ABSTRACT: We established a method for synthesizing a free cyclic peptide library via peptide array synthesis to demonstrate the sequence activity of cyclic peptides. Variants of the cyclic nonapeptide oxytocin (OXT) were synthesized via residue substitution. Natural amino acids (AAs) were classified into eight groups based on their physical properties and the size of their side chains, and a representative AA from each group was selected for residue substitution. All OXT variants were systematically evaluated for agonist/antagonist activity. Consequently, no improvement in agonist activity was observed, although substitution of the P4 and P8 residues resulted in decreased activity due to AA substitution. A few OXT variants exhibited antagonistic activity. In particular, the variants with P2 Leu residue substitution (Y2L) and Phe substitutions at residues 4 (Q4F), 5 (N5F), and 7 (P7F) showed high antagonistic activity. Variant Y2W was found to have the highest inhibitory effect, with a dissociation constant of 44 nM, which was comparable to that of the commercial antagonist atosiban (21 nM). Therefore, a free cyclic peptide library constructed via substitution with a natural AA residue was confirmed to be a powerful tool for bioactive peptide screening.

■ INTRODUCTION

Many bioactive peptides have shown bioactivity by binding to various proteins, such as intracellular soluble proteins, membrane-binding proteins, and so on. Such bioactive peptides have received much attention as promising chemicals for pharmaceuticals and food materials. Some high-affinity cytokine-like peptides are known to have cyclic structures. It has been reported that cyclic peptides have a high affinity for their target proteins because there is less entropy loss upon protein–peptide binding if the rigid structural peptide closely fits a target protein.

One of the important problems to be solved for bioactive screening is the magnitude of the sheer number of peptide variants. A new expression screening method for bioactive peptides, such as the random peptide-integrated discovery (RaPID) display and split-intein circular ligation of peptides and proteins (SICLOPPS), has been proposed, and a large number of peptide libraries have been prepared.

A typical method for establishing a chemically synthesized peptide library is a peptide array method established by Frank. Linear peptides are simultaneously synthesized on an activated cellulose membrane in an array format. The synthesized peptide library can then be utilized for in situ binding assays with the proteins of interest. A methodology for the circularization of synthesized linear peptides has also been reported. Compared with the above expression screening method, the library size for this peptide screening method is small (approximately $10^2$ to $10^4$) because peptide synthesis is performed via machine manufacturing. A peptide microarray for high-throughput binding detection on a solid surface has also been proposed. Usui et al. reported a peptide microarray method on the nanoliter scale using a robotic spotter for proteomic analyses and ligand screenings. However, the synthesis of many peptide variants is a rate-limiting step in the production of chemically synthesized peptide arrays.

Recently, computationally associated peptide screening has been proposed for the assessment of the activities of various bioactive peptides, such as antimicrobial peptides, antiox-
idative peptides,\textsuperscript{10} and therapeutic peptides,\textsuperscript{11} driven by the relationship analysis of their amino acid (AA) sequences and bioactivities. Peptide array screening is preferable to this method because quantitative activity data on all peptides can be gathered. It should be noted that the effect of AA residue substitution at any position can be investigated in detail because the sequence design is systematically planned.

In our group, bioactive peptide screening using a peptide array was performed.\textsuperscript{12−15} It was confirmed that peptide activity could be improved via AA residue substitution. A cyclic peptide library was recently established and applied for the assay of NGR cyclic cell adhesion peptides.\textsuperscript{16} Previously, we also established a free peptide library of linear peptides using a photocleavable linker.\textsuperscript{17}

Oxytocin (OXT) is a natural cyclic nonapeptide with the sequence CIYQNCPLG, which generates a disulfide bond between the two Cys residues. OXT bioactivity is reported to have diverse roles mediated by specific oxytocin receptors (OXTRs). Its role during uterine contraction at childbirth and milk ejection from the mammary glands is well known. It is also known to regulate several physiological functions such as vascular and cardiac relaxation and interferes with salt and water balance.\textsuperscript{18} OXT is a G-protein-coupled receptor (GPCR) expressed in both the myometrium and endometrium of the uterus and the myoepithelial cells of the mammary glands. OXTR is also expressed in the central nervous system, and there are many studies on the relationship between OXT function as a neurotransmitter and social recognition, anxiety, autism, and so on.\textsuperscript{19,20}

