Binding affinities of oxytocin, vasopressin, and Manning Compound at oxytocin and V1a
receptors in Syrian hamster brains

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Conceived the project: KEM, APR, HEA; Conducted pilot experiments: KEM, APR; Conducted experiments: JHT; Data analysis: JHT; Wrote the first draft of the manuscript: JHT; Edited manuscript: KEM, APR, HEA, JHT; Approved final manuscript: KEM, APR, HEA, JHT; Provided funding: HEA

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Conflict of Interest Statement

The authors declare no conflict of interest, real or perceived.

Data Availability Statement

Data are available upon reasonable request to the corresponding or senior authors.

ABSTRACT

Oxytocin (OT) and arginine vasopressin (AVP), as well as synthetic ligands targeting their receptors (OTR, V1aR), are used in a wide variety of research contexts, but typically their pharmacological properties are determined in only a few species. Syrian hamsters (*Mesocricetus auratus*) have a long history of use as a behavioral and biomedical model for the study of oxytocin and vasopressin, and more recently, hamsters have been used to investigate behavioral consequences of OT-mediated activation of V1aRs. We sought to determine the binding affinities of OT, AVP, and the selective V1aR antagonist, Manning compound, in OTRs and V1aRs found in hamster brains. We performed saturation binding assays to determine the K_d values for the selective OTR and V1aR radioligands, [125I]OVTA and [125I]LVA in hamster brains. We then performed competition binding assays to determine K_i values for OT, AVP, and Manning compound at both the OTR and V1aR. We found that OT and AVP each had the highest affinity for their canonical receptors (OT-OTR K_i=4.28 nM; AVP-V1aR K_i=4.70 nM), and had the lowest affinity for their non-canonical ligands (OT-V1aR=495.2nM; AVP-OTR K_i=36.1 nM). Manning compound had the highest affinity for the V1aR (MC-V1aR K_i=6.87 nM; MC-OTR K_i=213.8 nM), but Manning compound was not as selective for the V1aR as has been reported in rat receptor. When comparing these data to previously published work, we found that the promiscuity of the V1aR in hamsters with respect to oxytocin and vasopressin binding is more similar to the promiscuity of the human V1aR than the rat V1aR receptor. Moreover, the selectivity of oxytocin at hamster receptors is more similar to the selectivity of oxytocin at human receptors than the selectivity of oxytocin at rat receptors. These data highlight the importance of determining the pharmacological properties of behaviorally relevant compounds in diverse models species.

Key Words: radioligand binding assay, binding affinity, comparative, g protein-coupled receptor, GPCR

The nonapeptide hormones oxytocin (OT) and arginine vasopressin (AVP) and their cognate receptors are widely used in a variety of therapeutic and research contexts, including the study of social behavior. Perhaps due to their roles in modulating a wide variety of social behaviors across mammalian species, OT and AVP have been studied in many species, each with its own behavioral, practical, and ethological considerations^{1,2}. Moreover, because OT and AVP can bind and activate both the OT receptor (OTR) and the AVP V1a receptor (V1aR)³, there are dozens of pharmacological compounds that have been developed to selectively target each receptor⁴. Despite the widespread study of OT and AVP, the binding affinities of OT and AVP with OTRs and V1aRs have been determined in only a handful of species, with the majority of data limited to human, mouse, and rat receptors^{4,5} *c.f.*^{6,7}. Comparative studies, in which receptors from multiple species are probed at the same time using exactly the same methodology, show that there are differences in binding affinities (and subsequent activation potencies) even among closely related species^{6,7}. However, for many of the species that are currently being studied in the context of OT- and AVP-mediated social behavior, the binding affinities at the OTR and V1aR for these peptides, as well as for human-made pharmacological compounds, are not known.

