

Neuropeptide Y Receptor Binding Profiles of Endogenous Ligands in the Presence and Absence of Sodium: A Panoramic View

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Cite This: *ACS Pharmacol. Transl. Sci.* 2026, 9, 2497–2512



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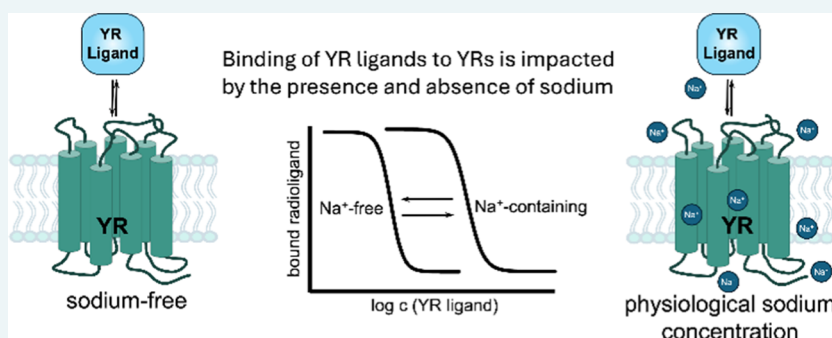
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ABSTRACT: Neuropeptide Y (NPY) receptors (YRs) play a key regulatory role in essential human physiological processes such as bone metabolism, feeding, anxiolysis, and pain modulation, making them potential therapeutic targets. Nonetheless, radiochemical ligand binding studies needed for the development of therapeutics acting at YRs, have been conducted inconsistently in the presence or absence of sodium, the latter representing a nonphysiological-like condition that may lead to inaccurate interpretations. As such, this study aimed to provide an in-depth overview of the binding affinities of endogenous YR agonists, namely NPY, peptide YY (PYY), and pancreatic polypeptide (PP), to the various YR subtypes, Y_1R , Y_2R , Y_4R , and Y_5R , in the presence and absence of sodium using recently developed tritium-labeled radioligands. Additionally, YR subtype-selective antagonists, BIBP3226 (Y_1), JNJ31020028 and BIIE0246 (Y_2), UR-MK188 (Y_4), and CGP71683A (Y_5), were studied. Significant differences in YR binding of the agonists, observed for Y_1R , Y_4R and Y_5R binding, were indicated by higher binding affinities in the absence of sodium. The effect was most pronounced for Y_1R binding of PP (ca. 100-fold difference in binding affinity). While Y_2R binding of the Y_2R antagonist BIIE0246 and Y_4R binding of the putative Y_4R antagonist UR-MK188 were not significantly influenced, a significantly decreased binding affinity was observed for the antagonists BIBP3226 (Y_1R) and CGP71683 (Y_5R) for binding under sodium-free conditions compared to the sodium-containing buffer. Overall, the determined YR binding affinities were largely consistent with literature data. Findings from this study confirmed sodium's allosteric modulation on YR agonist binding, although this modulatory effect was shown to be dependent on the type of ligand bound.

KEYWORDS: neuropeptide Y receptors, binding affinity, sodium-free buffer, radioligand binding studies

The neuropeptide Y (NPY) receptors (YRs) and their endogenous ligands are abundant in mammals' CNS and peripheral tissues. Their abundance in humans can be linked to their involvement in important physiological activities such as regulating anxiety, food intake, inflammation, cardiovascular activities, circadian rhythm, and gastrointestinal motility.^{1–3} This class A family of G-protein coupled receptors, consisting of the Y_1R , Y_2R , Y_4R , and Y_5R subtypes in humans, forms a multireceptor or multiligand system with the endogenous ligands NPY, peptide YY (PYY), and pancreatic polypeptide (PP), largely due to the sequence similarities between the ligands themselves and the YR subtypes (Figure 1).⁴ This situation complicates the design and development of ligands (particularly agonists) selectively targeting an individual YR subtype.