The OXT/OXTR system has also received attention as a pharmaceutical target for neuropsychiatric disorders, including schizophrenia and autism spectrum disorder (ASD).\textsuperscript{21} So far, many researchers have investigated OXT substitution variants and OXT analogues for drug development or activity structure analyses. There are a few reports on OXT variants generated via natural AA substitutions. Replacement of the asparagine residue at P5 to Ala, Gln, Ser, Orn, or Val has been reported to have a deleterious effect on its biological activity.\textsuperscript{21} There have also been reports of OXT analogues using δ-amino acids. For example, δ-Cys analogues substituted at P6 showed high antagonist activity, while replacement of Tyr at P2 with an aromatic δ-amino acid increased the uterotonic activity of OXT. Even now, studies on OXT analogues have been actively carried out.\textsuperscript{22−24}

In the present study, we aimed to establish a free cyclic peptide library to systematically evaluate the agonist/antagonist activity of OXT variants. Therefore, we determined the quantitative activity of OXT variants substituted with one AA residue using a transforming growth factor-α (TGF-α) shedding assay (Figure S1) and investigated the effect of the substituted residues on the relationship between the AA sequence and bioactivity. Consequently, no improvement in agonist activity was observed, but OXT variants with high antagonist activity were obtained. A free cyclic peptide library constructed via natural amino acid substitution is a powerful tool for screening bioactive peptides.

### RESULTS

**Construction of the Free Cyclic Peptide Evaluation System.** The cyclic peptides used in this study were synthesized on a cellulose membrane using a spot synthesis method. The reaction conditions for disulfide bond formation were established as described in our previous papers.\textsuperscript{7,12,16} When we preliminarily synthesized a free cyclic OXT peptide and analyzed the released peptide via mass spectrometry, the peak of the intermolecular dimer was detected (Figure S2). To prevent intermolecular oxidation, the photolinker concentration in the peptide array synthesis was decreased. When the concentration was adjusted to 10% of the original concentration (25 mM), a single peak of cyclic OXT was obtained. It was concluded that intermolecular dimerization was eliminated and disulfide bonds were formed in all molecules.

To assure the bioactivity of the cyclic OXT synthesized on the peptide array, its binding activity to OXTR was evaluated using a TGF-α shedding assay. The amount of AP-TGFα released based on G-protein signaling was observed in a dose-dependent manner (Figure S3). Thus, we confirmed the system construction to accurately evaluate the activity of cyclic peptides against GPCRs.

In addition, the detection range of the OXT concentration used in this evaluation system was 500 pm to 5 μM. Therefore, the peptide was eluted with 200 μL of Hank’s balanced salt solution (HBSS) buffer from the peptide array and then the solution diluted in five volumes was used for the assay.

**Evaluation of the Agonist Activity of OXT Variants Generated via AA Residue Substitution.** First, we tried to find an OXT variant sequence with higher biological activity than CIYQNCPLG, the original OXT sequence. AA substitution at seven positions was planned, except for the two Cys residues at P1 and P6.

To reduce the number of substituted AA residues and increase the screening efficiency, the number of AA residues used for substitution was decreased from 19 to 8. Natural AAs were classified into eight groups based on their physical properties and the sizes of their side chains, and a representative AA from each group was selected for residue substitution (Table 1). Ala (A), Leu (L), Phe (F), Ser (S), Asn (N), Asp (D), Lys (K), and Pro (P) were used for OXT variants as representative AAs in each group.

### Table 1. Grouping of 19 Amino Acids and Representative Amino Acid in Each Group

| group | representative amino acid | member |
|-------|--------------------------|--------|
| hydrophobic and small-sized amino acids | alanine (A) | G, A |
| hydrophobic and large-sized amino acids | leucine (L) | V, L, I, M |
| aromatic/Aromatic amino acids | Phenylalanine (F) | F, W, Y |
| Hydroxy amino acids | Serine (S) | T, S |
| Amide amino acids | Asparagine (N) | Q, N |
| Negatively charged amino acids | Aspartic acid (D) | D, E |
| Positively charged amino acids | Lysine (K) | H, K, R |
| Proline sole | Proline (P) | P |

None of the OXT variants showed higher agonist activity than the original sequence (Figure 1). Among them, AA substitution at P2, P3, P5, P7, and P9 resulted in a significant decrease in agonist activity.