The use of diverse animal models, each with its own unique constellation of social behavior, provides a comprehensive yet complex view of the roles of OT and AVP in modulating social behavior⁸. A recent comparative analysis shows that, with respect to receptor distribution, OTR and V1aR are quite variable within *Rodentia*⁹. Even among closely related species, there are differences in the OTR and V1aR amino acid sequences, which may translate to differences in ligand binding and signaling. Syrian hamsters are widely used as an animal model in social and biomedical research, and are preferred over other rodent models for many applications, including acute social stress^{10,11}, circadian rhythms^{12,13}, reproductive biology¹⁴, sex differences in physiology and behavior^{15,16}, and infectious diseases^{17,18}. Hamsters are phylogenetically similar to rats and mice, but display distinct patterns of social behavior and ecology. Despite a long history of use in the context of AVP- and OT-mediated behavior^{19,20}, the binding affinities at the hamster OTR and V1aR for OT, AVP, and other compounds are not known. This is particularly notable given the potential for behavioral effects of OT acting at the V1aR, and AVP acting at the OTR, as it has been demonstrated that some V1aR-mediated behavior in this species may be via OT, and not AVP²¹. Data regarding the binding affinities at the OTR and V1aR for

these peptides, as well as specific agonists and antagonists, may hold explanatory power for *in vivo* behavioral pharmacology, particularly when AVP and OT are administered in regions in which OTR and V1aR distributions overlap, or when OT and AVP are compared to selective agonists and antagonists.

There are a variety of methods used to determine the binding affinity for ligands at G protein-coupled receptors (GPCRs), including radioligand binding assays (RBAs). All RBAs require some type of tissue that has been selectively enriched with the GPCR of interest. Isolated membrane and whole cultured cell preparations are popular methods, but an underused method of determining binding affinity is to carry out an RBA in intact, native tissue that is naturally enriched in the GPCR of interest. This method is simple, provides a cellular context that is as close as possible to *in vivo* conditions, and many laboratories that study OT- and AVP-mediated social behavior are already equipped to perform these RBAs. Here, we demonstrate the use of an RBA in intact brain sections to determine the binding affinities of the OTR and V1aR in Syrian hamsters. We predicted that if Syrian hamster OTRs and V1aRs function like rat and mouse receptors, then we should obtain binding affinities for the hamster receptors using various compounds that are on the same order of magnitude as have been reported for mouse and rat receptors. We tested the binding affinities for OT and AVP at their cognate receptors, as well as at each other's receptors. We also tested the binding affinity of Manning Compound, a commonly used antagonist that is generally regarded as selective for V1aR in rat receptors, but not human or mouse^{4,5}.

Methods

Tissue preparation and quantification Adult male hamsters were deeply anesthetized with isoflurane. Upon induction of complete analgesia (measured via a toe pinch), hamsters were decapitated and the brain was rapidly removed and snap frozen in isopentane chilled on dry ice. Brains were stored at -80° C until sectioning. Brains were hemisected, sectioned at 20 µm on a cryostat, thaw mounted onto Superfrost plus slides, and then returned to -80° C until the day of assay. Brains were cut into series of eight to nine, such that consecutive sections were mounted onto consecutive slides. Matching sets were generated by mounting contralateral sections on separate slides. On the day of assay, slides were allowed to come to room temperature and dry

completely. Then, groups of four hemi-sections (n = 4 technical replicates) were roughly outlined with a wax pencil, and 250 μ L of binding solution (radioligand plus 50 mM Trizma Base, 21mM MgCl, 1% (w/v) BSA, 0.5%(w/v) bacitracin, pH 7.4) was pipetted directly onto each set of four sections. Slides were left at room temperature for one hour, after which excess binding solution was removed, and the slides were washed via two five-minute washes and one 35-minute wash in buffer (50 mM Trizma Base, 21mM MgCl, pH 7.4). After washes, slides were allowed to dry completely, and then laid on film (Carestream BIOMAX MR) and given 2-3 days of exposure. All experiments were carried out on at least two different days using fresh aliquots of cold ligands. Images were captured using a camera mounted above a light box. Binding was quantified by comparing the exposure intensity of specifically bound nuclei to [14C] standards that were laid with each film, fitted to a Rodbard function. Radioactivity is expressed in the same units as the [14C] standards, DPM/0.4 mg. The nuclei that were quantified are shown in Figure 1. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Georgia State University Institutional Animal Care and Use Committee.

