In vivo actions of SCTR/AT1aR heteromer in controlling Vp expression and release via cFos/cAMP/CREB pathway in magnocellular neurons of PVN

Sarah O.K. Mak[1], Li Zhang[2], Billy K.C. Chow[1*]

[1]School of Biological Sciences, The University of Hong Kong, Hong Kong, China

[2]GHM Institute of CNS Regeneration, Jinan University, Guangzhou, China

*Corresponding Author

Corresponding author and person to whom reprint request should be addressed:

Billy K.C. Chow, PhD

4N-12, School of Biological Sciences, Kadoorie Biological Sciences Building,

The University of Hong Kong,

Pokfulam Road, Sai Ying Pun, Hong Kong SAR, China.

Phone: +852-22990850

Fax: +852-25599114

Email: bkcc@hku.hk

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Abbreviations

5HT1A-mGlu$_2$	Serotonin metabotropic glutamate 2 receptor heterocomplex
ACSF	Artificial cerebrospinal fluid
ANGII	Angiotensin II
AT1aR	Angiotensin II receptor type 1 subtype a
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCR2/CCR5	Chemokine receptor type 2/ chemokine receptor type 5
CREB	cAMP response element-binding protein
DMSO	Dimethyl sulfoxide
EIA	Enzyme immunoassay
GPCR	G-protein coupled receptor
HE	Hematoxylin and Eosin
I.c.v.	Intracerebroventricular
IF	Immunofluorescence
IHC	Immunohistochemistry
IR	Immunoreactive
LCM	Laser capture microdissection
MAPK	Mitogen-activated protein kinases
MnPO	Median pre-optic nucleus
PaLM	Lateral magnocellular
PaMM	Medial magnocellular
PaMP	Medial parvocellular
PKA	Protein kinase A
PVN	Paraventricular nucleus
RIPA	Radioimmunoprecipitation assay
SCT	Secretin
SCT$^{-/-}$	Secretin knockout
SCTR	Secretin receptor
SCTR$^{-/-}$	Secretin receptor knockout
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIADH	Syndrome of inappropriate antidiuretic hormone secretion
SON	Supraoptic nucleus

54	$SSTR_{1B}$	Somatostatin receptor type 1 subtype b
55	TM	Transmembrane
56	TM-m	Transmembrane-mutant
57	Vp	Vasopressin
58	WT	Wild type
59	δOR-μOR	Opioid receptor heteromer

Abstract

With an increasing body of evidence regarding G-protein coupled receptor (GPCR) oligomerization and its clinical implications over the last decade, studies on modulation and dynamics of GPCR homo- and hetero-oligomers is recently an area of intense research focus. Previously, our lab showed *in vitro* heteromer formation between angiotensin II 1a receptor (AT1aR) and secretin receptor (SCTR), which is involved in *in vivo* control of hyperosmolality-induced water drinking behaviour. As the SCT/SCTR axis is crucial to the central actions of angiotensin II (ANGII), and both SCT and ANGII are capable of triggering vasopressin (Vp) release from hypothalamus, we investigated here the *in vivo* role of SCTR-AT1aR heteromer in regulating Vp release in hypothalamus using transmembrane (TM) peptides as tools. We showed that SCTR-AT1aR heteromer mediates stimulatory actions of both SCT and ANGII in hypothalamic Vp expression and release as well as neuronal activities via the immediate early gene cFos. Result from this study not only is consistent with our hypothesis that SCT and ANGII interact at the receptor level to mediate their water homeostatic activities, but also provide evidence for *in vivo* functions of cross-class GPCR heteromers.

