Title

Transmembrane peptide 4 and 5 of APJ are essential for its heterodimerization with OX1R

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Running head

APJ forms a functional heterodimer with OX1R

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Abstract

Increasing evidence indicates some G protein-coupled receptors function as a heterodimer, which provide a novel target for therapeutics investigation. However, study on the receptor-receptor interaction interface, a potent target on interfering dimer formation, are still limited. Here, using bioluminescence resonance energy transfer (BRET) combined with co-immunoprecipitation (Co-IP), we found a new constitutive GPCR heterodimer, apelin receptor (APJ)-orexin receptor type 1 (OX1R). Both APJ and OX1R co-internalized when constantly subjected to cognate agonist (apelin-13 or orexin-A) specific to either protomer. Combined with BRET and immunostaining, the *in vitro* synthesized transmembrane peptides (TMs) interfering experiments suggests that TM4 and 5 of APJ act as the interaction interface of the APJ-OX1R heterodimer, and co-internalization could be disrupted by these peptides as well. Our study not only provide new evidence on GPCR heterodimerization, but address a novel heterodimerization interface, which can be severed as a potential pharmacological target.

Key words

GPCR, heterodimerization, interface, APJ, OX1R, co-internalization

Introduction

As the largest family of plasma membrane binding proteins, more than 800 genes in human genome encode G protein-coupled receptors (GPCRs)[1]. GPCR play a pivotal role for the cell in sensing the extracellular signal. The signal is transmitted into the cell due to the association of the GPCRs with heterotrimeric G proteins, which in turn activates an extensive array of signaling pathways to regulate cell physiology. GPCR mediated signaling usually occurs in so called lipid rafts-rich in cholesterol and sphingolipid-which provide a small membrane domain for the dynamically interaction between each of the signaling components. Indeed, besides interaction with G proteins or beta-arrestins, increasing evidences indicate that GPCRs could associate into dimers or oligomers at the plasma membrane[2]. Therefore, at the molecular level, GPCR signaling is not only determined by the agonist binding, but also allosterically modulated by other receptors. Further characterization of the GPCR-GPCR interactions is critical for providing more information on GPCR functions and on therapeutic targets with less adverse effects[1-3].

It has been suggested that GPCR heteromer should meet three criteria, including interaction (colocalization and physical interaction), gain of function (heteromers exhibit distinct properties from those of the protomers) and loss of function (disruption the heteromer should lead to loss of the heteromer-specific properties)[4]. Demonstrating the physical interaction is an important first step. Furthermore, if one can figure out the interaction interface

of the heteromer, it would be helpful to understand the interaction pattern of GPCRs, and on the other hand, to precisely target the interaction strength for the purpose of therapeutics. In the case of μ-opioid receptor (MOR) isoform μOR1D and gastrin-releasing peptide receptor (GRPR) heteromers, a membrane-permeable fusion peptide unique to μOR1D isoform targeting μOR1D-GRPR heteromer lead to a decrease in morphine-induced scratching without affecting morphine-induced analgesia[4, 5]. Similarly, systemically administration of transmembrane peptide of μOR1 fusion with TAT to interfering with the dimerization of μOR1 and δOR could enhance morphine analgesia and reduce the antinociceptive tolerance to morphine[6]. While in the brain, blockade the μOR1-δOR heteromerization abolished the antidepressant-like and anxiolytic-like actions of the δOR-agonist UFP-512[7]. In addition to heteromerization, homomerization could also be disrupted with synthetic peptides targeting the interaction interface such as rhodopsin[8]. Actually, dissection of the interaction interface and utility of synthetized transmembrane peptides to interrupt the dimer/oligomer is a potent tool to study the function of dimerization/oligomerization of GPCRs[4, 9]. However, study on interaction interface remains largely insufficient.

OX1R and OX2R, two homologs of orexin (orexin-A and orexin B) receptor, have a widespread distribution in human brain, especially at hypothalamus, thalamus, hippocampus and some nuclei in midbrain and hindbrain[10]. Besides regulation of motivated behaviors, orexinergic system act as a crucial

regulator in sleep-arousal, addiction, anxiety and depression[10-14]. APJ , with its cognate ligands apelin (apelin-13, apelin-17 and apelin-36), is another GPCR involved in controlling of neuroendocrine response to stress through hypothalamic-pituitary-adrenal (HPA) axis. In the brain, APJ are extensively distributed yet mainly at paraventricular nucleus (PVN) of thalamus, supraoptic nucleus (SON) of the hypothalamus, hippocampus and habenular nuclei[15]. Considering the comparable roles in humoral response to stress and partially overlapping distribution in the brain, we hypothesize that there may be an interaction between OXR and APJ.