While the endogenous ligands NPY, PYY, and PP generally bind and activate the different YR subtypes, they usually exhibit varying receptor binding affinities. To date, analogs of NPY, PYY, or PP, either radioiodinated, tritiated, or fluorescence labeled, and tritiated antagonists have been used to determine YR-ligand binding affinities. Additionally, these affinities are generally determined under different binding assay

Received: March 4, 2026

Revised: June 30, 2026

Accepted: July 2, 2026

Published: August 6, 2026



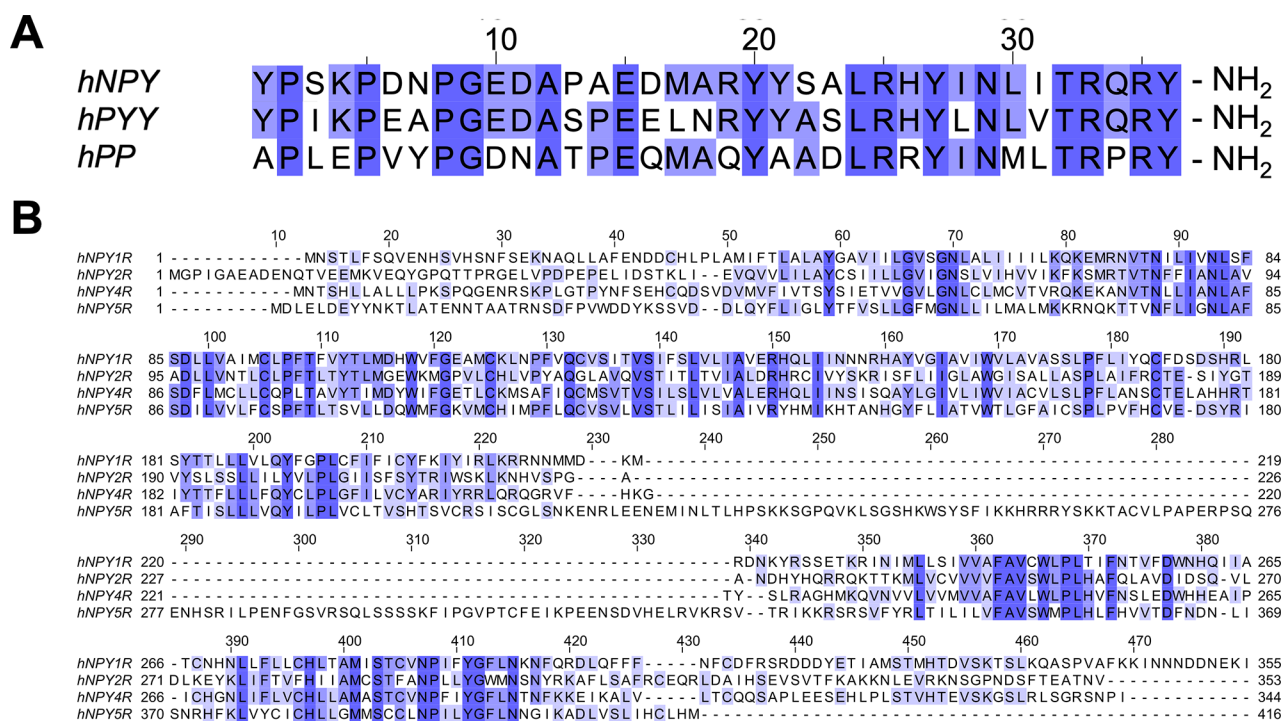


Figure 1. Multiple sequence alignment for the endogenous agonists (NPY, PYY, and PP) of YRs (A) and the different human YR subtypes (B). Sequence similarities are highlighted in blue (the color intensity directly correlates with amino acid similarities in the individual positions).

Table 1. Binding Assay Conditions (Sodium-Free vs Sodium-Containing Buffer) and Radioligands Commonly Used for Binding Studies at YRs