Keywords: Secretin receptor, Angiotensin II type 1a receptor, GPCR heteromerization, vasopressin, transmembrane peptides, *in vivo* analysis

Introduction

GPCR is one of the biggest gene families, acting as a key to relate messages from extracellular side to intracellular signalling events. Due to its significant involvement in physiological and pathological processes (1, 2), it is the most important target for therapeutic intervention accounting for approximately 50% of all current marketed drugs (3, 4). For the purpose of developing medicine targeting to GPCRs, it is clearly important that we understand how these receptors are activated and regulated. In the past, GPCRs have been assumed to exist in monomeric form, however, recent data from laboratories over the world have provided support to models in which some GPCRs can exist as homomers and/or heteromers carrying out numerous cellular functions that are not possible if they are in monomeric conformation (5, 6). Many studies have showed that GPCR oligomerization could confer structural and functional advantages and, in some cases, they are implicated in certain diseases (7). Oligomerization of class A rhodopsin-related receptors is the first direct structural evidence revealing that they can form higher-order oligomers within certain transmembrane interfaces, and these oligomers are functionally more efficient when compared to their monomeric forms (8, 9). An exciting example of GPCR oligomerization in clinical implication is the cross-class serotonin metabotropic glutamate 2 (5HT2A-mGlu$_2$) receptor heterocomplex in schizophrenia and antipsychotic drug action (10, 11).

Class A AT1aR and class B SCTR were found to share overlapping osmoregulatory functions (12, 13). Under hyperosmolality, ANGII and secretin (SCT) are released from the osmoregulatory centres of the brain to trigger the release of Vp (14, 15). More importantly, central administration by intracerebroventricular (i.c.v.) injection of ANGII was found ineffective in stimulating water intake and Vp release in SCT$^{-/-}$ and SCTR$^{-/-}$ mice, indicating that a functional SCT/SCTR axis is needed to mediate ANGII osmoregulatory actions (12). As these two receptors play overlapping activities in osmoregulation, as well as they are co-localized in the paraventricular nucleus (PVN) (12), we hypothesize that AT1aR and SCTR may interact in PVN to control central Vp expression and release. By bioluminescence resonance energy transfer (BRET) assays, our laboratory has previously demonstrated that these two receptors are able to form heteromer *in vitro* (13). Investigating *in vivo* biological actions of GPCR oligomers is technically challenging (16), and in this report, we tried to circumvent this by using TM peptides as a biochemical tool to modulate functions of SCTR/AT1aR heteromer. Since the lipid-exposed face of GPCR TM peptides are the major determinant for their oligomerization, therefore artificial corresponding TM segments of receptors are added as competitors to disrupt the formation of GPCR oligomers. TM peptides were used in the past in studying opioid receptor heteromer (δOR-μOR) (17, 18). We have also shown that, the first and fourth TM segments of AT1aR (ATM1 and ATM4) and the second and fourth TM segments of SCTR (STM2 and STM4) can reduce *in vitro* SCTR/AT1aR heteromer formation as shown in BRET assays (13). Moreover, central injection of ATM4 and STM2 can abolish hyperosmolality-induced water drinking behaviour in mice (13). As ANGII and Vp are the most important components in body osmoregulation, we seek to

investigate the mechanistic role of SCT/SCTR in bridging their actions in central. In this report, we analyzed the effects of SCT/ANGII-induced Vp release and expression within hypothalamus after i.c.v. injection of ATM4 or STM2. We demonstrated that SCTR/AT1aR heteromer could control Vp release and expression, as well as its involvement in activation of cFos gene and PKA pathway in PVN and supraoptic nucleus (SON) of the hypothalamus.

Materials and Methods

Animals

Procedures of animal care and handling were in accordance with the protocols approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. All experiments were carried out using adult mice (25-30 g) of ≥N10 generation, which were kept in a temperature-controlled room with a 12-h light-dark cycle and provided with standard (0.3% Na; TestDiet) rodent chow and water ad libitum prior to experimentation. As controls, C57BL/6N [wild-type (WT)] male mice were used in the experiments.

