist Candesartan (a) and the opioid μ/κ receptor antagonist Naltrexone (b) have no
1050 significant effect on seizure severity as compared to the pilocarpine control group. Furthermore,
1051 pre-treatment with Candesartan or Naltrexone does not abolish the anticonvulsive effect of Ang
1052 IV. **p < 0.01; ns, not significant compared to the pilocarpine control group (ANOVA followed by
1053 Bonferroni's Multiple Comparison Test). TSSS, total seizure severity score; Ang IV, angiotensin
1054 IV; LVV-H7, LVV-Hemorphin 7.

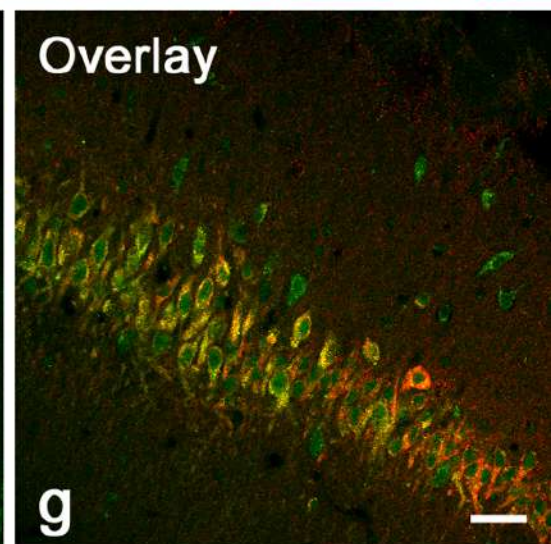
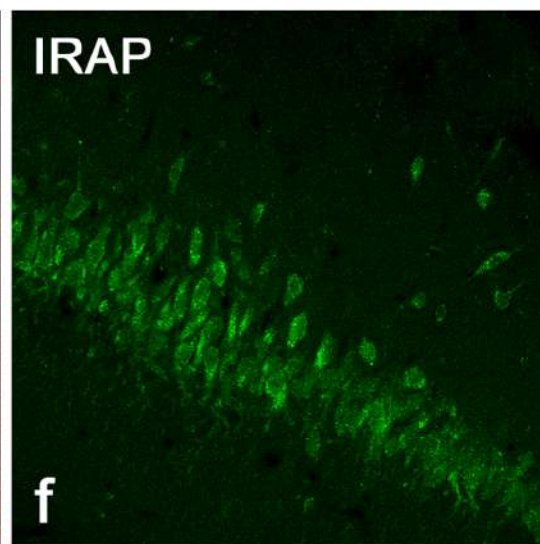
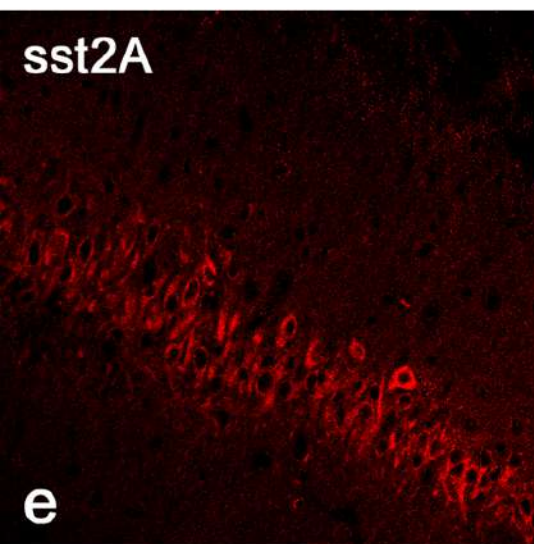
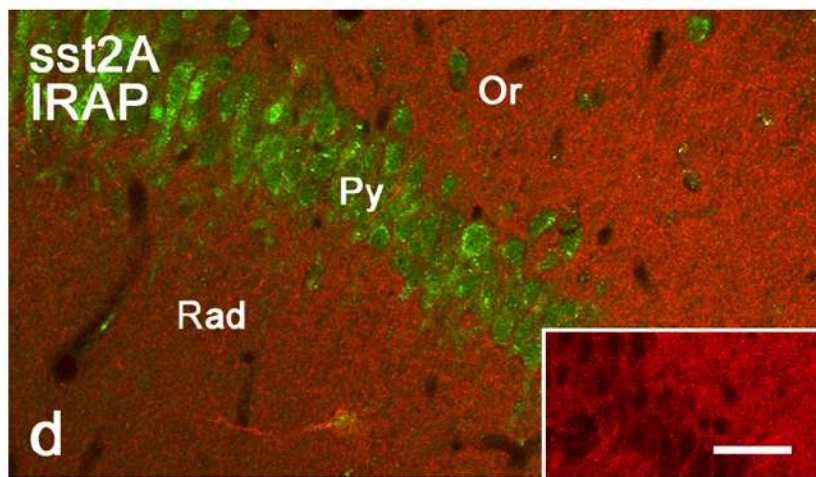
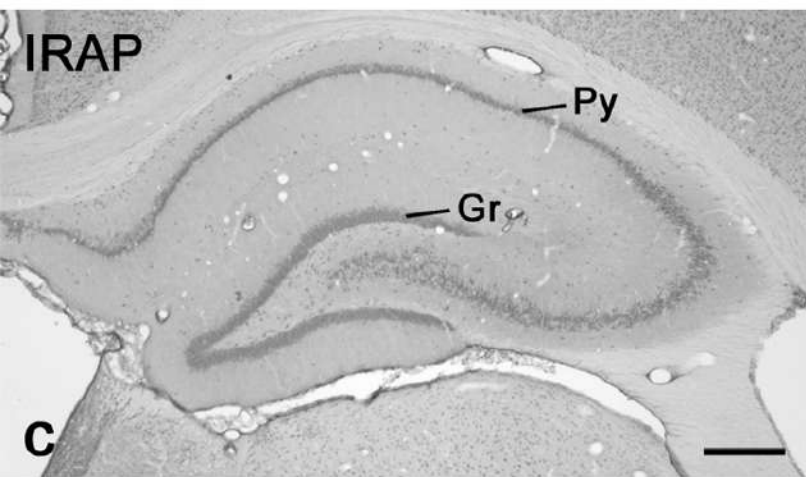
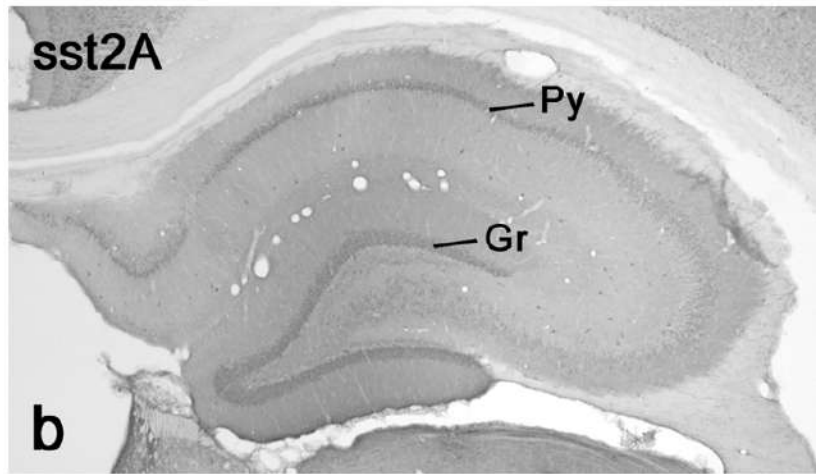
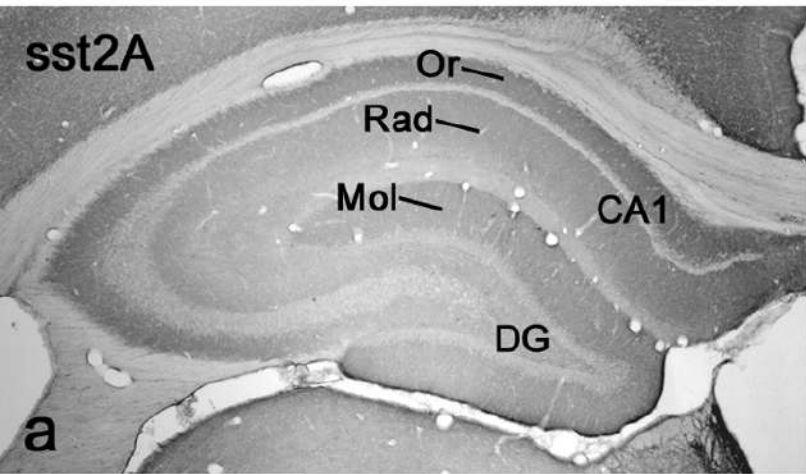
1055
1056 **Figure 13. Administration of Ang IV and LVV-H7 does not decrease hippocampal SRIF**
1057 **concentration.** Administration of Ang IV or LVV-H7 has no effect on SRIF concentration of