To test this hypothesis, using co-immunoprecipitation (Co-IP) and bioluminescence resonance energy transfer (BRET), we found that OX1R and APJ form a heterodimer, in which transmembrane (TM) α-helix 4 and 5 of APJ may work as an interface. In the stimulation experiments, either orexin-A or apelin-13, the corresponding cognate ligands of the two receptors, could result in the internalization of the heteromers, but the process can be disturbed by the TM peptides 4 and 5 of APJ. Together, these results robustly suggest that OX1R dimerize with APJ, in which TM 4 and 5 of APJ serve as an interface.

Materials and methods

Plasmid constructs

Among all the plasmids used in the study, 3xHA-APJ, APJ-Venus[16], OX1R-Rluc (*Renilla* luciferase), KOR-Rluc, OX1R-Venus[17], KOR196-Rluc,

NTSR1-Venus[18] were constructed in our laboratory previously. For constructs of OX1R-Myc and KOR-HA, *hOX1R* was amplified using primers (5'-CCGCTCGAGATGGAGCCCTCAGCCACC-3' and 5'-GGAATTCTCAGAGATCCTCTTCTGAGATGAGTTTTTGTTCGGGCAGCACTGTGGTGACG-3') and cloned into *Xho* I / *Eco*R I of pcDAN3.1 (-), while hKOR amplified using primers (5'-CCGCTCGAGATGGACTCCCCGATCCAGA-3' and 5'-CCCAAGCTTTCAAGCGTAGTCCGGAACGTCGTACGGGTATGGTTTATTCATCCCATCGA-3') but cloned into *Xho* I / *Hind* III of pcDAN3.1 (-). Primers sequences underlined are tags.

Cell culture and transfection

HEK293T cells were cultured in DMEM medium (Gibco, 12100046) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 ^{o}C in an atmosphere of 5% CO_2. Cells were plated in a 6-well plate at a concentration of 1×10^6 cells/well or in 10-cm-diameter flasks and cultured overnight prior to transfection. Cells were transiently transfected using Lipofectamine™ 2000 transfection reagent (invitrogen) according to manufacturer's instructions.

Agonist treatment

For concentration dilution series, HEK293T cells co-transfected with OX1R-Rluc and OX1R-Venus were treated with apelin-13 or orexin-A in a concentration series from 10^{-5} to 10^{-12} M by ten-fold dilution for 18 min before BRET assay. While for time series, the same cells were treated with 10^{-6} M

apelin-13 or orexin-A for 2, 6, 9, 13, 16, 23 min before BRET assay.

TM peptide treatment

The seven TM peptides of APJ were synthesized as described previously with a TAT sequence at N or C terminus to ensure peptides incorporating into the plasma membrane in correct direction[6, 19], as shown in supplemental table 1. Immediately before use, the peptides were solubilized in dimethyl sulfoxide (DMSO) and diluted in the DMEM medium to yield a final concentration of 1% DMSO. We verified that, no effect on cell viability was observed for each tested concentration of DMSO alone. Cells were incubated with the peptides mentioned above at 37 ^{o}C for 2 h prior to performing BRET analysis.

BRET^{1} assay

The BRET^{1} assay was performed as Pfleger *et al.* described[20]. In saturation assay, HEK293T cells were transiently co-transfected with constant amounts (1 μg) of plasmids encoding for OX1R-Rluc and increasing amounts (0.5–8 μg) of plasmids encoding for APJ-Venus. Forty-eight hours after transfection, the cells were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions were distributed in duplicate into 96-well microplates; black plates (Corning 3651) for fluorescence measurement or white plates with white bottom (Corning 3600) for BRET determination. For BRET^{1} ratio measurement, coelenterazine h substrate (Molecular Probes) was added at a final concentration of 5 μM. Readings were performed after 1 min and the BRET signal was detected using the Mithras LB941 plate reader

(Berthold Technologies, Bad Wildbad, Germany) that allows the sequential integration of the signals detected with two filter settings. Data were then represented as a normalized netBRET[1] ratio versus the fluorescence value obtained from the Venus, normalized with the luminescence value of OX1R-Rluc expression 10 min after coelenterazine h incubation. The normalized netBRET[1] ratio was defined as the BRET ratio for co-expressed Rluc and Venus constructs normalized against the BRET ratio for the Rluc expression construct alone in the same experiment: netBRET[1] ratio = [(Venus emission at 530 ± 10 nm) / (Rluc emission 485 ± 10 nm)] – cf. The correction factor, cf. corresponds to (emission at 530 ± 10 nm) / (emission at 485 ± 10 nm) found with the receptor-Rluc construct expressed alone in the same experiment. BRET isotherms were fitted using a nonlinear regression equation assuming a single binding site, which provided BRETmax and netBRETmax values. The maximal value of BRET (BRETmax or netBRETmax) corresponds to the situation when all available donor molecules are paired up with acceptor molecules.