I.c.v. incannulation and drug administration

WT mice were anesthetized and surgery was conducted in aseptic conditions. The coordinates of cannula implantation were determined according to mouse brain atlas of Paxinos and Franklin (19). The cannula (11 mm long, 21-gauge stainless steel tubing) was stereotaxically placed into lateral ventricle (Bregma: 0.5 mm, lateral: 1.0 mm, depth: 2.0 mm) and secured using Vetbond (3M Animal Care Products, St Paul, MN, USA) and dental acrylic. Animals were allowed to recover for 3 days before peptide injection. SCT (500 ng/5 μl; 60677; AnaSpec, Fremont, CA, USA) and ANGII (100 ng/2 μl; 002-12; Phoenix Pharmaceuticals, CA, USA) were centrally injected at the beginning, while TM peptide (4 μg in 5 μl) was i.c.v. injected after 1 hr. The TM peptides were synthesized by GenScript (Piscataway, NJ, USA) or AnaSpec (Fremont, CA, USA) with >90% purity. The sequences of TM peptides were as previously described (13). Artificial cerebrospinal fluid (ACSF; 5 μl) was centrally injected as basal control, dimethyl sulfoxide (DMSO; 20%) as vehicle control and TM-mutant (TM-m) peptide (4 μg in 5 μl) as negative control. Blood was collected from left jugular vein at 15 min or 30 min after i.c.v.-TM peptide injection. Vp levels from time 0 to 90 at 15 min intervals were monitored, and to eliminate the effects on blood volume regarding to Vp release, each data point was sampled only from 1 animal (n=6). Vp protein concentrations from blood serum were measured by the [Arg^8]-Vasopressin EIA kit (EK-065-07; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA).

Laser capture microdissection (LCM) and quantitative real-time PCR

Cells of PVN were captured by LCM as described in our previous studies (12, 14, 20). Total RNA of captured cells on the high-sensitivity caps (CapSure LCM Caps, ThermoFisher Scientific, USA) was isolated by Trizol. Total RNA was precipitated overnight with absolute ethanol, 0.3M ammonium acetate, yeast tRNA and glycogen. The precipitated RNA was then washed by 70% ethanol and resuspended in RNase-free water for first strand cDNA synthesis following protocol from HiScript®II Q RT SuperMix (Vazyme biotech co., ltd, China). Vp mRNA expression in PVN was measured by real-time PCR using SYBR Green PCR kit (Applied Biosystems, CA, USA) with 18S as an internal standard. Fluorescence signals were measured during the extension step by the

7300 Real-Time PCR System (Applied Biosystems, CA, USA). The threshold cycle (C_t) was defined as the fractional cycle number at which the fluorescence signal reaches 10-fold SD of the baseline (from cycles 2 to 10). The ratio change in the target relative to 18S control was determined by the $2^{-\Delta\Delta Ct}$ method (21). Primer sequences were: Vp, forward 5'-ATGCTC GCCAG GATGC TCA-3', reverse 5'-GGAGAC ACTGT CTCAG CTC-3'; PKA, forward 5'-TGGGAG ACCCC TTCTC AGA-3', reverse 5'-GCTTCA GGTCC CGGTA GAT-3'; 18S, forward, 5'-CTCTAG ATAAC CTCGG GCC-3', reverse, 5'-GAACCC TGATT CCCCG TCA-3'.

Immunohistochemistry (IHC) Staining

Brains isolated from mice were fixed in 4% formalin, dehydrated with graded ethanol, embedded in paraffin, and sectioned (8 μm). Immunostaining was performed with a Leica Bond-Max automatic immunostainer (Leica Bannockburn, IL, USA) according to the recommended procedure using rabbit anti-cFos antibody (1:500 dilution; Santa Cruz Biotechnology, Texas, USA). Images were captured with Nikon Eclipse Ni-U upright microscope (Nikon H550L). cFos-immunoreactivities (IR) positive nuclei expressed in PVN and SON were counted in 3 sections at the same Bregma level -0.82/-0.94 mm, which includes lateral magnocellular (PaLM), medial magnocellular (PaMM) and medial parvocellular (PaMP) neurons, and evaluated with ImageJ 2.0. Statistical comparisons were made between the number of cFos-IR positive cells in these brain areas in control and treatment groups using Student's *t*-test with significance set at $P<0.05$.