**Relationship between Substitutions at the Fourth and Eighth Residues and Agonist Activity.** From the results shown in Figure 1, we further synthesized and validated different AA substitution variants only at positions 4 and 8. The results are shown in Figure 2. For the fourth residue, the Asn substitution variant in the same side-chain structure as the original Gln residue showed approximately 35% of the agonist
activity of the original OXT. The Ser substitution variant, which has the same uncharged hydrophilic property as Gln, showed approximately 8% agonist activity of the original sequence. For the eighth residue, the Ile substitution variant, which retained the highest activity, showed 82% of the agonist activity of the original sequence, the Val substituent showed 55%, followed by 22% for Asn, and 18% for Ala. However, none of them showed higher agonist activity than the original sequence.

Next, we demonstrated the importance of the ring structure. In our previous report, we succeeded in forming heterodimers via intramolecular SS bonding using cysteine residues. For this reason, we decided to introduce ring structures at specific residues.

Figure 1. Relative activity in the agonist assay. Statistical significances were assessed against the data of OXT syn; *p < 0.05.

Figure 2. Relative activity in the agonist assay using substituents at fourth (A) and eighth (B) residues. Statistical significances were assessed against the data of OXT syn; *p < 0.05, **p < 0.01. In (A), no data with significance was observed because OXT as a positive control exhibited a large error bar.
purpose, we used Fmoc-Lys(ivDde)-OH to synthesize peptides with different main and side chains and introduced a disulfide bond into the interior of both peptides. This method was used to synthesize a heterodimer that is cleaved at any peptide bond within its ring structure. Although all heterodimers were assayed, the results showed that none of the variants showed any activity (data not shown).

**Evaluation of the Antagonist Activity of OXT Variants Generated via AA Residue Substitution.** We were unable to obtain OXT variants with higher agonist activity than the original OXT. However, OXTR antagonists are required as pharmaceutical interventions to prevent premature births. Therefore, we also investigated the antagonist activity of OXT variants. For this purpose, an approximately 15 times larger amount of OXT variants than that in the agonist assay was used in the TGF-α shedding assay. In the experiment, 10 μL of HBSS solution containing the substituted variant was first added to a culture medium containing OXTR-expressing HEK293 cells, and subsequently, 10 μL of 10 nM OXT was added to evaluate the inhibitory effect of the substituted variant.

The results are shown in Figure 3. Of the 53 substituted variants, 18 variants showed a relative activity of less than 0.5. The variants with extremely low activity included three Phe substituents (Q4F, N5F, and P7F), and the observed inhibition was attributed to antagonist activity.

In contrast, 12 variants showed a relative activity of more than 2, which was the enhancement effect, not the inhibitory effect. Three variants, Q4N, L8A, and L8S, demonstrated at least tenfold greater activity than the control. The enhancement effect was observed in some variants substituted at the fourth residue and eighth residue. A high enhancement effect was predicted to be correlated with high activity in the agonist assay, as shown in Figure 1.

To define the relationship between agonist and antagonist assays, the activity of the variants listed in Figures 1 and 3 is described in Figure 4. A moderate positive correlation was observed, and three groups containing variants with significantly different activity levels were defined. Group A consisted of variants showing significantly low activity in the antagonist assay (p < 0.01) and low activity in the agonist assay. These were Y2L, Q4F, NSF, and P7F, and they are defined as antagonists. Group B consisted of six variants with significantly high enhancement activity in the antagonist assay (p < 0.05) (L8S, L8A, L8N, L8K, Q4S, and Q4N), and group C consisted of three variants showing significantly high enhancement activity in the antagonist assay (p < 0.05) and low activity in the agonist assay (L3D, Q4D, and N5D). A major contributor to the high level of enhancement observed in the antagonist assays is the 15 times larger amount of OXT variants used in these assays as compared with the one used in agonist assays. However, the activity of L8A and L8S variants was significantly high even though a large amount of these variants was added. The observed high activity level may be explained by the additive or synergistic effect toward the agonist activity of OXT.

![Figure 3. Relative activity in the antagonist assay. Statistical significances were assessed against the data of OXT (10 nM); *p < 0.05, **p < 0.01.](image)

![Figure 4. Correlation between agonist and antagonist assays.](image)
It should be noted that all variants identified in group C were Asp (D) substitutes. Other D substitutes, such as Y2D, I3D, Q4D, N5D, P7D, L8D, and G9D, exhibited no agonist activity with the singular exception of L8D; however, all D substitutes, with the exception of P7D, exhibited enhancement activity (e.g., relative activity >1). It may be considered to be the allosteric effect by the structure change of the OXT receptor. Further investigation is required to gain clarity about the mechanisms involved.