Coimmunoprecipitation (Co-IP)

Co-IP was conducted according to a standard method with minor modifications[21]. In brief, 10 μg plasmids were transfected into HEK293T cells, followed by culturing for 48 h. For cell lysis, cells were washed with ice-cold PBS for 3 times before adding the cell lysis buffer (Beyotime，P0013). Detach cells with cell scraper and lyse them using ultrasonic cell disrupter (100-200 w,

work for 30 s and pause for 1 min, 3-4 times). To completely dissolve the membrane proteins, the cell lysis was turned upside down and centrifuged 18,000 g for 30 min both in a 4 ^{o}C circumstance. Determine the protein concentration using a BCA protein concentration kit (TIANGEN, PA115) according manufacturer's instructions. Co-IP was conducted using a Pierce Anti-HA Agarose kit (Thermo Scientific, 26181) as instructed. Dilute the 5× SDS sample buffer with precipitate suspended in 200 μl TBS buffer and then incubate the mixture for 10 min in a 37 ^{o}C water bath. Subsequently, conduct a Western Blot with a protocol described in Molecular Cloning: a laboratory manual (4th edition). The antibodies of HA (CST, 3724), Myc (CST, 2278) and beta-actin (CST, 3700) were diluted in 1:10,000, 1:500 and 1:2,000, respectively. While the HRP-conjugated secondary antibodies (Invitrogen, G21040 and G21234) were all diluted in 1:5,000.

Immunostaining

Culture the transfected cells overnight, and at the same time, treat the clean round coverslips with 0.1 % (w/v) poly-L-lysine (PLL) overnight as well. Passage cells to coverslips for a continuous culture for 24 h. Using 4 % formaldehyde diluted in warm PBS to fix cells for 15 min at room temperature and proceed with rinsing with PBS for three times, 5 min each. For immunostaining, Block specimen in blocking buffer (1× PBS, 5% normal serum 5% BSA, 0.3% Triton™ X-100) for 60 min, and rinse the specimen briefly with PBS. Incubate the primary antibody of HA (CST, 2367) with dilution 1:100

overnight at 4 °C followed by rinse slips three times in 1× PBS for 5 min each. Next, incubate the specimen with cy3-conjucgated secondary antibody (Invitrogen, A10521) with dilution of 1:50 for 2 h away from light at room temperature. After rinsing three times in 1X PBS for 5 min each, coverslip slides with Mounting medium with DAPI (Duolink, Art no 82040) and allow mountant to cure overnight protected from light at room temperature. Slides were scanned with confocal microscopy (Leica TCS SP8).

Statistical analysis

Statistical analysis was carried out with GraphPad Prism software, and all the data were showed as mean ± SEM. For comparing two groups of data, two-tailed, unpaired Student's t test was used. The difference was considered significant when $p<0.05$.

Results

1. APJ and OX1R form constitutive heterodimers

To investigate whether APJ and OX1R dimerize or not, we employed bioluminescence resonance energy transfer (BRET)[1] assay. OX1R-Rluc (*Renilla* luciferase) and APJ-Venus were co-expressed in HEK293T cells, with combination of KOR-Rluc and APJ-Venus, KOR196-Rluc and NSTR1-Venus as the positive and negative control, respectively[17, 18]. BRET saturation assay showed that APJ and OX1R could dimerize with each other (Fig. 1A). To verify the BRET results, co-immunoprecipitation (Co-IP) was implemented.

From Fig. 1B, we can find that when 3×HA-APJ and OX1R-Myc were expressed in the same HEK293T cell, OX1R-Myc could be found in sedimentation precipitated with anti-HA antibody (Fig 1B). However, not any OX1R-Myc could be co-precipitated using the same method when the two fused protein expressed solo then mixed together (Fig 1B, mix). Co-IP assay indicated that APJ and OX1R constitute a complex. These results robustly demonstrated that APJ and OX1R could heterodimerize.

We wonder the effect of ligand binding on dimerization of APJ and OX1R. First, different concentration of apelin-13 (the agonist of APJ) and orexin-A (the agonist of OX1R) were applied on cells expressing both APJ-Venus and OX1R-Rluc following by BRET assay. However, no correlation trend could be found between BRET ratio and the concentration gradient (Fig. S1A), suggesting that ligand binding didn't affect the dimerization of these two receptors. In addition, there is no effect on the time series either (Fig. S1B). Together, these results indicate that APJ and OX1R can constitutively form a heterodimer.