Double Immunofluorescence (IF) Staining

Paraffin sections (8 μm) were dewaxed and rehydrated in degraded ethanol. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Microwave antigen retrieval was performed with 10 mM sodium citrate buffer at pH 6.0 for 10 min, followed by blocking of non-immunological binding with 5% bovine serum albumin (BSA) for 2 hr. Sections were then incubated overnight at 4°C with rabbit anti-Vp (1:100 dilution; Abcam, USA). After several washes with PBS, sections were incubated with Alexa Fluor 594 donkey anti-rabbit IgG (1:500 dilution; Invitrogen, USA) for 75 min, blocked again by 5% BSA for 90 min, and detected by rabbit anti-cFos (1:500 dilution; Santa Cruz Biotechnology, Texas, USA) and Alexa Fluor 488 donkey anti-rabbit IgG (1:500 dilution; Invitrogen, USA). Sections were counterstained with Hoechst 33258 (Invitrogen, USA). Images were captured using Nikon 80i fluorescent microscope (Nikon, Tokyo, Japan).

Hematoxylin and Eosin (HE) staining

Paraffin sections (8 μm) were prepared as same as the above immunostaining. HE staining was performed using Leica ST5020 Multistainer according to recommended procedures. Briefly, the sections were dewaxed and rehydrated in degraded ethanol. After washing with distilled water (diH_2O), the sections were stained with hematoxylin for 10 min, followed by rinsing with tap water for 1 min and staining with eosin for another 1 min.

HE-stained sections were washed by diH_2O and allowed to air dry before mounted with Histomount (Invitrogen, USA). PVN images were captured using Nikon Eclipse Ni-U upright microscope (Nikon H550L).

Western Blot analysis

Control or treated mice brain were decapitated, washed and chilled in ice-cold ACSF carbogenated with 95% O_2 and 5% CO_2. The brain was instantly sectioned serially in 300 μm from bregma to lambda by vibrotome. PVN tissue was then micropunched following Palkovits's microdissection procedure as previously described (22, 23). The tissues were collected from both sides of the PVN of each individual mouse. Tissue samples were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with freshly added proteinase inhibitors (Roche Diagnostics, Germany) and phosphatase inhibitors (Roche Diagnostics, Germany). The lysates were incubated overnight at -80°C and then centrifuged at 13, 000 ***g*** for 10 min at 4°C. Total protein concentration was determined by the method of Bradford (Bio-Rad, USA). Protein lysates with an equal amount were loaded and separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The membrane was blocked in standard Tris Buffered Saline-Tween-20 (TBST) (20 mN Tris-HCl, pH 7.4, 150 mM NaCl with 0.05% Tween-20) containing 5% non-fat milk powder, followed by incubation in primary antibodies overnight at 4°C against total CREB (1:1000, Abcam, USA), phospho-cAMP response element binding (pCREB 1:5000, Abcam, USA) and GADPH (1:1000, Cell Signaling Technology, USA). Membranes were then washed three times in TBST and incubated with HRP-conjugated anti-rabbit IgG antibody (1:5000, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hr at room temperature. Immunoreactive signals were revealed using Western Lightning plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham, MA, USA) and the intensity was quantified by ImageJ 2.0 (National Institutes of Health, Bethesda, MD, USA) normalized by control GADPH.

Statistical analysis

All data are shown as means ± SEM. The deviations between groups were analysed using Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed using unpaired *t*-test when 2 groups under consideration, 1-way ANOVA when more than 2 groups under consideration, 2-way ANOVA when comparing groups that have been split on two independent variables, followed by *post hoc* Sidak comparison test.