Saturation Binding. Saturation binding experiments were carried out using eight to nine slide series. One hemisphere was used for total binding, while the contralateral section was used for non-specific binding. For OTRs, saturation binding occurred in the presence of doubling concentrations of [125I] OVTA (Perkin Elmer, NEX254010UC) ranging from 7.6 pM to 1000 pM. Non-specific binding was defined as 1×10^{-5} M OT. For V1aRs, saturation binding occurred in the presence of doubling concentrations of [125I] LVA (Perkin Elmer, NEX310050UC) ranging from 7.6 pM to 2000 pM. Non-specific binding was defined as 1×10^{-5} M AVP. Saturation curves were generated using curve fitting software (Graphpad Prism) and binding affinities (Kd) determined as the half maximal concentration. Reported Kd values and SEM were determined by averaging across biological replicates (n = 6-7). The potential for ligand depletion was assessed by pipetting 10 μ L of each ligand concentration at the beginning and the end of the binding assay onto filter paper. These were then laid onto film with the tissue slides and compared for changes in film exposure.

Competition Binding. Competition binding experiments were carried out using eight to nine slide series. For OTRs, competitive binding occurred using 200 pM [¹²⁵I]OVTA in the presence or absence of competitor in concentrations ranging from 10⁻¹¹-10^{-4.5} M. For V1aRs, competitive binding occurred using 400 pM [¹²⁵I]LVA in the presence or absence of competitor in concentrations ranging from 10⁻¹¹-10^{-4.5} M. Technical replicates (n = 4 hemisections per experiment) were averaged and treated as biological replicates (n = 4-9). Competition curves were generated using curve fitting software (Graphpad Prism) incorporating the measured K_d and concentration of the radioligand (i.e. a Cheng-Prusoff correction), and binding affinities (K_i) determined as the half maximal concentration. A Bonferroni-corrected cutoff ($p = .05 \div 3 = .0167$) was used to determine statistically significant differences in K_i values. The competitor ligands were OT (CYIQNCPLG-NH₂ ; Bachem), AVP (CYFQNCPRG-NH₂ ; Sigma) and Manning Compound (d(CH₂)₅[Tyr(Me)²]AVP ; Sigma)

Results

Saturation Binding. Saturation binding assays for [¹²⁵I]OVTA and for [¹²⁵I]LVA yielded K_d values that were similar to those reported in humans and rats (Table 1). The average K_d for [¹²⁵I]OVTA in hamster tissue was 0.0945 (± .002) nM, with a B_{max} of 2957 (± 55.03) DPM/0.04 mg. The average K_d for [¹²⁵I]LVA in hamster tissue was 0.2619 (± 0.067) nM, with a B_{max} of 5230 (± 1144) DPM/0.04 mg. Importantly, [¹²⁵I]OVTA and [¹²⁵I]LVA produced binding patterns that were visually distinct, even at the 2000 pM concentrations, indicating their selectivities as probes (Figure 1). Ligand depletion did not occur, as measured by 10 uL blots of radioligand buffer pipetted onto filter paper before and after binding had occurred.

Representative saturation curves are shown in Figure 2.

Competitive Binding. Competitive binding assays yielded K_i values for each ligand that were within the expected ranges, based on previous reports in other species (Table 1, Figure 3). At the hamster OTR, OT had an average K_i of 4.28 nM, AVP had an average K_i of 36.06 nM, and Manning Compound had a K_i of 213.8 nM. The binding affinity of OT at the OTR was significantly lower than that of AVP ($F_{1,140} = 17.55, p < .0001$) and Manning compound ($F_{1,119} = 61.94, p < .0001$). The binding affinity of AVP at the OTR was significantly lower than that of

Manning compound ($F_{1,111} = 17.55$, $p = .0148$). At the V1aR, OT had a K_i of 495.2 nM, AVP had a K_i of 4.70 nM, and Manning Compound had an average K_i of 6.87 nM. The binding affinity of AVP at the V1aR was significantly lower than that of OT ($F_{1,88} = 13.37$, $p = .0004$), but was not significantly different than that of Manning compound ($F_{1,82} = 224$, $p < .638$). The binding affinity of Manning compound at the V1aR was significantly lower than that of OT ($F_{1,60} = 55.39$, $p < .0001$). Importantly, these data show that in hamsters, OT and AVP bind with the highest affinity (i.e. lowest K_i) to their cognate receptors.