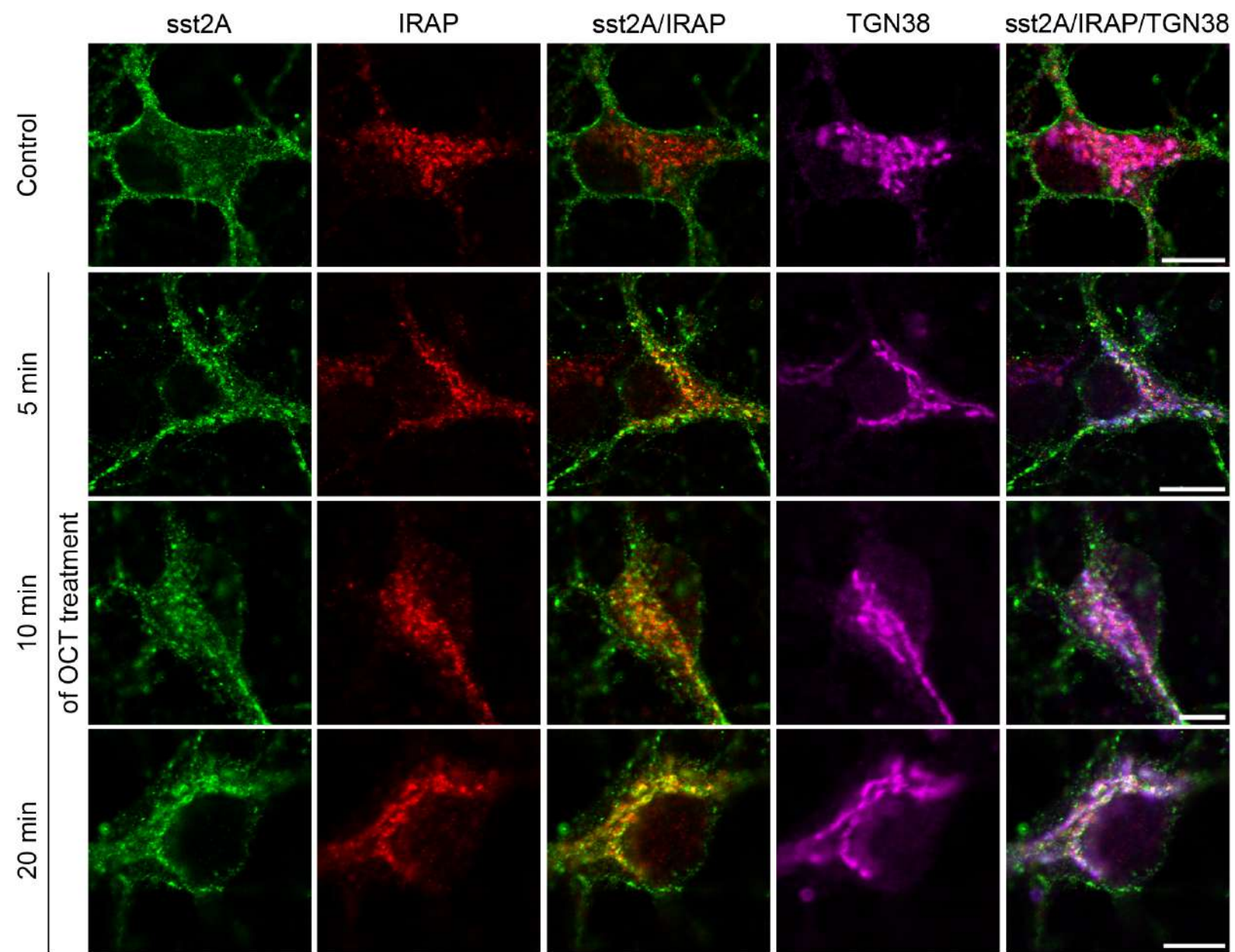
1058 hippocampal microdialysates (two-way ANOVA followed by Bonferroni's Multiple Comparison
1059 Test: Ang IV or LVV-H7 vs. aCSF control group). aCSF, artificial cerebrospinal fluid; Ang IV,
1060 angiotensin IV; LVV-H7, LVV-Hemorphin 7.

1061
1062 **Figure 14. Ang IV potentiates the anticonvulsive effect of SRIF in the focal pilocarpine**
1063 **model.** A combination of subthreshold concentrations (i.e. ineffective on pilocarpine-induced
1064 seizures) of Ang IV (1 μ M) and SRIF (0.1 μ M) significantly reduces behavioral seizures (a)
1065 compared to the pilocarpine control group. In rats treated with both Ang IV and SRIF, EEG
1066 seizure duration is not significantly different (b), while the latency to first EEG seizure is
1067 significantly higher (c) compared to the pilocarpine control group. The TSSS is positively
1068 correlated with EEG seizure duration (c) and negatively correlated with EEG seizure latency (d).
1069 Representative EEG traces are shown in (f) with (g) illustrating baseline EEG activity (left
1070 panels) and typical spike-wave activity 40 min following the onset of pilocarpine administration
1071 (right panels). * $p < 0.05$; ** $p < 0.01$ compared to the pilocarpine control group (ANOVA followed
1072 by Bonferroni's Multiple Comparison Test). TSSS, total seizure severity score; Ang IV,
1073 angiotensin IV; LVV-H7, LVV-Hemorphin 7.

1074
1075 **Figure 15. Ang IV increases *in vivo* the density of sst2A receptors at the plasma**
1076 **membrane in pilocarpine-treated rats.** a,b) In pilocarpine control rats, many sst2A receptor
1077 immunoparticles are intracellular (arrows) in dendrites (a) and dendritic spines (inset of a) of
1078 CA1 pyramidal neurons. In the pilocarpine group which received intra-hippocampal perfusion of
1079 Ang IV, numerous sst2A receptor immunoparticles are located at the plasma membrane
1080 (arrowheads) of dendrites (b) and dendritic spines (inset of b) of CA1 pyramidal neurons. Scale
1081 bar: 500 nm. c) In pilocarpine control rats, the largest proportion of sst2A receptor
1082 immunoparticles are intracellular in CA1 pyramidal cell dendrites, only 28.6% of them are
1083 associated with the plasma membrane. By contrast, in the pilocarpine group treated with Ang IV,

1084 70.5% of sst2A receptor immunoparticles are at the plasma membrane of CA1 pyramidal cell
1085 dendrites. d) Statistical analysis reveals that the density of immunoparticles is significantly higher
1086 at the plasma membrane and lower intracellularly in the Ang IV-treated pilocarpine group as
1087 compared to the pilocarpine controls. Note that the total number of immunoparticles is not
1088 different between the two groups. Ten dendrites/animal were analyzed in 3 control and Ang IV-
1089 treated rats. Values are expressed in relation to an arbitrary unit (100%) of the control values.
1090 **p < 0.01; ***p < 0.001; ns, not significant (Mann-Whitney U test). Ang IV, angiotensin IV; IC,
1091 intracellular; PM, plasma membrane.