The inhibitory group A peptides were found to have only one residue substitution from OXT. The P2 residue variant substituted with Leu (Y2L) and the P4, P5, and P7 residue variants substituted with Phe (Q4F, N5F, P7F) exhibited antagonistic activity. Given that some researchers reported the antagonistic activity of the P2 residue substituent,25,26 we then performed a detailed evaluation of the activity of the P2 residue variants.

Antagonist Activity of P2 Residue Substitution Variants. We evaluated the antagonistic activity of the P2 residue substitution. Tyr was substituted in 18 amino acids, except for Tyr and Cys in the original sequence. The results are presented in Figure 5. Ile and Trp substitutions showed similar inhibition to the Leu variant.

Dose Dependence of the Inhibitory Effect of P2 Residue Substitution. To evaluate the antagonistic activity of the P2 variant substituted with Leu, Ile, and Trp, these three P2 variants were obtained by outsourcing to GL Biochem Ltd. Cyclic nonapeptides with a purity of more than 95% and a single peak of mass spectrometry were supplied (Figure S4). Using these peptides, we investigated the dissociation constant of the variant–OXTR complex. Substituted peptides at any concentration were first administered to OXTR-expressing HEK293 cells, and their activities under different OXT concentrations were evaluated. As a result, an OXT dose-dependent decrease in antagonist activity was observed for all of the variants (Figure 6). The results suggest that the P2 variant substituted with Leu, Ile, and Trp showed inhibitory activity against OXTR, and their antagonistic activity was determined to be via competitive inhibition. The dissociation constants ($K_d$) of each variant were calculated from the sigmoid curves obtained using a Schild plot (Table 2).27 Among the variants tested in this study, Y2W was found to have the highest inhibitory effect. The dissociation constant for Y2W was 44 nM, which is comparable to that for the commercial antagonist atosiban (21 nM, as shown in Figure S5).

DISCUSSION

Agonist Activity of OXT Variants. To date, our group has successfully discovered highly active peptides by synthesizing residue substitutions of bioactive peptides. To find sequences with higher biological activity than the original sequence of OXT, we synthesized AA substitutions in seven positions, excluding the two cysteine residues.

Unexpectedly, none of the substitutions showed a similar or higher agonist activity than OXT. As shown in Figure 1, the residues at positions 2, 3, 5, 7, and 9 showed almost no activity upon substitution, suggesting that these residues are already optimized. Substitution of the P5 Asn residue with other AAs has been reported to decrease agonist activity.21 When the side-chain structure of the P3 Ile residue was substituted with seven kinds of functional groups, none of the OXT analogues showed uterotonic potency.28 Vrachnis et al. reported that OXT substitutions showing agonist activity include the Thr substitution of the P4 Gln residue and its analogues.23 These results partly coincide with the results presented here.

As a comparison, we found that substitutions at the P4 and P8 residues showed some agonist activity. We considered that the molecular evolution of OXT might be responsible for the fact that the AA residues at P4 and P8 are more tolerant of substitutions than those at other positions. Koehbach et al. summarized the variety of AA sequences during the molecular evolution of the OXTR ligand in humans (CYIQNCPLG, original OXT sequence).29 The AA sequences of ligands are usually conserved across species. The OXT sequence is perfectly conserved in primates, even-toed ungulates, and rodents, but the P2, P4, and P8 residues have changed through evolution. Therefore, we assumed that the ligand remained tolerant of substitution at P4 and P8 because the
corresponding receptor-side mutation sites have not yet been fully optimized.

Recently, Waltenspühl et al. elucidated the structure of OXTR and reported the results of a structural analysis of the receptor and its synthetic ligand, retosiban. RETOSIBAN corresponds to CYIQ in terms of structural similarity to OXT, and it was reported that the 11 residues on the receptor side were in contact with retosiban. Eight of the 11 had the same AA residue as the vasopressin receptor (V1AR), and the three residues in the OXTR active site (I201, I204, and A318), which are not conserved in V1AR, were hydrophobic. The amino acid difference between OXT and vasopressin CYFQNCPRG has occurred at P3 and P8. Therefore, it is reasonable that the Ile residue at P3 was necessary to maintain high hydrophobic binding and did not allow substitution. Structural analyses of retosiban and OXTRs showed that the side chains of the P3 Ile residue and the P4 Gln residue of OXT were located close to each other. Therefore, it is also reasonable that the Gln residue at P4 allows substitution of the nonpolar small residues, Asn, Thr, or Ser (Figure 2A).