2. APJ dimerize with OX1R mainly through its transmembrane peptide 4 and 5

The interactive interface of GPCRs remains to be clarified, especially for heterodimers[4]. To dissect the interactive interface of APJ and OX1R, the seven transmembrane peptides (TMs) of APJ used to disrupt the interaction of

APJ and OX1R were *in vitro* synthesized (supplemental table 1). Combined with BRET technique, the mixture of all seven TMs (10, 20 or 40 μM each) was exerted to HEK293T cells expressing OX1R-Rluc and APJ-Venus, and we found that 20 and 40 μM could interfere with the heterodimer formation (Fig. 2A). With the same method, 20 μM of each TM was applied separately instead of a mixture, we successfully found out that TM 4 and 5 of APJ could disrupt the dimerization of APJ and OX1R significantly (Fig. 2B). These results demonstrated that TM 4 and 5 of APJ mainly constitute the interaction interface with OX1R.

3. APJ and OX1R could co-internalize subject to either apelin-13 or orexin-A

Given the heterodimerization of APJ and OX1R, we speculated that the heterodimer may co-internalize when stimulate with the agonists. To test this, we first co-express OX1R-Venus and 3×HA-APJ in HEK293T cells following by immunostaining with HA antibody. As expected, both OX1R-Venus and 3×HA-APJ co-localize on the plasma membrane (Fig. 3). Subsequently, the cells were incubated with apelin-13 or orexin-A, or both agonists for 30 min. Interestingly, either apelin-13 or orexin-A could result in internalization of the heterodimer (Fig. 4A). Dual agonists incubation lead to even more serious internalization, whereas the vehicle saline had not any internalization with the same incubation time (Fig. 4A). These results indicate that the dimer of APJ and OX1R could co-internalize subject to either agonists (apelin-13 or

orexin-A).

4. Dimerization is essential for co-internalization of APJ and OX1R

We next explored if the interface peptides could impede co-internalization of heterodimers. As shown above, both application of orexin-A and apelin-13 could cause the co-internalization of the dimer between APJ and OX1R (Fig. 4A). However, when TM 4 and 5 of APJ were applied simultaneously, the co-internalization of the dimer was disrupted, instead, application of either apelin-13 or orexin-A only internalize the corresponding protomer after incubation for 30 min (Fig. 4B). This suggests that the dimerization is essential for co-internalization of APJ and OX1R, and TM 4 and 5 of APJ worked as the interaction interface, consistent with the preceding results.

Discussion

In this study, we found that APJ and OX1R could form a heterodimer, which served as an entity for co-internalization in HEK293T cells. In addition, TM 4 and 5 of APJ may be the interface in its dimerization with OX1R and these in vitro synthesized peptides could disrupt the co-internalization of the heterodimers, further demonstrating the significant roles of APJ's TM 4 and 5 in its interaction with OX1R.

It has been known that GPCR signaling can be modulated at multiple levels, in which one of the most important modulations is the internal interactions of

GPCRs themselves[22, 23]. Interactive GPCR can form a homomer or heteromer that can exhibit properties distinct from those of the protomers, such as altering the binding properties of protomer-selective ligands, changing the signal transduction and leading to novel trafficking properties[4]. Here we found a completely new GPCR heteromer of APJ and OX1R, and more intriguingly, agonist of either protomer can stimulate the co-internalization of both protomers (Fig. 1 and 4). Similar to this discovery, studies have reported that morphine triggers the internalization of MOR1D and GRPR and that sst_{2A}-selective ligand L-779976 induces internalization of sst_{2A}-MOR1 heterodimer[5, 24]. However, it should be noted that co-internalization of both above heterodimers can only be induced by the agonist specific to one of the protomer but not by the other, distinct to APJ-OX1R heteromers. The internalization of GPCRs are initiated with phosphorylation of receptors and then preceded with recruiting arrestins[25]. We speculate that one of the possible mechanisms is APJ-OX1R co-internalize as an entirety due to the stable interaction albeit only one protomer is phosphorylated, the other possibility is conformation of one protomer is allosterically modulated by the other, resulting that both protomer are phosphorylated by the same kinase. Further work needs to be addressed on phosphorylation of APJ and OX1R when co-internalized.

Nevertheless, sufficient study on dimerized interface is not only crucial to reveal the molecular rules on GPCR-GPCR interactions, but provides potential

tools to manipulate them for pharmacology. In general, the hydrophobic helices work as the interaction interface but sometimes it depends. In terms of previous study, three transmembrane domains (TMD) are interactive hotspot, which are TM1-2, TM4-5, and the C terminus. Specifically, TM1-2 mainly participate in the homodimerization, typically in homodimers such as neurotensin 1 receptor, M_3 muscarinic receptor, rhodopsin, and APJ[8, 19, 26-28]. Except for homodimers of M_3 muscarinic receptor, rhodopsin and angiotensin II type 1 receptor[8, 27-29], TM4-5 are directly involved in heteromerization of A2A Adenosine and D2 Dopamine Receptor[3]. The C terminus of MOR1D, D1 and DOR act as the interface of heterodimerization with GRPR, D2 and MOR[6, 7, 30]. Using bioluminescence resonance energy transfer (BRET) and immunostaining, we found that TM4-5 of APJ may serve the dimerization interface of APJ with OX1R (Fig. 2 and 4). This finding added new evidence that TM4-5 are significant interfaces in GPCR heteromerization. In contrast to APJ's homomerization, in which TM1-4 rather than TM 5-7 are indispensable for the formation of homomers[19], TM4 and 5 of APJ involve in the heteromerization with OX1R. We propose that the interactive TM domains differentially participate in homomerization and heteromerization for the same GPCR, although in this case TM4 is essential both for homomer and heteromer formation of APJ.