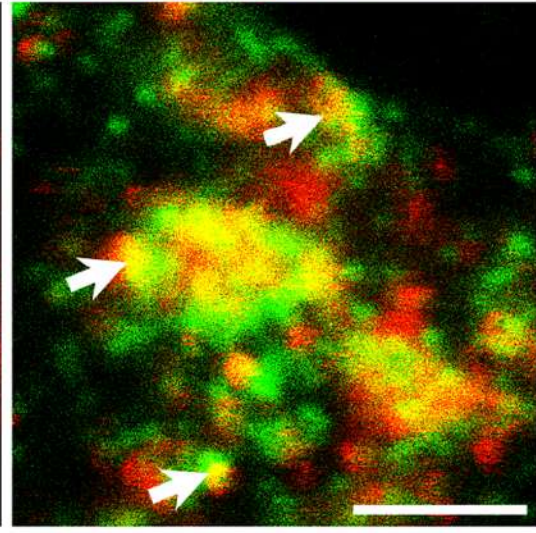
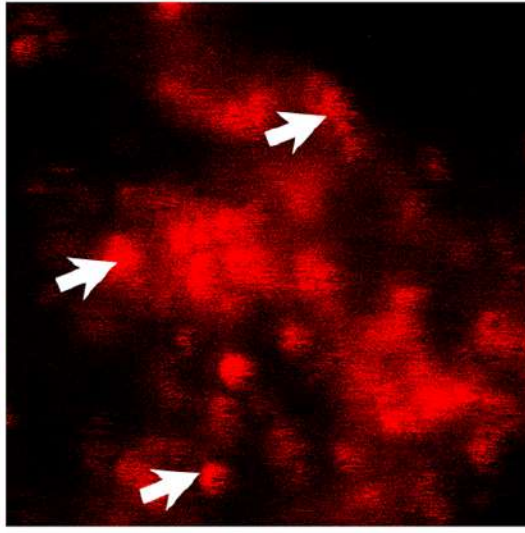
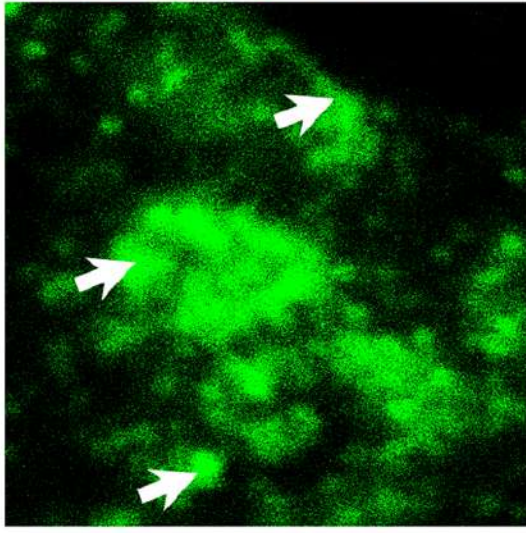
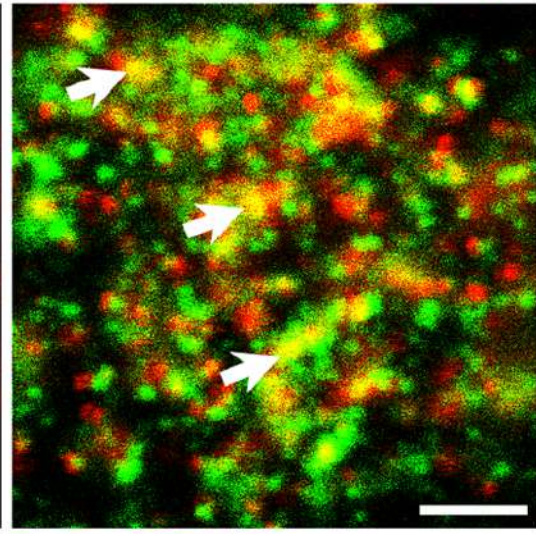
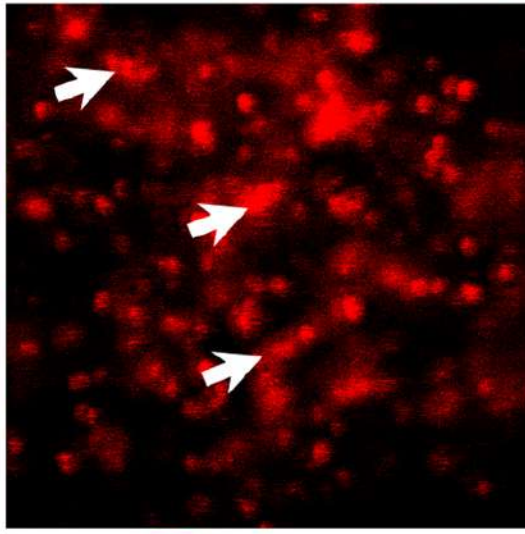
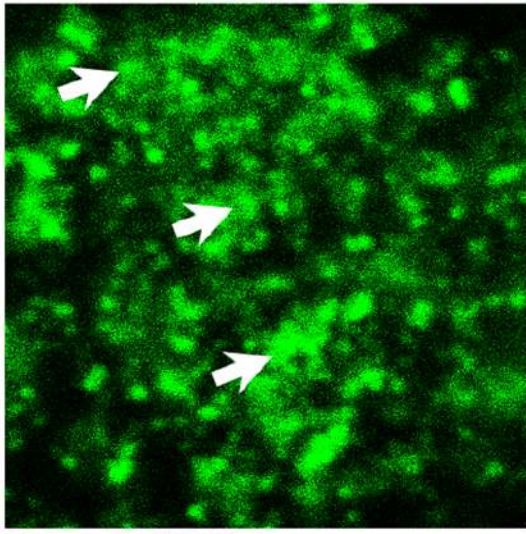
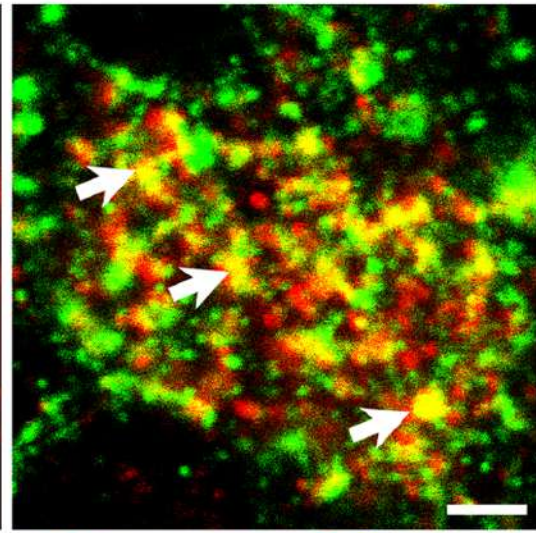
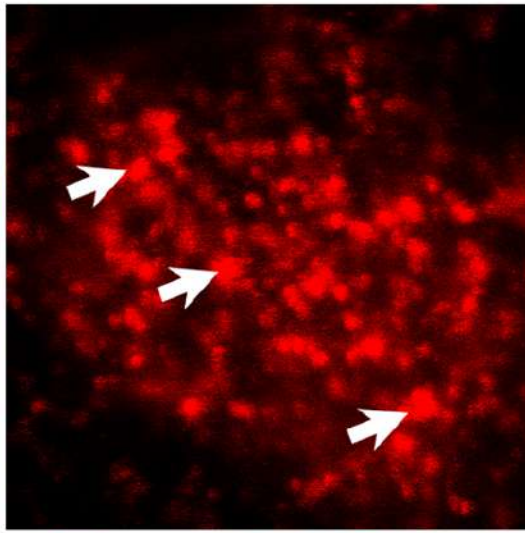
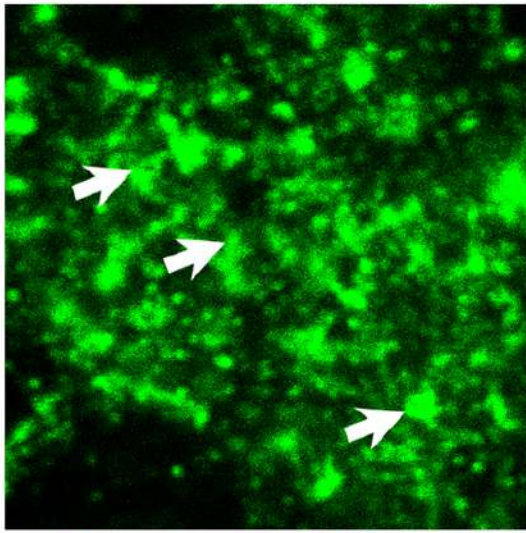