Results

SCTR/AT1aR heteromer regulates Vp release into circulation

To establish that central SCT and ANGII can stimulate Vp release, we performed i.c.v. injection of SCT or ANGII and measured Vp concentration from blood sampled from left jugular vein at 15 min intervals to 90 min post-injection. Consistent with our previous findings (12), we found that both peptides could significantly elevate plasma Vp release at 15 min after injection, and this stimulated release of Vp was steadily increased to 90 min (SCT: 1.85-fold and ANGII: 2.15-fold at 15 min; SCT: 3.56-fold and ANGII: 4.01-fold at 90 min; **Fig. 1A**) when compared to ACSF control. We have shown previously that specific TM peptides can modulate *in vitro* activities of SCTR-AT1aR heteromer, therefore we next test if central administration of these peptides have a direct effect on Vp release. In this study, ATM4, ATM1, STM2, or STM4 were centrally injected into the lateral ventricle of WT mice without any prior stimulation, followed by the evaluation of the plasma Vp concentrations sampled directly from left jugular vein at 15 min, 30 min and 45 min post-injection. Our data showed that there were no significant changes of plasma Vp levels at 15 min, 30 min and 45 min after i.c.v. injection of ATM4, STM2 or STM4, and a statistically difference after i.c.v injection of ATM1 **(Fig. 1B)** compared with ACSF control in WT mice under normal osmolality condition. This and previous data from our group collectively suggest that formation of SCTR-AT1aR heteromer in *in vivo* is ligand-induced or occurs only under stimulated conditions. We therefore tested the actions of these TM peptides and their mutant forms in Vp release after SCT or ANGII stimulation. The experimental setup is shown in **Fig. 2A**. At time 0, SCT or ANGII was i.c.v.-injected into the lateral ventricle, and at time 60 min, TM or TM-m peptide was administered. From time 0 to 60 min, we observed steady increase of Vp in circulation upon i.c.v. SCT or ANGII consistent with data from **Fig 1A**. ATM4 significantly reduced SCT and ANGII-induced plasma Vp release 15 and 30 min after peptide injection (SCT+ATM4: 44.5% drop, $p = 0.0016$ & ANGII+ATM4: 62.8% drop, $p = 0.0254$ at 75 min; SCT+ATM4: 40% drop, $p = 0.0016$ & ANGII+ATM4: 49.7% drop, $p = 0.0161$ at 90 min; **Fig. 2B, C**). The effect of ATM4 was specific as both ATM4-m and DMSO vehicle controls were unable to induce significant release of Vp. Similarly, i.c.v. STM2 also significantly lowered SCT/ANGII-induced plasma Vp after 15 min and 30 min of injection (SCT+STM2: 37.6% drop, $p = 0.0141$ & ANGII+STM2: 53.5% drop, $p = 0.0061$ at 75 min; SCT+STM2: 55.1% drop, $p = 0.0006$ & ANGII+STM2: 61.6% drop, $p = 0.0019$ at 90 min; **Fig. 2D, E**), while both STM2-m and DMSO vehicle controls showed minimal effects. These and our previous data taken together revealed that formation of SCTR/AT1aR heteromer in central is mechanistically important to carry out *in vivo* actions of both SCT and ANGII. Modulations of SCTR/AT1aR heteromer by specific TM peptides corresponding to either receptors could attenuate plasma Vp release, thus providing evidence to understand how SCT mediate actions of ANGII in central and in the overall osmoregulatory axis.

SCTR/AT1aR heteromer regulates Vp expression in PVN

As ANGII controls also Vp expression in the hypothalamus, we next seek to investigate if SCTR/AT1aR heteromer may also mediate gene expression of Vp in PVN. Central Vp is primarily synthesized in PVN, where it receives the neuronal signal from upstream osmoregulatory centres, such as subfornical organ (SFO) and median pre-optic nucleus (MnPO) (24). In this study, by laser-capturing only the PVN region and coupled to real time-PCR, we measured changes in Vp transcript levels at time 75 min or 90 min in PVN after SCT or ANGII stimulation for 60 min followed by and TM peptide injection at time 60 min (**Figure 2A** for experimental setup). Again, we found that SCT was able to stimulate Vp gene expression at time 75 and 90 min (**Fig 3A-D**; ACSF vs SCT+ACSF). Similar to what was observed in plasma Vp release, SCT-induced Vp expression was significantly reduced at 75 min and 90 min after i.c.v. injection of ATM4 (79.9% drop, $p = 0.0148$ & 73.8% drop, $p = 0.0275$; **Fig. 3A, B**) and STM2 (61.5% drop, $p = 0.0419$ & 56.1% drop, $p = 0.0381$; **Fig. 3C, D**). In summary, these data showed that activating SCTR/AT1aR heteromer by either SCT or ANGII is key to regulating Vp gene expression in PVN, while disruption of heteromer's actions by functional TM peptides reduces both Vp expression and release.