Our results suggested that apelin and orexin system may work synergistically. To investigate this question, first of all, one must figure out if

APJ and OX1R could dimerize in vivo. Several methods can be employed, including antibody-aided or ligand-aided time-resolved fluorescence RET (TR-FRET), antibodies-aided proximity ligation assays and co-immunoprecipitation and so on[4]. To address the physiological roles of the dimer especially roles in stress response[10, 15], a direct method is to interrupt the dimer. Interfering peptides discovered in this study may provide a choice.

To summarize, we found that in HEK293T cells, APJ and OX1R could form a heterodimer, which internalized when subject to agonist to either protomer of the dimer. Co-inernalization of the dimer could be disrupted by interfering peptides of TM4 and 5 of APJ, which serves as the interactive interface of APJ-OX1R heterodimer. These findings suggest that APJ and OX1R system may mutually affected and provide more clues on the study of GPCR dimerization interfaces.

Disclosure

All the authors declared no competing interests.

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Figure legends

Figure 1. Characterization of the heterodimerization between APJ and OX1R in HEK293T cells. (A) Saturating BRET assay. In cells expressing a fixed amount receptor1-Rluc and increasing amounts of receptor2-Venus, BRET was measured. (B) Co-IP. 3×HA-APJ and OX1R-Myc were co-expressed in the same cells, or expressed individually and mixed together as the negative control, before performing immunoprecipitation (IP) with HA antibody. Pairs of OX1R and kappa opioid receptor (KOR) were used as positive control. Arrows indicate positions of anticipated bands. IB, immunoblot.

Figure 2. Transmembrane peptide (TM) 4 and 5 of APJ are involved in the

dimerization with OX1R. (A) Minimal concentration of TM that could effectively disrupt the heterodimerization was determined using a concentration gradient of gross TMs as indicated to incubate with HEK293T cells co-expressing OX1R-Rluc and APJ-Venus before BRET was measured. (B) 20 μM of each TM of APJ were incubated with HEK293T cells co-expressing OX1R-Rluc and APJ-Venus following BRET measurement. Data represent the mean ± SEM of at least three independent experiments. The statistical significance between vehicle and TM was assessed using an unpaired student's t-test (*P < 0.05). ns, not statistically significant.

Figure 3. APJ and OX1R co-localize on the plasma membrane of HEK293T. OX1R tagged with Venus fluorescent protein and APJ tagged with 3×HA were expressed individually or together in HEK293T cells, followed by immunostaining. DAPI (blue), Venus (green) and Cy3 (red) show nuclei, OX1R and APJ, respectively.

Figure 4. Co-internalization of APJ-OX1R heterodimer. (A) apelin-13 or orexin-A stimulate the co-internalization of the dimer. HEK293T cells co-expressing OX1R-Venus and 3×HA-APJ were stimulated for 30 min with 100 nM of apelin-13 and/or 1 μM of orexin before imaging. (B) Blockage the dimerization prevents the co-internalization. HEK293 cells co-expressing OX1R-Venus and 3×HA-APJ were incubated with one kind of agonists

combined with the TM 4 and 5 of APJ at the same time.

Supplementary figure 1. The effect of ligand stimulation on APJ and OX1R heterodimerization. (A) Different concentration of ligands has no effect on heterodimerization reflected by BRET ratio when stimulated for 18 min. (B) 10^{-6} M ligands have no effect on heterodimerization either.

Supplementary table 1. Amino acid sequences of synthetic peptides derived from the transmembrane domains of human APJ