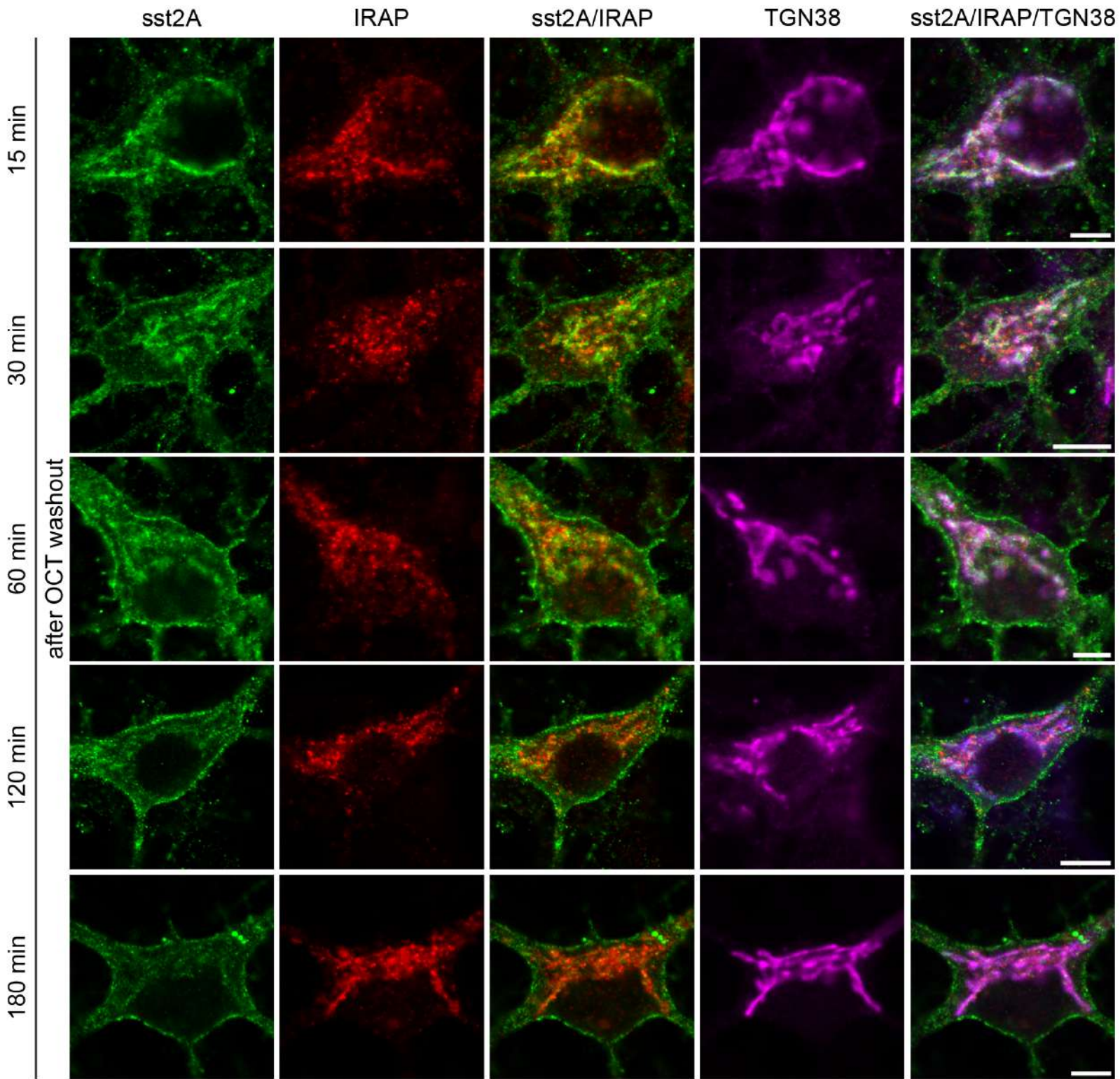


sst2A

IRAP

sst2A/IRAP



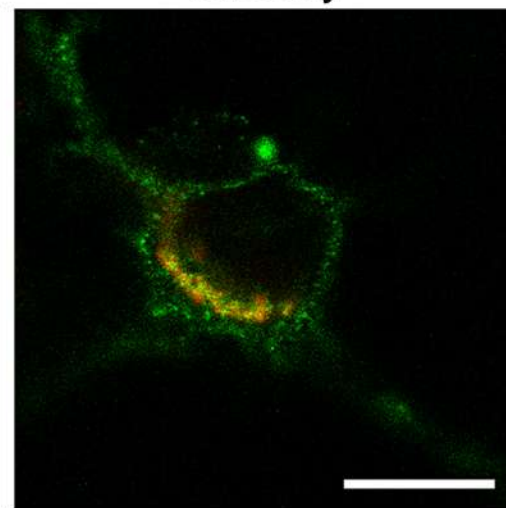
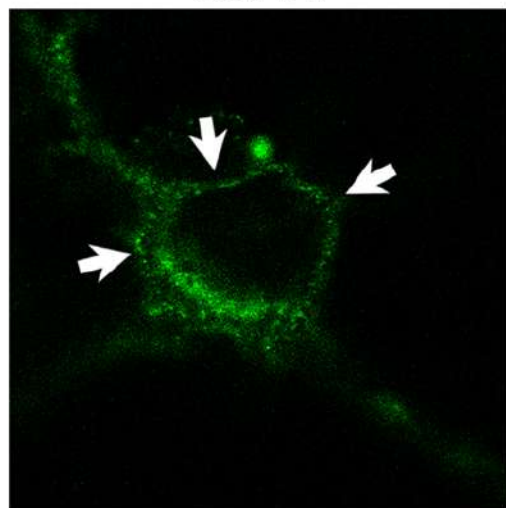