SCTR/AT1aR heteromer in hypothalamic Vp synthesis via the cFos-PKA-CREB pathway

Vp is produced in magnocellular and parvocellular neurons in PVN and SON (24), and cFos is an immediate early gene which is routinely used as a marker to detect neuronal activation (25). To investigate how SCTR/AT1aR heteromer controls Vp synthesis and release in the hypothalamus, by immunohistochemical staining, we examined the stimulation of cFos gene expression in PVN and SON after SCT stimulation followed by injection of STM2 or STM2-m peptides. Compared with ATM4, STM2 can only disrupt the formation of SCTR/AT1aR heterodimerization but not homodimerization (13), therefore STM2 would be the best tool to study the functional specificity of SCTR/AT1aR heteromer. High density and intensity of cFos-immunoreactive (IR) positive cells were found in SCT-stimulated PVN and SON (**Fig. 4A, C**), and STM2, but not STM2-m, could suppress SCT-induced cFos expression in both PVN and SON (51.5% drop, $p = 0.0017$ & 40.9% drop, $p = 0.0391$; **Fig. 4A-D**). Double immunofluorescent staining was then carried out to demonstrate neuronal activation of vasopressinergic neurons in PVN after SCT and STM2 treatment. Meanwhile, we compared the expression patterns in two neuronal cell types in PVN after the treatment. Magnocellular neurons primarily reside in lateral PVN with a larger soma compared to parvocellular neurons **(Fig. 4E)** which is the principal site of vasopressinergic cells projecting to the adenoneurohypophysis to control hormone release and behavioural response, such as controlling water drinking behaviour under hyperosmolality condition (26). While parvocellular neurons project to median eminence and mediate blood pressure changes (27, 28). In our data, we found co-localization of cFos- and Vp-IR positive cells (**Fig. 4F**, yellow cells) mainly in magnocellular neurons after i.c.v. SCT stimulation, while less yellow cells were found in parvocellular neurons **(Fig. 4F).** I.c.v injection of STM, but not STM2-m, reduced SCT-induced Vp-IR as well as co-localization of cFos/Vp in magnocellular

cells **(Fig. 4F).** Together with previous immunohistochemical and *in situ* staining showing AT1aR and SCTR proteins and transcripts in magnocellular neurons of PVN (12, 14, 29), our data collectively indicate activated AT1aR/SCTR heteromer in magnocellular cells of PVN is largely responsible for carrying actions of these peptides. These data also explained the finding that SCTR/AT1aR stimulates water drinking under hyperosmotic stress (13).

Transcriptional regulation of Vp gene is primarily regulated by cAMP/PKA and PKC/MAPK pathways (30, 31), in which the cAMP/PKA/CREB pathway is responsible to activate Vp gene transcription both in basal and stimulated conditions (32, 33). To elucidate the downstream signaling mechanism of SCTR/AT1aR heteromer in modulating Vp gene expression in PVN, activities of these signaling molecules were examined in the same experimental setup. Upon SCT pre-stimulation, we found that PKA transcript was significantly elevated compared to control in PVN after i.c.v. ACSF By LCM coupled to real time PCR, (3.23-fold, $p < 0.0001$ vs control ACSF; **Fig. 5A**). Again, this stimulation was significantly attenuated in the presence of STM2 (65.6% drop, $p = 0.0051$ vs control SCT+ACSF), but less and insignificant in STM2-m (37.7% drop, $p = 0.4865$ vs control SCT+ACSF; **Fig. 5A**). Our data showed that SCT-induced PKA gene expression in PVN was lessened when SCTR/AT1aR heteromers were disrupted. These data are consistent with earlier *in vitro* studies indicating SCTR/AT1aR heteromer utilizes the cAMP pathway (13). To confirm, we next showed by Western blot analysis that SCT-induced phosphorylation of pCREB was significantly lowered in the presence of STM2 but not the mutant peptide (47.1% drop; $p = 0.0111$; **Fig. 5B**). In summary, our data revealed that SCTR/AT1aR heteromer regulates Vp gene transcription via cFos and PKA/CREB in magnocellular PVN cells.