Figure 1

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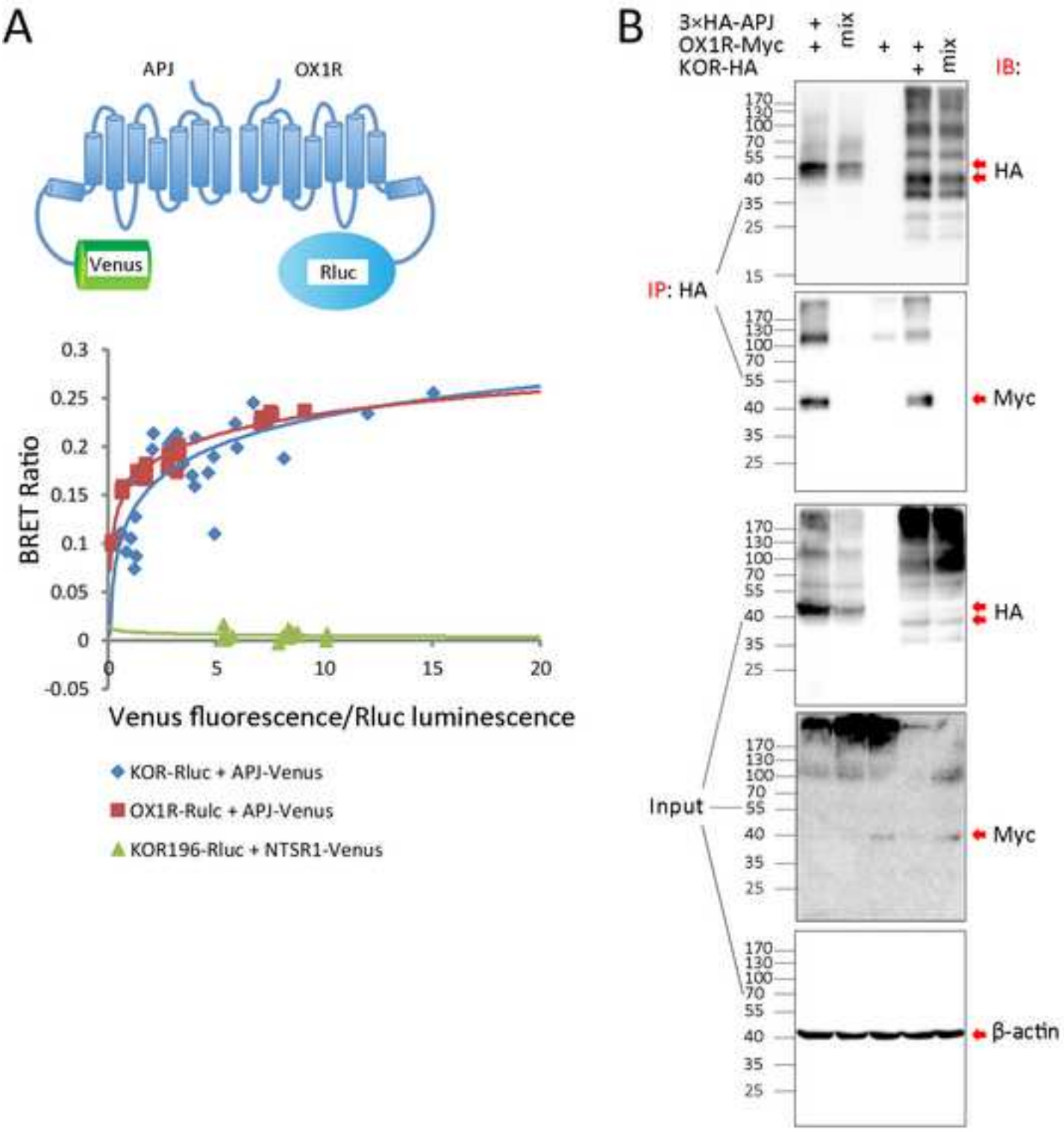