**Antagonist Activity of OXT Variants.** The antagonist activity of OXT substituents was also examined. The antagonist activity of the P2 Leu residue substituent (Y2L) and the Phe substituents at P4 (Q4F), P5 (N5F), and P7

| Table 2. Dissociation Constant of 1-Amino Acid Substituted Peptides at the Second Residue and Atosiban |
| peptide | dissociation constant (Kd) [nM] |
| Y2L     | 424   |
| Y2I     | 101   |
| Y2W     | 44    |
| atosiban | 21    |

Figure 6. Dose–response curve of oxytocin vs 1-amino acid substituted peptides at the second residue: (A) Y2L, (B) Y2I, and (C) Y2W.
(P2F) was high. As for Phe substitutions, these residues may bind to the hydrophobic pocket of OXTR, increasing their hydrophobicity, leading to increased antagonist activity.

On the other hand, when the P2 residue of OXT was substituted with hydrophobic AAs such as G, A, V, I, and L, those with larger side chains exhibited antagonistic activity (Figure 5). This tendency was also observed for aromatic ring AAs, His, Trp, and Tyr (in the original OXT sequence). This result suggests that the second residue should be highly hydrophobic or have relatively large side chains, which are important for antagonist activity. As reported by Magafa et al., substitutions at the P2 residue to D-Tyr (Et) or D-1-Nal become strong antagonists.26 This indicates that the aromatic ring of the P2 residue is essential for the antagonist activity. This coincides with the results obtained.

We also found a dose-dependent effect of antagonist substitutions, Y2I, Y2L, and Y2W, with inhibitory activity. Consequently, the P2 residue was characterized by the fact that the receptor was not activated by AA substitutions in the evaluation of antagonist activity. As reported by Magafa et al., substitutions at the P2 residue to D-Tyr (Et) or D-1-Nal become strong antagonists.26 This indicates that the aromatic ring of the P2 residue is essential for the antagonist activity. This coincides with the results obtained.

**CONCLUSIONS**

In the present study, AA residue substitutions in OXT were comprehensively investigated using a TGF-α shedding assay. We succeeded in constructing a system for the comprehensive search of free cyclic peptides using peptide arrays. We evaluated various substituted peptides using this search system and found that agonist activity was maintained even after substituting Gln at P4 and Leu at P8. In particular, the L8I variant (CYIQNPCIG) showed approximately 84% agonist activity compared to OXT. Substitution of the P2 residue, Tyr, abolished agonist activity but retained its binding to the receptor, indicating that it functions as an antagonist. In particular, the Y2I, Y2L, and Y2W variants showed high antagonist activity, indicating that their binding to the receptor was higher than that of the original OXT. Hence, using the free cyclic peptide search system developed in this study, it is possible to optimize the sequence of various cyclic peptides to improve their biological activity.

**MATERIALS AND METHODS**

**Peptide Array Synthesis.** Peptide arrays were prepared on cellulose membranes and synthesized via Fmoc solid-phase synthesis using a peptide autospotter (APS222, Intavis, Cologne, Germany).6 Cellulose membranes (grade 542; Whatman, Maidstone, U.K.) were activated using β-alanine as the N-terminal basal spacer. The Fmoc photolinker (sc-294977A, SANTA CRUZ) was first conjugated with β-alanine. Then, the concentration of the Fmoc photolinker was prepared to be 25 mM to eliminate intermolecular disulfide bond formation. Consequently, peptide synthesis was performed using Fmoc-protected amino acids (Watanabe Chemical Industry Co., Ltd., Hiroshima, Japan). Each Fmoc amino acid was spotted three times on the membrane to complete the dehydration synthesis. After peptide synthesis, the side-chain-protecting groups were removed for 2.5 h using a mixture of trifluoroacetic acid (TFA, A00025; Watanabe), m-cresol (034-06466; Wako), 1,2-ethanedithiol (A00057; Watanabe), and thiouanisole (T0191; Tokyo Chemical Industry, Tokyo, Japan) at a volume ratio of 40:1:3:6. Finally, the membrane was washed thoroughly with diethyl ether and methanol in a consecutive manner.

**Construction of a Free Cyclic Peptide Library.** The deprotected peptide array was incubated in 20% dimethylsulfide oxide (DMSO, 043-07216; Wako) in phosphate-buffered saline (PBS) (pH 7.2–7.4) at 25 °C for cyclization via oxidation between two Cys thiols.26 After 24 h, the peptide array was washed three times with PBS and three times with methanol and air-dried.