sst2A

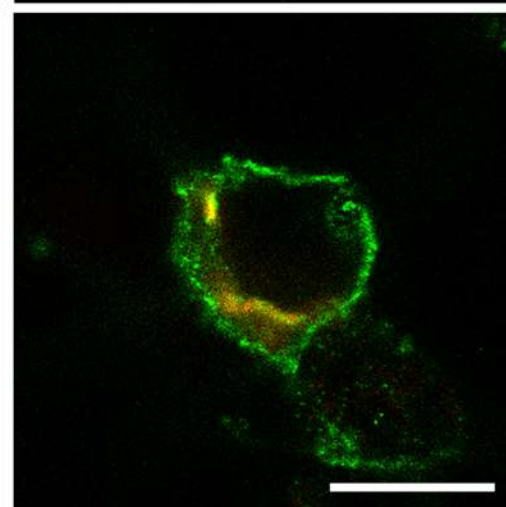
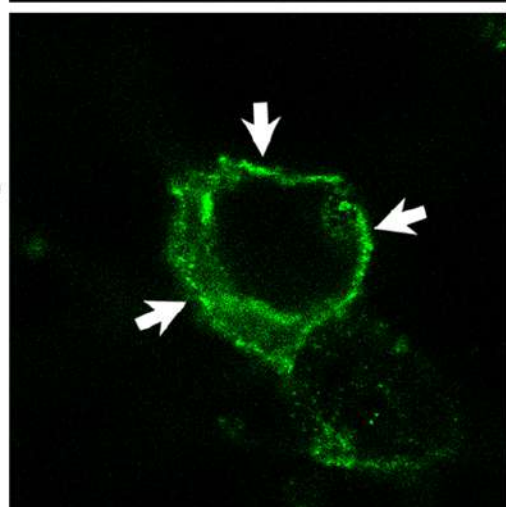
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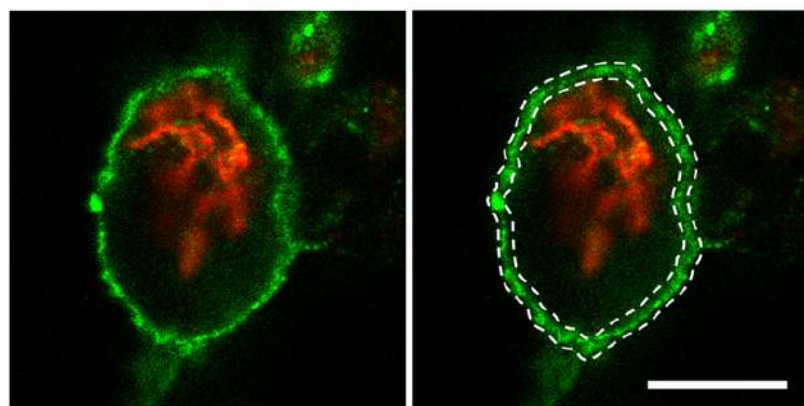
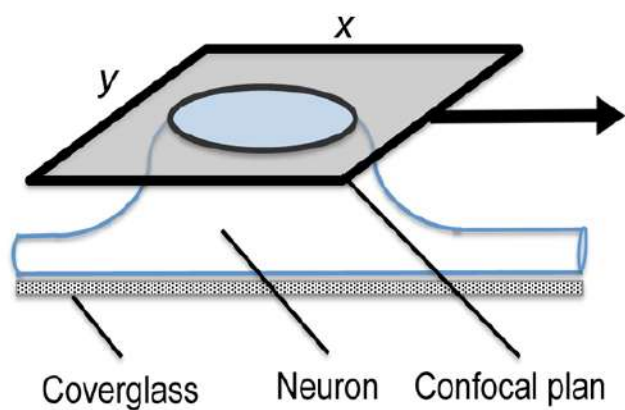
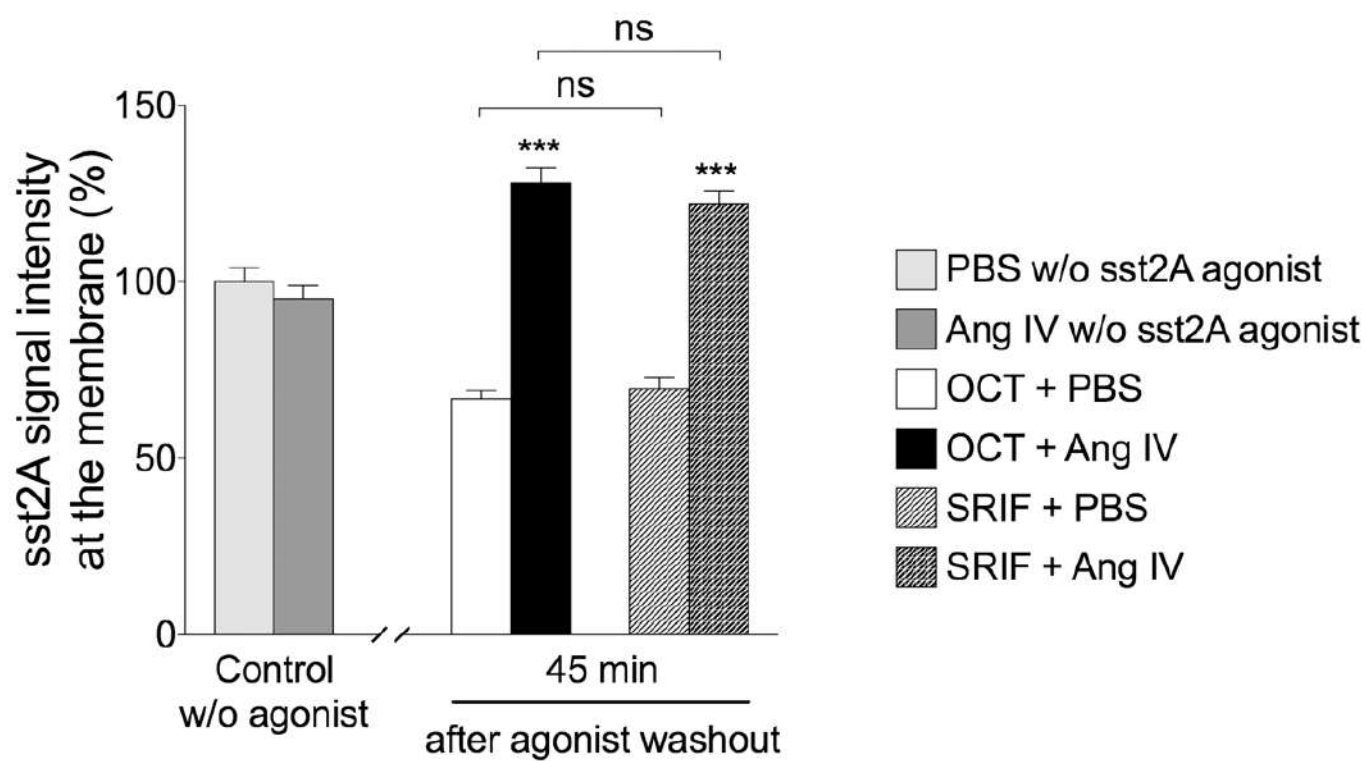
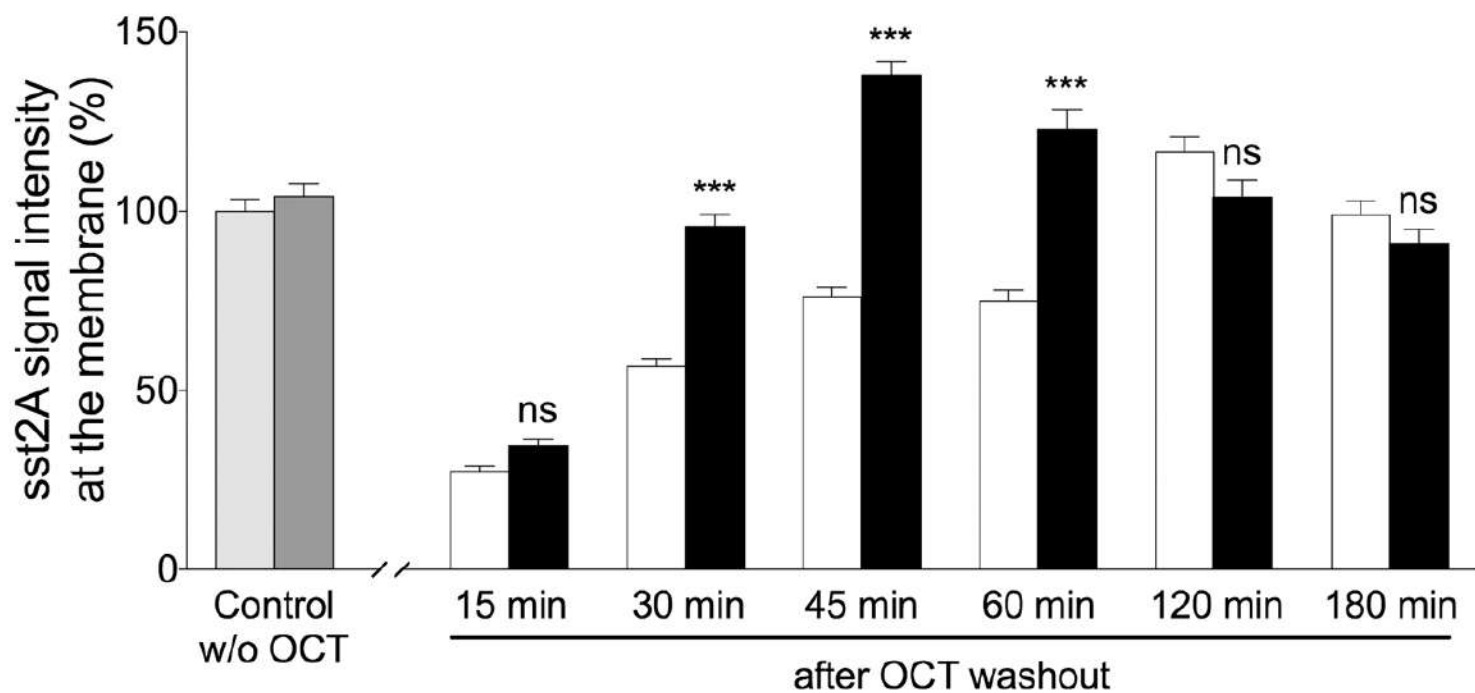
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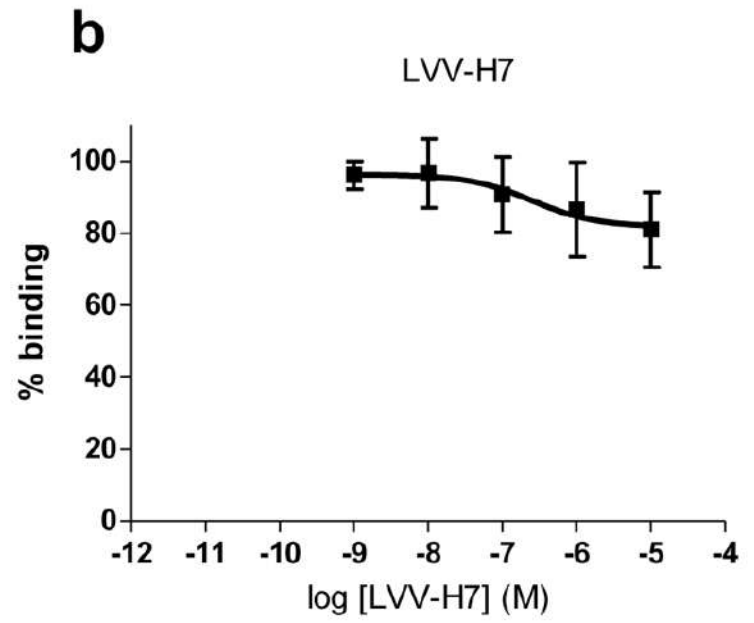
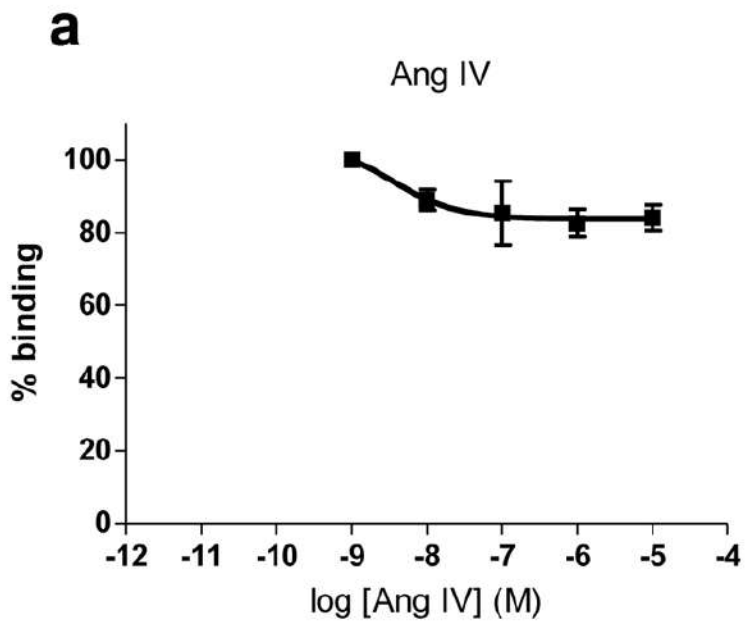
OCT + PBS