YR subtype	receptor species	buffers used	commonly used radioligands	references
Y ₁ R	human or rat	both sodium-free and sodium-containing	[¹²⁵ I]PYY, [³ H]propionyl-NPY, [¹²⁵ I][Leu ³¹ , Pro ³⁴]NPY	Gerald et al., ¹¹ Söll et al., ¹² Krause et al., ¹³ Loh et al., ¹⁴ Lundell et al. ¹⁵
Y ₂ R	human or rat	both sodium-free and sodium-containing	[¹²⁵ I]PYY, [³ H]propionyl-NPY, [¹²⁵ I]NPY, [¹²⁵ I]PYY(3–36)	Gerald et al., ¹¹ Krause et al., ¹³ Söll et al., ¹² Rose et al., ¹⁶ Dumont et al. ¹⁷
Y ₄ R	human, rat or mouse	mostly sodium-free	[¹²⁵ I]PP, [¹²⁵ I]PYY	Walker et al., ¹⁸ Berglund et al., ¹⁹ Söll et al., ¹² Balasubramaniam et al., ²⁰ Yan et al., ²¹ Eriksson et al. ²²
Y ₅ R	human or rat	mostly sodium-containing	[³ H]propionyl-NPY, [¹²⁵ I]pPYY, [¹²⁵ I][hPP(1–17), Ala ³¹ , Aib ³²]NPY, [¹²⁵ I][cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	Söll et al., ¹² Borowsky et al., ²³ Dumont et al., ²⁴ Dumont et al. ²⁵

conditions with respect to the presence or absence of sodium in the binding buffer, which in the latter case, are mostly hypotonic. Radioligand binding studies at the Y₁R and Y₂R have been generally conducted using both sodium-free and sodium-containing buffers. By contrast, Y₄R binding assays, until recently, have been primarily performed in hypotonic sodium-free buffers. On the other hand, sodium-containing buffers have been almost exclusively used for Y₅R binding studies. A summary of the assay conditions and the most often used radioligands is provided in Table 1.

Using different buffers with varying sodium concentrations for the determination of YR–ligand binding affinities can lead to discrepancies in the determined binding data. Assay conditions using buffers containing sodium at physiological concentrations (130–175 mM) tend to produce lower binding affinities of agonists (compared to sodium-free conditions), including radiolabeled agonists, since sodium acts as a negative allosteric modulator for numerous class A GPCRs including YRs.^{5–8} This effect is clearly observed in Y₄R binding, where the presence of sodium in binding assays, compared to sodium-

free conditions, can lead to a decrease in the agonist's binding affinity by a factor of more than 10.^{7,9,10} This might be the reason why sodium-free buffers have been used for radioligand binding studies at YRs: a higher radioligand affinity implies lower radioligand concentrations in the experiment, which in turn means lower consumption of radioligand and lower nonspecific binding.

As the use of sodium-free buffers does not mimic physiological-like conditions, receptor–ligand binding affinities should generally be determined in buffers containing sodium at a physiological concentration to improve the translatability of the data. For this reason, we recently introduced the tritium-labeled radioligands [³H]UR-JG102¹⁰ and [K⁴-[³H]-propionyl]hPYY²⁶ (structures see Figure S1, Supporting Information), representing useful probes for binding studies in sodium-containing buffer at the Y₄R and Y₂R, respectively.

To create a comprehensive overview of YR subtype binding profiles of the endogenous agonists NPY, PYY and PP, including their comparison in sodium-free and sodium containing buffers, we determined the binding affinities of