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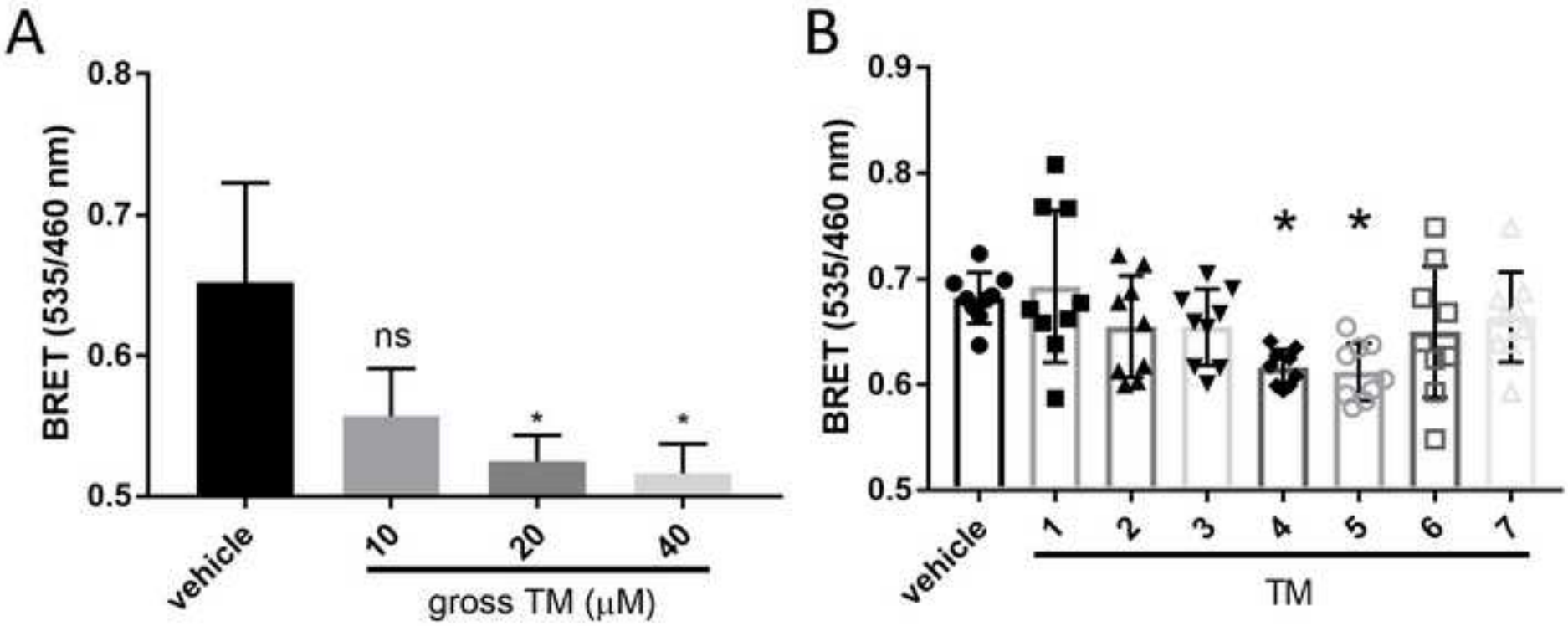

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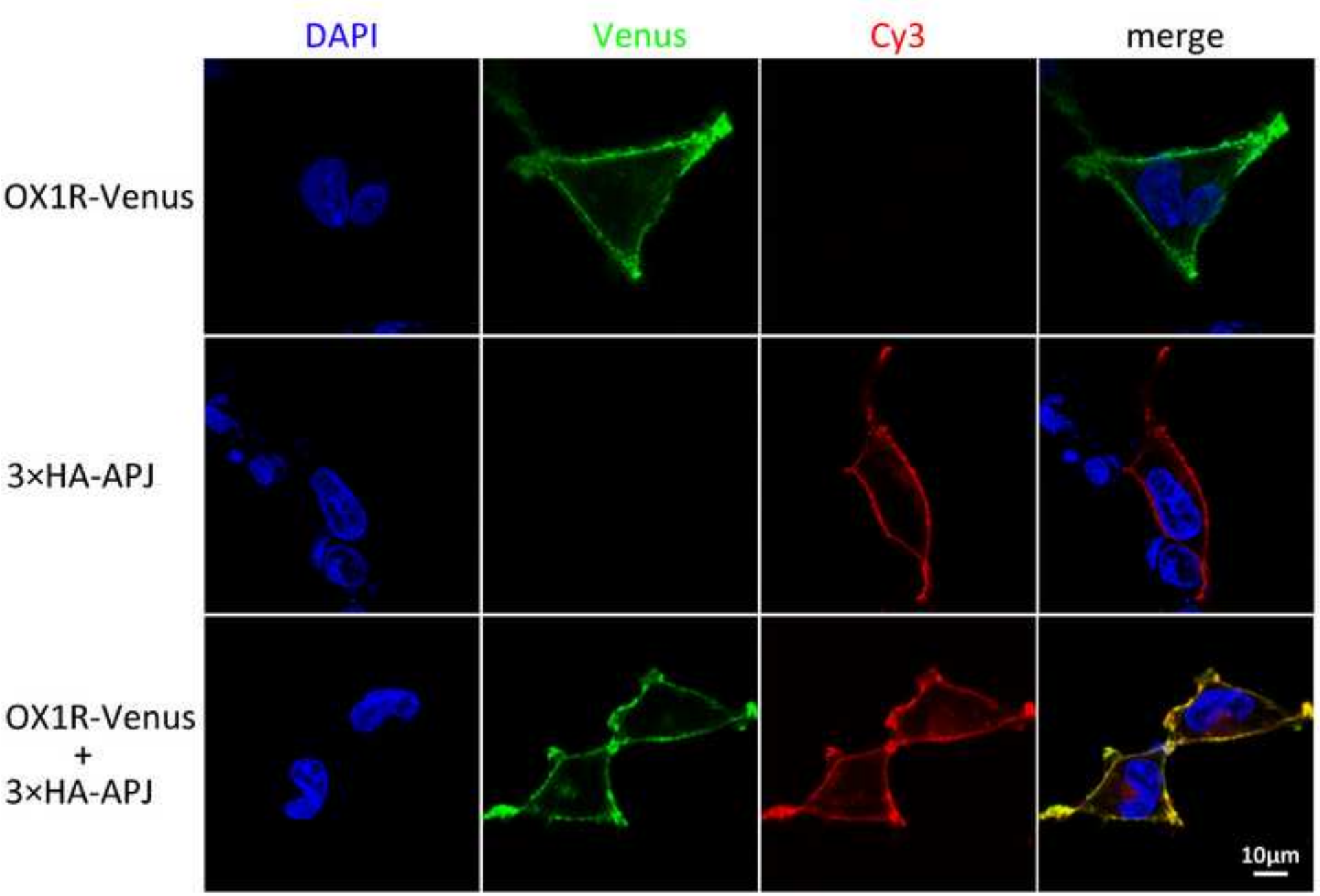