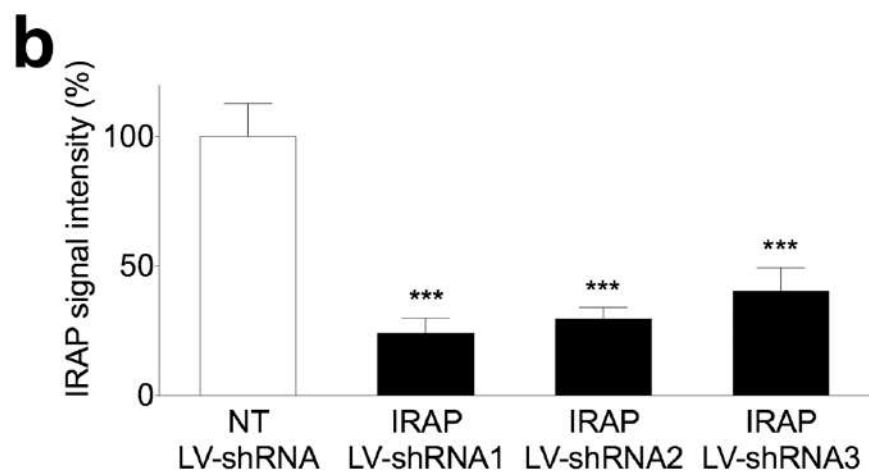
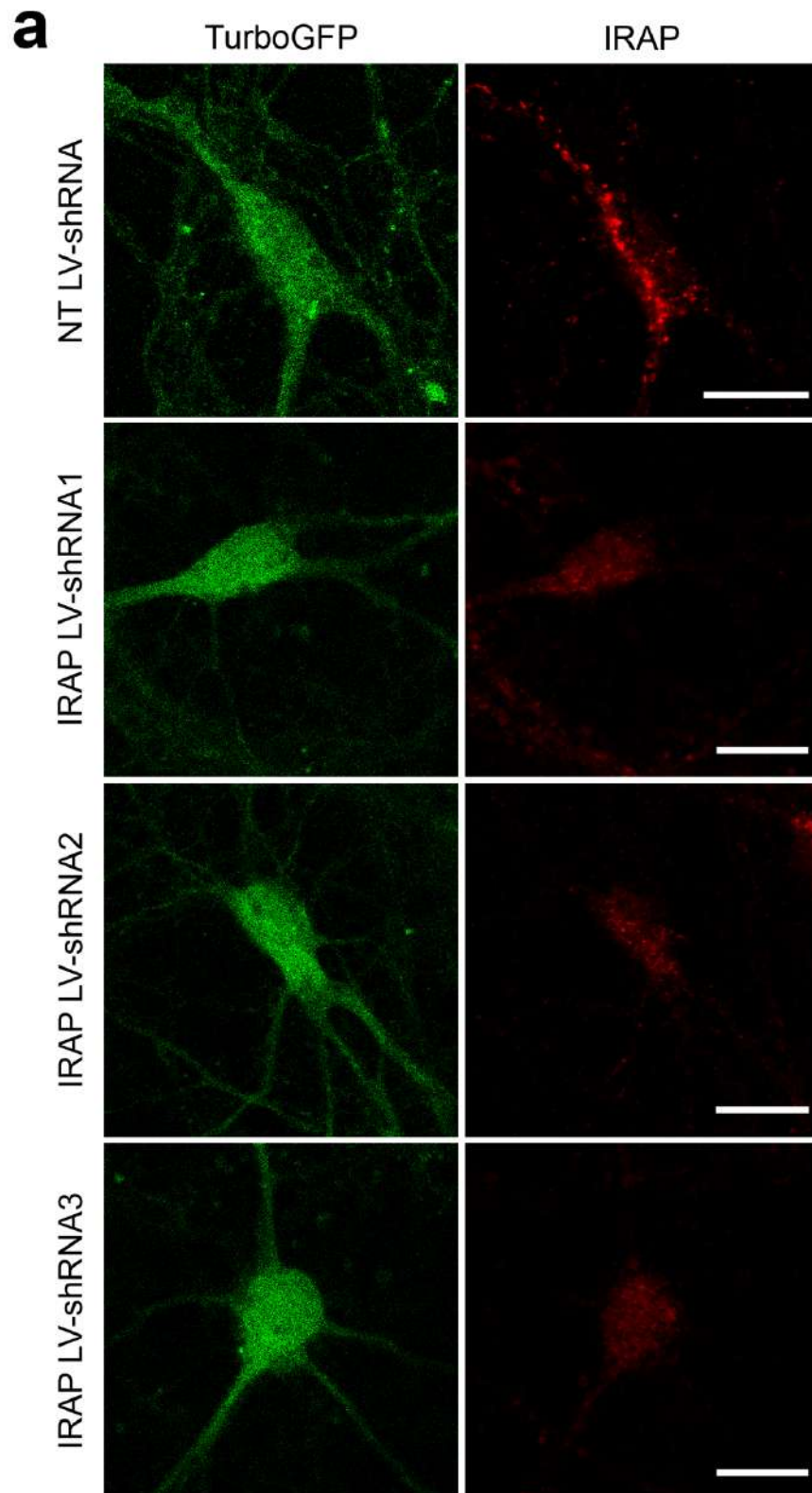