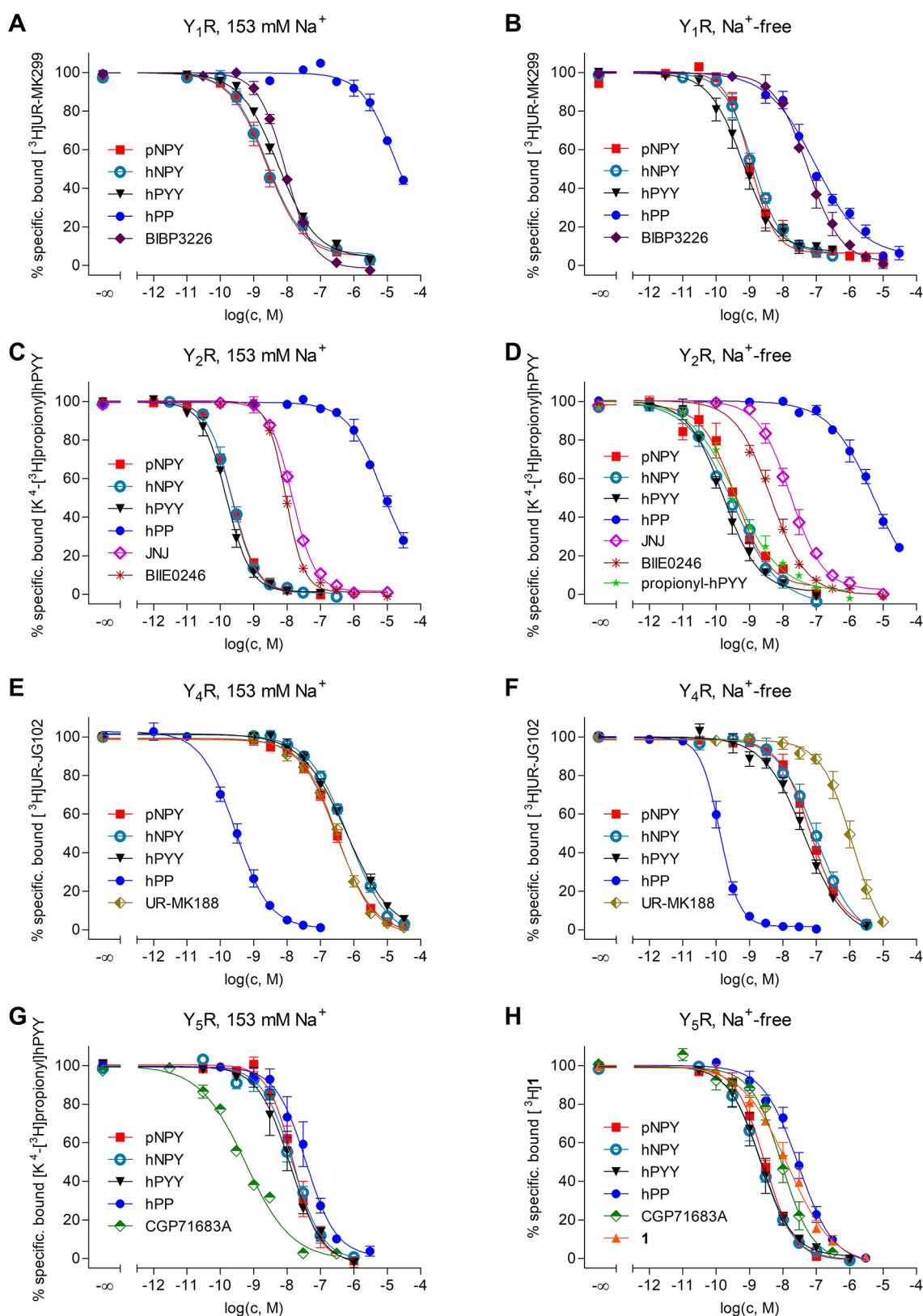


Figure 2. Radioligand displacement curves obtained from competition binding experiments at the different YR subtypes performed in sodium-containing buffer (DPBS, 153 mM Na⁺; A, C, E, and G) and sodium-free buffer (buffer Ia (Y₁R) or buffer Ib (Y₁R, Y₂R, Y₅R); B, D, F, and H). Used cells and radioligands: (A,B) SK-N-MC cells, [³H]UR-MK299 (DPBS, $K_d = 0.084$ nM, $c = 0.1$ nM; buffer Ib, $K_d = 0.41$ nM, $c = 0.5$ nM). (C,D) CHO-hY₂R cells, [³H]-[³H]propionylhPYY (DPBS, $K_d = 0.17$ nM, $c = 0.4$ nM; buffer Ib, $K_d = 0.078$ nM, $c = 0.12$ nM). (E,F) CHO-hY₄-G_{q15}-mtAEQ cells, [³H]UR-JG102 (DPBS, $K_d = 0.11$ nM, $c = 0.25$ nM; buffer Ia, $K_d = 0.012$ nM, $c = 0.03$ nM). (G,H) HEC-1B-hY₅R cells, [³H]-[³H]propionylhPYY (DPBS, $K_d = 14$ nM, $c = 10$ nM), [³H]-[³H]propionylHis-hPYY ([³H]1) (buffer Ib, $K_d = 20$ nM, $c = 15$ nM). Data represent mean values $\pm$ SEM of at least three independent experiments performed in triplicate. JNJ: JNJ31020028.