After cyclization, the peptide arrays were irradiated with UV light at 365 nm for 3 h using a transilluminator (DT-20LCP; Atto, Tokyo) to cleave the membranes. Each spot on the peptide array was punched out into a 96-well plate, and the peptides were eluted with Hank’s balanced salt solution (HBSS) containing 15 mM HEPES (pH 7.4) for 1 h at 25 °C to construct a free cyclic peptide library.

**Peptide Preparation for the Ligand Activity Assays.** For the agonist assay, one spot of the peptide array was eluted with 200 μL of HBSS for 1 h, and a 1:5 v/v dilution of the peptide solution was used for the assay. For the antagonist assay, three spots were eluted with 100 μL of HBSS. For the dose–response assay, oxytocin and its variants (CIIQNCPLG, CIIQNCPLG, CIIQNCPLG) were purchased from GL Biochem Ltd. (Shanghai, China) and purified to a purity of >95% using high-performance liquid chromatography (HPLC).

**Cell Culture.** Human embryonic kidney cells (HEK293 cells) were used as the expression system. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (DMEM, 08458-16, Nacalai Tesque, Kyoto) containing 10% fetal bovine serum (FBS, F7524, Sigma-Aldrich) and 1% penicillin–streptomycin (PS, 168–23191, Fujifilm Wako Pure Chemical Co.) in a humidified 5% CO2 incubator at 37 °C.

**TGF-α Shedding Assay.** In the agonist/antagonist assay of oxytocin and its variants, we performed the TGF-α shedding assay as reported by Inoue et al.,31 with some modifications (Figure S1).

HEK293 cells were seeded in a 24-well plate at a density of 1.0 × 105 cells/well and incubated for 24 h. For each well, a mixture of 125 ng of AP-TGFα plasmid and 50 ng of oxytocin receptor (OXTR) plasmid was transfected using PEI (49553-93-7, Polysciences, Warrington, PA) and incubated for 24 h. The AP-TGFα expression vector (pCAGGS/AP-TGFα) was kindly provided by Dr. Inoue (Tohoku University). The OXTR plasmid (pcDNA3.1++/C-(K)-DYK vector) (NM_000916.3, GenScript) was used as the expression vector for OXTR. The next day, 80 μL of transfected HEK cells was reseeded in 96-well plates. For the agonist assay, 20 μL of OXT variant solution (dissolved in HBSS in 15 mM HEPES) was added to each well 1 h after reseeding. The OXT variant solution was prepared from one spot after UV irradiation by elution in 200 μL of HBSS. A fivefold dilution of the eluted sample was then used for the assay. After 1 h of adding the ligand solution, 80 μL of the supernatant containing AP released by the reaction was collected from each well and transferred to another 96-well plate. The alkaline phosphatase activity was measured by adding 80 μL of 10 mM p-
nitrophényl phosphatase (pNPP, 34045, Thermo Fisher Scientific) solution to both the adherent cell plate and the supernatant plate. The absorbance difference at 405 nm (ΔOD_{405}) was measured using a microplate reader before and after 30 min of incubation at 37 °C. The absorbance was calculated as the amount of AP-TGFα released using the following equation

\[
\text{AP-TGFα release (\%)} = \frac{(\text{ΔOD}_{405} \text{ in supernatant plate})}{(\text{ΔOD}_{405} \text{ in supernatant plate}) + (\text{ΔOD}_{405} \text{ in cell plate})}
\]

To evaluate the agonist activity of the variants, the AP-TGFα release of the OXT variants was converted to a relative value based on the ligand activity of synthesized OXT, which was determined from a calibration curve using OXT purchased from GL Biochem Ltd. After 10 min of adding the OXT solution, 80 μL of the supernatant plate was collected from each well and transferred to another 96-well plate, and the alkaline phosphatase activity was measured as described above.

Each experiment is performed in triplicate, and the data are presented as the mean and standard deviation (SDs).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04982.

Schematic of the TGF-α shedding assay; mass spectrometry of synthesized OXT peptides under linker concentrations of 250 mM and 25 mM; dose–response of the TGF-α shedding assay by synthesized peptides; mass spectrometry of outsourcing peptides, Y2I, Y2L, and Y2W; and dose–response curve of oxytocin vs atosiban (PDF)

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**Notes**

The authors declare no competing financial interest.

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