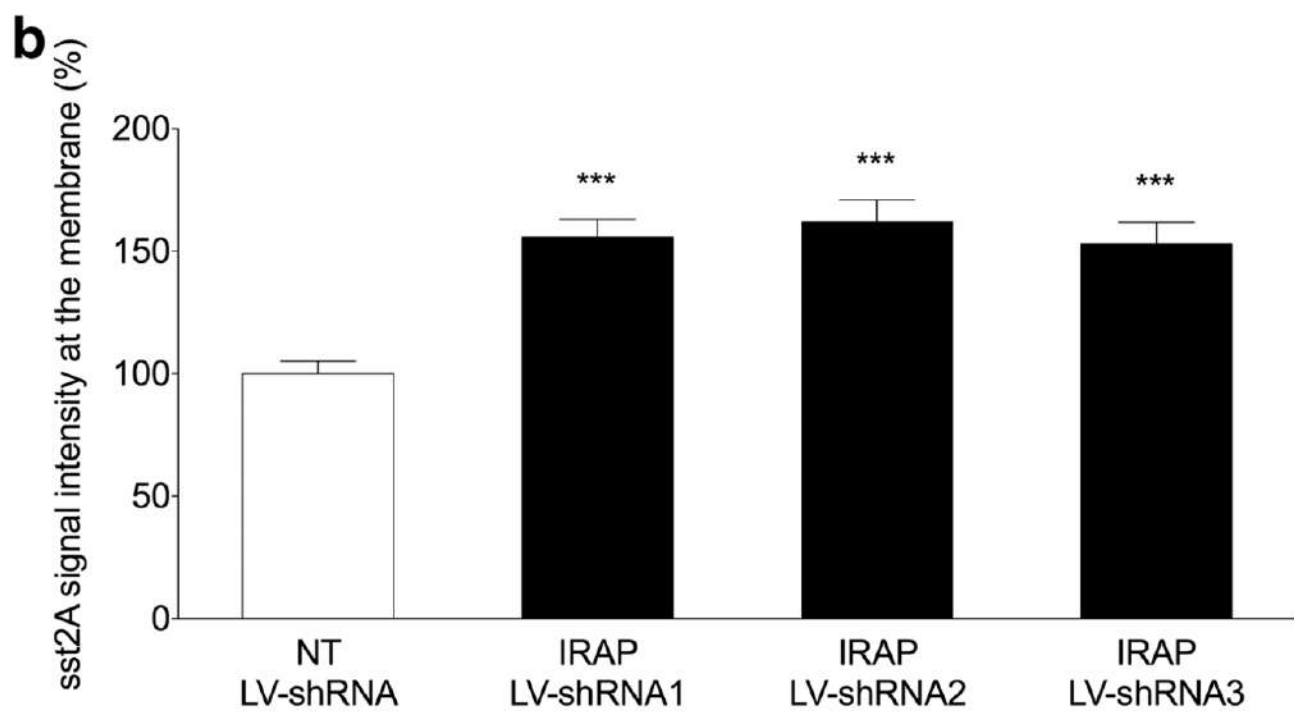
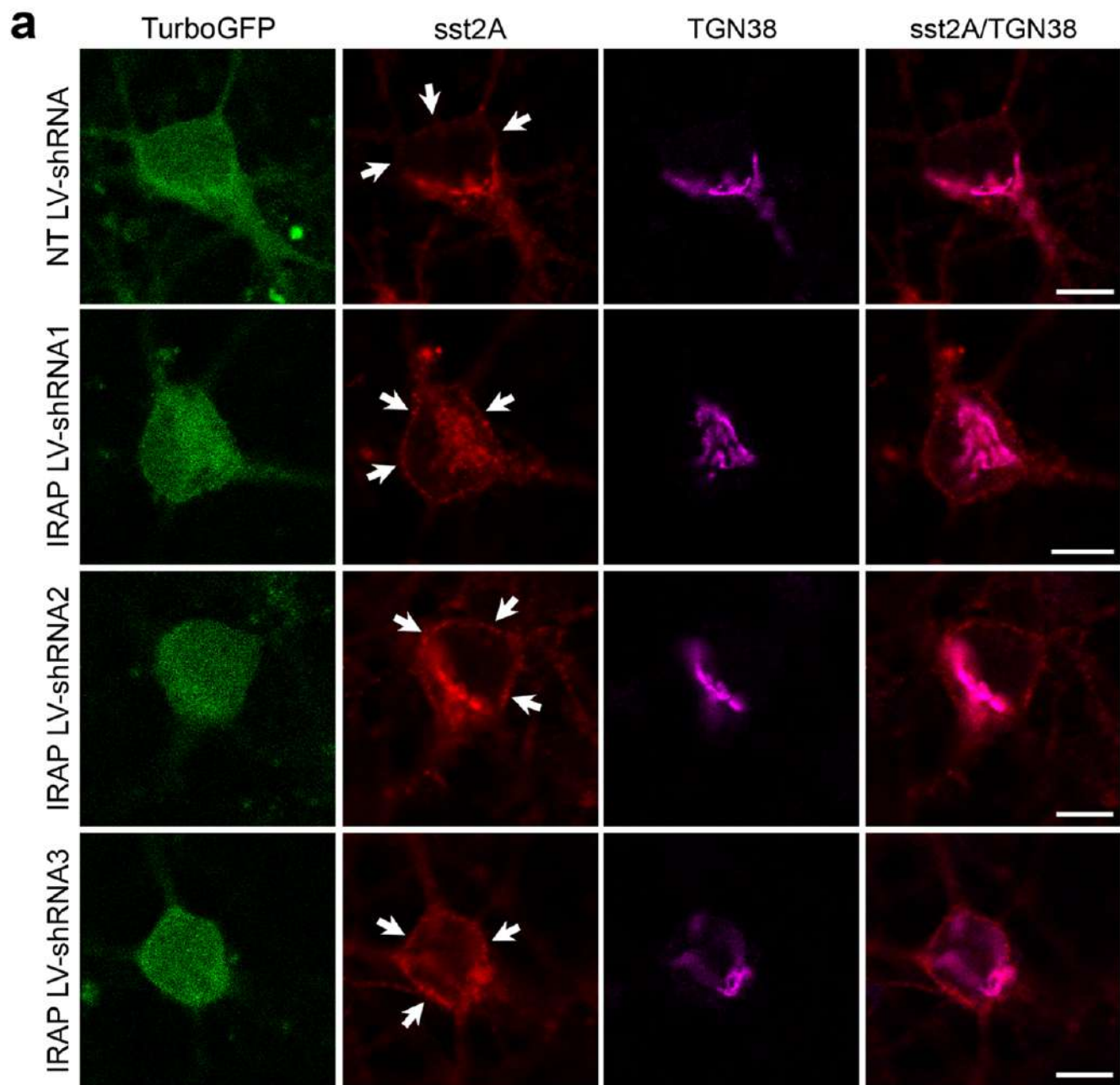
OCT + Ang IV