Table 2. YR Binding Affinities of the Endogenous Agonists pNPY, hNPY, hPYY, and hPP Determined in DPBS (153 mM Na⁺) and Sodium-free Buffer^{a,b}

compd.	pK _i ± SEM (this study), pK _i (lit.) or pIC ₅₀ (lit.)					
	hY ₁ R			hY ₂ R		
	with Na ⁺	Na ⁺ -free	with Na ⁺	with Na ⁺	Na ⁺ -free	with Na ⁺
pNPY	determined	8.95 ± 0.13	9.29 ± 0.12	10.18 ± 0.03	9.90 ± 0.09	7.72 ± 0.10*
	literature	^a 10.18 ¹¹	10.07 ³⁶	^b 9.92 ¹²	9.68 ³⁶	^b 7.02 ± 0.07*
		^b 10.92 ¹²	9.22 ³⁷	^a 9.07 ¹¹	9.10 ³⁹	^b 6.42 ³⁵ (r)
		^b 8.59 ³⁵ (r)		^b 10.40 ³⁸	9.40 ⁴⁰	^b 8.22 ²²
hNPY	determined	8.97 ± 0.02*	9.26 ± 0.06*	10.20 ± 0.08	9.89 ± 0.13	7.56 ± 0.12**
	literature	^a 10.06 ^{11,41}	9.30 ²⁰	^b 9.40 ³⁵ (r)	9.60 ³⁶	8.01 ¹⁵
			9.75 ³⁶	^a 9.27 ¹¹	9.70 ²⁰	^a 8.53 ⁴⁴
			9.40 ⁴²	^b 10.34 ± 0.04	10.16 ± 0.13	7.92 ⁴³
hPYY	determined	8.61 ± 0.03**	9.56 ± 0.13**	10.34 ± 0.04	10.16 ± 0.13	7.89 ± 0.04**
	literature	^a 9.77 ^{12,41}	9.85 ³⁶	^a 9.47 ¹¹	9.85 ³⁶	8.84 ¹⁵
		^b 8.72 ³⁵ (r)		^b 10.03 ⁵ (r)	9.70 ²⁰	9.03 ⁴⁵
						9.51 ⁴³
hPP	determined	5.05 ± 0.09***	7.43 ± 0.08***	5.55 ± 0.07	5.83 ± 0.12	10.44 ± 0.07
	literature	^a 7.10 ^{11,42}	7.72 ³⁶	^a <6.0 ¹¹	6.0 ³⁶	^a 10.29 ⁴⁵
		^b 6.36 ⁴⁶	9.30 ²⁰	^b <6.0 ³⁵ (r)	<5.5 ³⁹	10.86 ¹⁵
		^b <5.5 ⁹	6.36 ³⁹		<5.5 ⁴⁶	10.10 ²⁰
		^b 6.44 ³⁵ (r)			9.35 ⁹	^a 8.85 ²³
					10.41 ²²	^b 7.77 ⁴⁶
						^b 8.68 ³⁵ (r)

^a10–30 mM of Na⁺ is contained in the binding buffer. ^b100–175 mM of Na⁺ is contained in the binding buffer. r and rh indicate that pK_i values were obtained at rat and rat-human chimeric YR, respectively. Mean values ± SEM (pK_i) were obtained from at least three independent experiments performed in triplicate. Data reported as IC₅₀ or K_i were converted to pIC₅₀ or pK_i. *Not available for hPYY and human, mouse or rat Y₃R. **p < 0.05, ***p < 0.005 or ****p < 0.0005 (Welch *t*-test). Data displayed in italics represent pIC₅₀ values.

Table 3. YR Binding Affinities of the Antagonists BIBP3226 (Y₁R), JNJ31020028 (Y₂R), BIIE0246 (Y₂R), UR-MK188 (Y₄R) and CGP71683A (Y₅R) Determined in DPBS (153 mM Na⁺) and Sodium-free Buffer^{a,b}