Discussion

Terrestrial animals often experience osmotic stress, and hence their bodies evolved sophisticated systems for defending the body cells from osmotic changes and maintaining the constancy of *milieu interieur* within the body all the time. Peripheral and central sources of ANGII and Vp were found to be the key mediators to rapidly correct body osmolality back to normal (15, 34, 35). In the past decade, there were emerging evidences indicating that SCT may serve as a bridge connecting ANGII and Vp via SCTR and AT1aR interactions in central nervous system. We earlier revealed that SCTR/AT1aR heteromer in osmoregulatory brain centers participates in hyperosmolality-induced water intake (13), however there was no information in the past concerning the potential interactions of SCTR with AT1aR to control Vp expression and release. In this study, using TM and mutant TM peptides as biochemical tools to dissect actions of the SCTR/AT1aR heteromer in PVN, we firstly demonstrate the *in vivo* actions of SCTR/AT1aR heteromer in regulating plasma Vp release and expression via cFos/cAMP/CREB pathway in magnocellular neurons of PVN. Our data also explained the result of previous studies indicating 1) co-localization of SCTR and AT1aR in PVN, particularly in magnocellular neurons, 2) central actions of ANGII requires an intact SCT-SCTR axis, and 3) actions of the SCTR/AT1aR heteromer is largely dependent on the cAMP pathway (13, 36, 37). Of note, the functional specificity of SCTR/AT1aR heteromer was fully demonstrated by i.c.v. injection of STM2 because it can only disrupt SCTR/AT1aR heterodimerization but not SCTR or AT1aR homodimerization. It is noteworthy also to mention that i.c.v injection of TM peptides alone into mice could not affect basal Vp release, indicating that similar to the ligand induction of oligomerization processes observed in β_2-adrenoceptors, somatostatin receptor type 1 subtype b ($SSTR_{1B}$), chemokine receptor type 2/ chemokine receptor type 5 (CCR2/CCR5) receptors as well as dopamine D_2 receptors (38-41), functional SCTR/AT1aR complex requires the presence of either ligands.

GPCRs are major drug targets, constituting more than 50 percent of existing therapeutic agents (5). SCTR is prototypic of class B GPCRs (42) and we have recently established its role in lipid metabolism (43), water and salt homeostasis (14) and motor control and learning (44). Together with its role in gut pH (45) and insulin secretion (46), these diverse roles SCT/SCTR make it a potential drug target. Identification of receptor heteromer *in vivo*, understanding their precise dynamics and regulation, and even their implications in pathophysiological processes are the prerequisites for developing novel drugs in this direction. Unfortunately, there are very limited current techniques available for studying GPCR oligomerization. To confirm the existence and exact location of oligomers in native tissues, co-immunoprecipitation or fluorescent-tagged ligands can be used to detect and analyze GPCR oligomers interaction endogenously. However, in the case of SCTR/AT1aR heteromer, co-immunoprecipitation is extremely challenging because of the low abundance of receptors in native tissues. Moreover, antibodies for GPCR are not well-established yet for sufficiently high resolution detection of the heteromer (16, 47). Recently, TM peptides are exploited as a biochemical tool in present study. This strategy has

been demonstrated in analyzing the activity of mGluR2-5HT2aR heteromer (10, 48), in which the TM4 and TM5 of mGluR2 were found to be able to compete with receptor for interacting with 5HT2aR. Additionally, same approach was applied on the study of rhodopsin dimerization (49). We have also demonstrated that specific TM peptides can modulate cAMP responses of SCTR in the presence or absence of an active form of AT1aR (13). More importantly, we have reported the *in vivo* effects of these TM peptides to prevent hyperosmolality-induced drinking. Similarly, in current research, specific TM peptides were successfully used in studying the *in vivo* role of SCTR/AT1aR heteromer in central Vp release and expression. This and other studies therefore indicate the potential of using TM peptides in modulating actions of specific GPCRs. Syndrome of inappropriate antidiuretic hormone secretion (SIADH) is a pathologic state showing hyponatremia in patients. It is resulted from the inappropriate secretion of Vp and subsequently leading to impaired water retention in kidney (50). One of the causes of SIADH can be due to malfunction of hypothalamus in mediating Vp synthesis and secretion, therefore this report may shed a light for scientists to explore the use of TM peptides as drug to mediate hypothalamic Vp release and solve this pathophysiological state. Second generation TM peptides and analogues may have great potential in the future as a novel drug development direction by modifying actions of GPCRs that require higher order structures to explicit their functions.