Discussion

The data presented here indicate that many properties of V1aRs and OTRs in Syrian hamsters more closely resemble those of human receptors than those of more closely related rodent species (i.e. rats and mice), and demonstrate the usefulness of hamsters and less commonly used animal models for pharmacological experiments. Translational relevance is crucial for preclinical research using animal models. While mouse and rat models of human behavior and disease are beneficial because of the plethora of genetic tools available, these models often fall short of providing ethologically- or translationally-relevant readouts. Thus, the use of diverse model systems is necessary for providing a better understanding of the molecular mechanisms underlying behavior and disease. For examples, hamsters are a preferred model for investigating reproductive biology¹⁴, tumor growth^{22,23}, and more recently in studying properties of infectious diseases, including Ebola, Zika, and influenza viruses^{17,18,24,25}. Furthermore, the human stress response system is more similar to the hamster system than to that of other rodent models and both male and female hamsters readily exhibit aggressive behavior, making hamsters an excellent model to investigate behavioral, physiological, and molecular responses to social stress in both sexes^{10,11,16}. The data presented here indicate that many properties of V1aR and OTR in Syrian hamsters more closely resemble those of human receptors than those of more closely related rodent species, suggesting that further work in hamsters may help to reveal more translationally-relevant details on how these ligands interact with their receptors to facilitate behavior.

While it is not prudent to directly compare the binding affinities obtained here to the binding affinities obtained through other methods, it is valuable to compare the *relationships* between binding affinities of different ligands at different receptors. For example, our results

indicate that the degree of promiscuity of the hamster V1aR, but not the hamster OTR, is more similar to the promiscuity of human receptors than to rat receptors. We found that the affinity of OT at the OTR was ~8.4 fold better than the affinity of AVP at the OTR. In rats, this same relationship is only ~1.7 fold, while in humans, it is ~2.1 fold different⁴. Likewise, the affinity of AVP at the hamster V1aR was ~105 fold better than the affinity of OT at the V1aR. In rats, this relationship is only ~27.3 fold, while in humans and other primates it is >100 fold^{4,6,7}. Interestingly, when focusing on the selectivity of the ligands rather than the selectivity of the receptors, we found that the selectivity of OT, but not AVP in hamsters more closely resembles that in humans than rats. OT is ~116 fold more selective for the hamster OTR than for the V1aR. In rats, this relationship is ~71 fold, while in humans it is ~150 fold different⁴. Likewise, AVP is ~7.7 fold more selective for the hamster V1aR than for the OTR. In rats, this relationship is ~2.167 fold, while in humans it is only ~1.54 fold. Thus, with respect to the promiscuity of the *receptors*, the OT/AVP relationship in the hamster V1aR is more similar to the same relationship in humans than in rats; however, with respect to the *ligands*, the selectivity of OT in hamsters is more similar to the same relationship in humans than in rats. Furthermore, Manning compound is ~31 fold more selective for the hamster V1aR than for the OTR, which is not considered “selective” by the definition of a 100 fold difference in selectivity used by⁴, a criteria which Manning compound meets in rat receptors. However, this compound is more selective for the V1aR in hamsters than it is in humans, and slightly more selective than it is in mice⁵. Taken together, these findings highlight the need to carefully evaluate a ligand’s pharmacological properties when applying it to new model species, whether that be through an RBA or through other bio- or behavioral assays.