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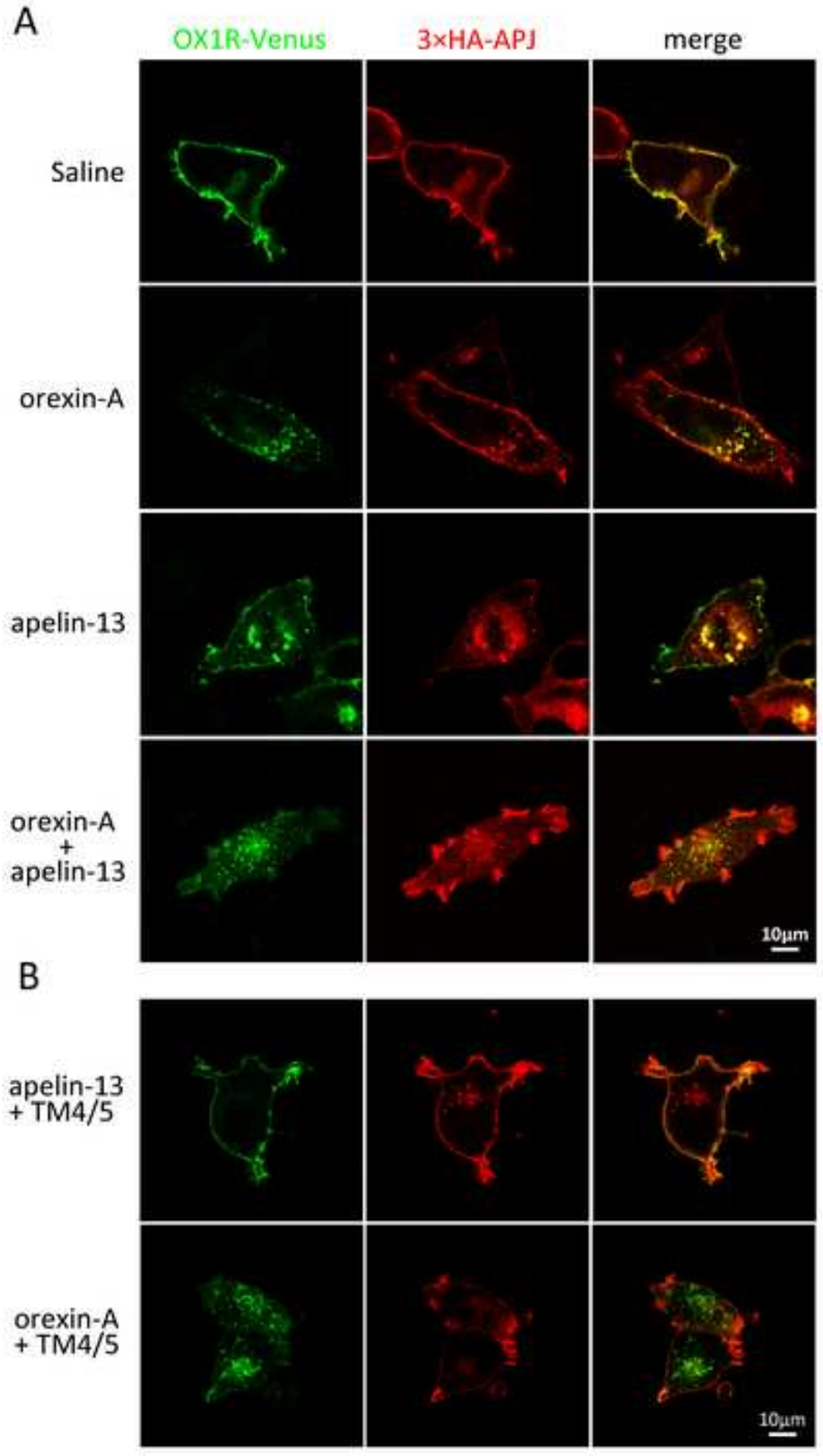

Electronic Supplementary figure 1

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