compound	pK _i ± SEM (this study), pK _i (lit.) or pIC ₅₀ (lit.)							
	hY ₁ R		hY ₂ R		hY ₄ R		hY ₅ R	
	with Na ⁺	Na ⁺ -free	with Na ⁺	Na ⁺ -free	with Na ⁺	Na ⁺ -free	with Na ⁺	Na ⁺ -free
BIBP3226	determined	<i>b</i> 8.39 ± 0.04*	7.56 ± 0.15*	-	-	-	-	-
	literature	<i>b</i> 8.82 ⁴⁸	8.92 ⁵¹	-	-	-	-	-
		<i>b</i> 8.89 ⁴⁹						
		<i>b</i> 8.19 ⁵⁰						
		<i>b</i> 7.97 ⁵⁰						
JNJ31020028	determined	-	-	<i>b</i> 8.37 ± 0.03	8.17 ± 0.14	-	-	-
	literature	-	-	<i>b</i> 8.07 ⁵²	n.a.	-	-	-
BIIE0246	determined	-	-	8.56 ± 0.04	8.76 ± 0.08	-	-	-
	literature	-	-	<i>b</i> 8.48 ³⁸	8.59 ⁵³	-	-	-
				<i>b</i> 7.92 ⁵²	7.77 ⁵⁴			
UR-MK188	determined	-	-	-	<i>b</i> 7.00 ± 0.06	6.47 ± 0.13	-	-
	literature	-	-	-	<i>b</i> 6.62 ⁹	6.53 ⁹	-	-
CGP71683A	determined	-	-	-	-	-	<i>b</i> 9.49 ± 0.05*	8.31 ± 0.14*
	literature	-	-	-	-	-	<i>b</i> 9.52 ²⁵ (r)	8.72 ⁹
							<i>b</i> 8.80 ⁹	

^a10–30 mM of Na⁺ is contained in the binding buffer. ^b100–175 mM of Na⁺ is contained in the binding buffer. r indicates that pK_i values were obtained at rat YR. Mean values ± SEM (pK_i) were obtained from at least three independent experiments performed in triplicate. Data reported as IC₅₀ or K_i values were converted to pIC₅₀ or pK_i. *Mean pK_i values (Na⁺-free buffer compared to Na⁺-containing buffer) are significantly different with *P* < 0.05 (Welch *t*-test). Data displayed in italics represent pIC₅₀ values. n.a.: not available.

pNPY, hNPY, hPYY, and hPP for the different human YR subtypes in a sodium-free buffer and in DPBS with physiological levels of sodium (153 mM Na⁺). Additionally, at least one standard antagonist was included for each receptor subtype. All competition binding assays were performed with intact cells stably expressing human wild-type YRs using well-characterized tritiated ligands (Y₁R, Y₂R, Y₄R) and one newly prepared tritium-labeled radioligand (Y₅R). As pNPY, in contrast to hNPY, contains no oxidation-prone methionine and is more frequently used than hNPY for pharmacological investigations, it was also included in the study. Functional characterization of the endogenous peptides NPY, PYY, and PP was not included in the present study since it is well-known that these peptides act as full agonists and functional assays have generally been performed in sodium-containing buffers (for reported potencies of NPY, PYY, and PP, determined in various functional YR assays see Table S1, Supporting Information).

RESULTS AND DISCUSSION

For the purpose of this study, tritium-labeled radioligands were used to determine the binding affinities of the various endogenous agonists and reported antagonists to the human YR subtypes Y₁R, Y₂R, Y₄R, and Y₅R. Typically, iodinated (I-125) peptidic radioligands are used for YR binding studies. However, iodinated derivatives of NPY, PYY or PP, unlike tritium-labeled analogs, have several disadvantages such as the different radiolabeled species obtained by iodination,²⁷ adverse effect on the physicochemical properties of the ligand,²⁸ and lower stability.²⁹ Therefore, the recently reported tritium-labeled high-affinity ligands [³H]UR-MK299³⁰ (Y₁R), [³H]-[³H]propionyl]hPYY²⁶ (Y₂R), and [³H]UR-JG102¹⁰ (Y₄R) were used in the present study. The radioligands [³H]-[³H]propionyl]hPYY and [³H]UR-JG102 were developed with the purpose of establishing Y₂R and Y₄R binding assays, respectively, using a sodium-containing buffer and a tritium-labeled radioligand.^{10,26} In contrast to [³H]-[³H]propionyl]-

hPYY, the binding of the previously reported radioligand [³H]-[³H]propionyl]pNPY to CHO-hY₂R cells used in the present study was not saturable in sodium-containing buffer.²⁶ In the case of Y₄R, all tritiated ligands reported before the introduction of [³H]UR-JG102, exhibit equilibrium dissociation constants >6 nM in sodium-containing buffer.¹⁰ [³H]UR-JG102 displays considerably higher Y₄R binding affinity in sodium-containing buffer (K_d = 0.11 nM), leading to lower nonspecific binding and reduced radioligand consumption.¹⁰