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Author Contributions

S.O.K. Mak and B.K.C. Chow designed the research; S.O.K. Mak performed the experiments; S.O.K. Mak and B.K.C. Chow contributed to data analysis; S.O.K. Mak, L. Zhang and B.K.C. Chow wrote the paper.

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Figure legend

Figure 1. Effect of SCT/ANGII/ TM peptides central injection on plasma Vp release. (A) Effects of i.c.v. SCT (500 ng/ 5μl), ANGII (100 ng/ 2 μl) on plasma Vp release. (B) Effects of i.c.v. ATM4, ATM1, STM2 and STM4 (4 μg/ 5 μl) on plasma Vp release. Data are presented as means ± SEM (n = 4-6/ group). ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 vs. ACSF control.

Figure 2. Effect of TM peptides on plasma Vp release upon ANGII/ SCT pre-treatment. (A) Schematic diagram showing the experimental design. ANGII (100 ng/ 2 μl) or SCT (500 ng/ 5μl) were i.c.v. injected initially, ATM4 or STM2 (4 μg/ 5 μl) were subsequently i.c.v. injected after 60 mins. Blood from mouse jugular vein was then collected at 15 mins or 30 mins upon TM peptides injection. **(B, C)** ATM4 was i.c.v. injected 1 hr after SCT or ANGII pre-treatment in WT mice. **(D, E)** STM2 was i.c.v. injected 1 hr after SCT or ANGII pre-treatment in WT mice. Data are presented as means ± SEM (n = 6/ group). ***P<0.001, **P<0.01, *P<0.05 v.s. respective plasma Vp concentration at 60 mins.

Figure 3. Effect of TM peptides on Vp mRNA expression in PVN upon SCT pre-treatment. (A, B) Effects of i.c.v. ATM4 (4µg/ 5µl) on mouse SCT-induced Vp expression in PVN at 75 min and 90 min. **(C, D)** Effects of i.c.v. STM2 (4µg/ 5µl) on mouse SCT-induced Vp expression in PVN at 75 min and 90 min. Data are presented as means ± SEM (n = 5-6/ group). *P<0.05 vs. SCT-induced Vp mRNA levels at 60 mins.

Figure 4. Effect of STM2 on cFos and Vp expression in hypothalamic nuclei upon 1hr SCT postinjection. cFos-IR in PVN **(A)**, SON **(C)** 15 mins after i.c.v. ACSF or SCT (500 ng/ 5μl) or STM2 (4 μg/ 5 μl) upon 1 hr SCT postinjection. Number of positive cFos neurons in PVN **(B)** and SON **(D)** were measured and compared with ACSF control. **(E)** Morphology of mouse PVN. Representative images of HE stained PVN section from wild type mice, where the red arrows indicated the magnocellular neurons and the black arrows indicated the parvocellular neurons. **(F)** Double immunofluorescence staining showed co-localization of cFos and Vp in PVN after i.c.v. ACSF or SCT or STM2 upon 1 hr SCT pre-treatment. 3rd V, third ventricle. OCH, optic chiasm. **P<0.01, *P<0.05 vs. respective controls. Data are presented as means ± SEM (n = 5-6/ group).

Figure 5. Effect of STM2 on signalling molecule in PVN upon 1hr SCT postinjection. (A) mRNA levels of PKA in PVN at 75 min after i.c.v. STM2, STM2-m or ACSF in WT mice pre-treated with central SCT injection. **(B)** Representative image of Western blot and protein expression of pCREB in PVN of WT mice 15 min after i.c.v. ACSF or STM2 upon 1 hr SCT pre-treatment. **P<0.01, *P<0.05 vs. SCT-induced PKA and pCREB expression. Data are presented as means ± SEM (n= 6/ group).