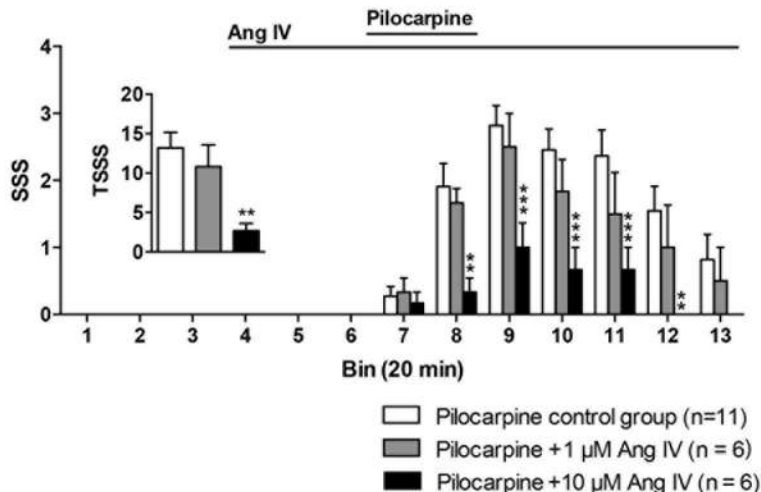
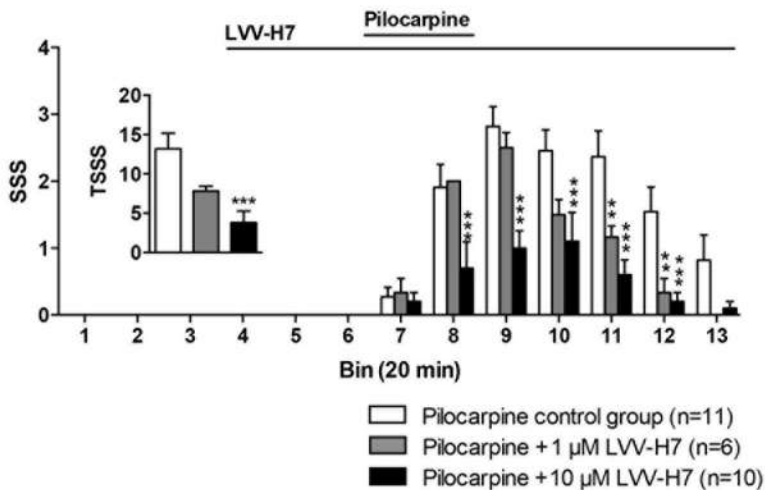


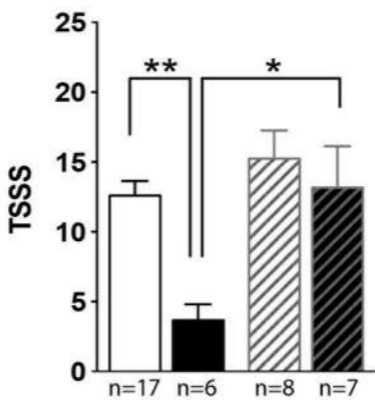
a**b**



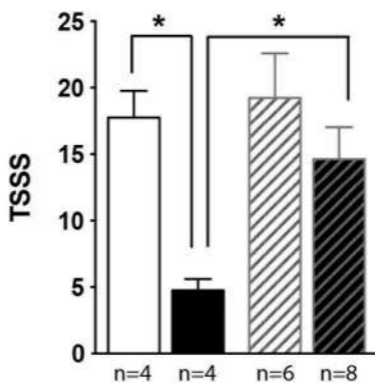




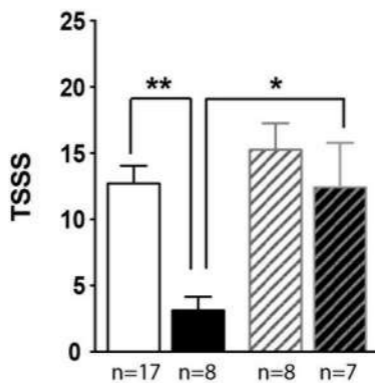
a**b**

a

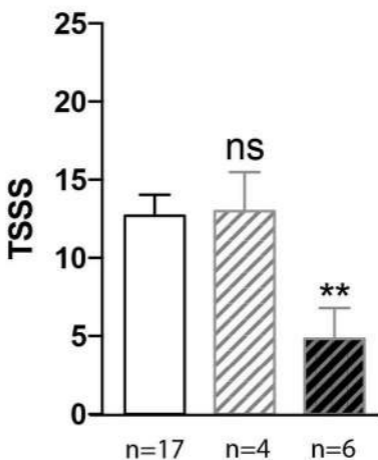
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Ang IV (10 μ M)	-	+	-	+
Cyanamid (0.1 μ M)	-	-	+	+

b

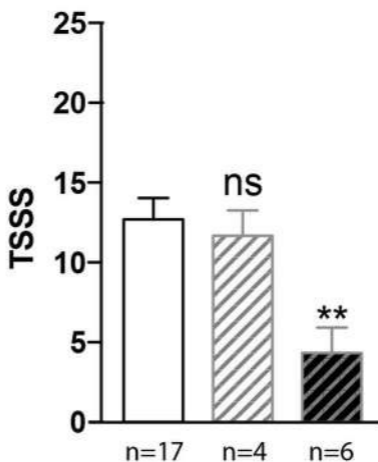
Pilocarpine	+	+	+	+
Ang IV (15 μ M)	-	+	-	+
BIM-23627 (0.1 μ M)	-	-	+	+

c

Pilocarpine	+	+	+	+
LVV-H7 (10 μ M)	-	+	-	+
Cyanamid (0.1 μ M)	-	-	+	+

a

Pilocarpine	+	+	+
Ang IV (10 μ M)	-	-	+
Candesartan (0.1 μ M)	-	+	+

b

Pilocarpine	+	+	+
LVV-H7 (10 μ M)	-	-	+
Naltrexone (0.1 μ M)	-	+	+