In this paper we showed that the binding affinities for commonly used pharmacological compounds can be quickly and easily measured in a species for which enriched tissue is available. Many labs that regularly utilize *in vivo* behavioral pharmacology already have the capacity to perform similar assays using their species and ligands of choice. As the number of model species for various behavioral inquiries grows, it is important to consider the possibility that ligands which are validated for use in mice and rats may not act on other species’ receptors in the same way, even in species that are phylogenetically similar. This method also provides a cellular context that is as close as possible to the binding conditions in living, native tissue. This is an important factor to consider, because there is the possibility that receptor heterodimers

influenced the binding affinities of each ligand at the OTR or V1aR. Both the OTR and V1aR form heterodimers with each other, as well as with the vasopressin V2 receptor, but these heterodimers exhibit only small differences in binding affinity for AVP²⁶. When all of these considerations are taken together, the potential for a myriad of complex influences makes this method an attractive alternative and adjunct to other RBA methods for studying ligand-receptor dynamics under conditions that are as close as possible to those *in vivo*.

Though these data provide new information on the pharmacological characteristics of commonly used ligands in a widely used model species, they do not provide any information on the ability of OT, AVP, or Manning Compound to activate one or more signaling cascades. Binding affinity and selectivity are the critical factors in the pharmacological properties of a true antagonist, but for agonists and inverse agonists, binding affinity and receptor activation can often tell quite different stories. For example, the binding affinity for AVP at the OTR for human and macaque receptors is similar, but AVP exhibits nearly 10 fold different potency for calcium mobilization between these two species⁷. Likewise, the affinity of OT at the human V1aR is ~15 fold better than the affinity of OT at the marmoset V1aR, but OT acts as a partial agonist/ partial antagonist for calcium mobilization at the human V1aR, and a superagonist at the marmoset V1aR⁶. Thus, just as it is important to evaluate how well ligands bind to the receptors of various animal models, it is also important to evaluate the cellular and molecular consequences of ligand binding.

We have shown here that the relationship in hamsters between OT, AVP, and their receptors in some ways more closely resembles those same relationships in humans than in rats, highlighting important differences in binding patterns of closely related species. The determination of the pharmacological properties of both native ligands and those used as research tools is an important component to understanding how these ligands affect behavior. As more diverse animal models continue to be developed, it will be critical to re-evaluate the compounds that are used, as well as highlight the differences and similarities between species and how these variations in pharmacological properties correlate to social behavior. In particular, comparative approaches to pharmacological experiments have the potential to provide a wealth of information that can help when translating to human research.

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Table 1. Binding affinities, ligand specificities, and receptor specificities

	Ligand	Ki/Kd nM (95% CI)		Ligand Specificity (OTR vs V1aR) ^a
		OTR	V1aR	
	OVTA	0.094495	<i>n.d.</i>	
	LVA	<i>n.d.</i>	0.262	
	OT	4.28 (2.9-6.3)	495.2 (198.5-1276)	115.67
	AVP	36.1 (12.4-97.0)	4.70 (1.5-14.1)	7.67
	Manning Compound (MC)	213.8 (117.3-392.7)	6.87 (4.0-11.9)	31.13
Receptor Specificity^a				
	OT vs AVP	8.42	105.3	
	MC vs AVP	5.93	1.461	
	MC vs OT	49.94	72.09	

Note.^asuperscript indicates ratio of ligands were calculated pairwise by dividing ligand with higher Ki by ligand with lower Ki.

Figure 1. Representative photomicrographs of sections bound to A,C) [125 I] OVTA or B,D) [125 I] LVA. Each ligand produced distinct patterns of binding, even at A,B) 2000 pM concentrations. Regions quantified are shown in yellow boxes in C,D. 1. dorsal endopiriform nucleus, 2. lateral septum, 3. anteroventral bed nucleus of the stria terminalis

Figure 2. Representative saturation curves for [125I] LVA (top) and [125I] OVTA (bottom) in hamster brain slices. Total = Total binding, NSB = Non Specific Binding

Figure 3. Competition curves for oxytocin (OT) vasopressin (AVP) and Manning compound (MC) at the hamster OT receptor (left) and vasopressin V1a receptor (right).