Since a tritium-labeled radioligand with high affinity (K_d < 10 nM) is still lacking for the Y₅ subtype, a newly synthesized tritiated hPYY derivative, [³H]-[³H]propionyl]His-hPYY ([³H] 1), was used to determine Y₅R binding in sodium-free buffer while [³H]-[³H]propionyl]hPYY was used for Y₅R binding studies in sodium-containing buffer.

All binding assays were performed using whole cells because whole-cell assays, irrespective of the composition of the used buffers and the incubation temperature, better mimic physiological conditions as compared to the use of cell membranes or homogenates. For Y₁R binding studies, human SK-N-MC neuroblastoma cells, a canonical cell line endogenously expressing moderate levels of Y₁R,³¹ were used. In the case of human Y₂R and Y₄R, stably transfected CHO cells, representing a widely used cell line for the expression of GPCRs, were used (CHO-hY₂R cells (PerkinElmer); CHO-hY₄-G_q5-mtAEQ cells³²). As the expression of the human Y₅R failed in CHO cells but was successful in the human endometrial cancer cell line HEC-1B,³³ this cell line (HEC-1B-hY₅R cells³³) was used for Y₅R binding studies. The competition binding curves originating from the binding studies at the four YR subtypes are presented in Figure 2 (sodium-containing buffer DPBS and sodium-free buffer) and in Figure S8 (Supporting Information) (sodium-containing buffer II).

Y₁R Binding

Binding studies at Y₁R were performed with [³H]UR-MK299.³⁰ Y₁R binding of this radioligand was originally

studied in *buffer II*, a magnesium-free buffer containing 175 mM sodium, yielding a K_d of 0.044 nM.³⁰ For the batch of [³H]UR-MK299 used for this study, a K_d of 0.058 nM was obtained.²⁶ In the present study, Y₁R binding assays were conducted in DPBS, containing 153 mM sodium, in *buffer II*, containing 175 mM sodium, and in sodium-free *buffer Ib*. DPBS was used because it contains magnesium (3.98 mM) and therefore better simulates physiological conditions. For DPBS and *buffer Ib*, [³H]UR-MK299 was first characterized by saturation binding, yielding K_d values of 0.084 nM and 0.41 nM, respectively (Figures S6A,B, Table S2; Supporting Information) before it was used in competition binding experiments.

The Y₁R binding affinity of hNPY was similar to that of pNPY for DPBS and *buffer Ib* (Table 2, Figure 2A,B). The pK_i values of both peptides obtained for *buffer Ib* were slightly higher compared to the pK_i values determined in DPBS ($\Delta pK_i = 0.34$ and 0.29, respectively). However, this difference was only significant for hNPY ($P < 0.05$). In the case of hPYY, the difference in binding affinity (DPBS vs *buffer Ib*) was more pronounced (9-fold higher in *buffer Ib*, $P < 0.005$). For hPP, a marked difference in Y₁R binding was observed (ca. 240-fold higher binding affinity in *buffer Ib*, $P < 0.0005$). When comparing the determined binding data of the endogenous ligands with reported Y₁R binding affinities, the discrepancies were generally more pronounced for sodium-containing buffers compared to the sodium-free conditions. The Y₁R antagonist BIBP3226³⁴ exhibited a lower Y₁R binding affinity (ca. 7-fold, $P < 0.05$) in *buffer Ib* (Na⁺ free) than in DPBS (153 mM Na⁺), being consistent with the Y₁R binding of the radiolabeled BIBP3226-derived Y₁R antagonist [³H]UR-MK299 studied in the different buffers (*buffer II*/DPBS: $K_d = 0.058/0.084$ nM, *buffer Ib*: $K_d = 0.41$ nM). This suggests that these antagonists may prefer the inactive Y₁R conformation, which is stabilized in the presence of sodium.^{5,8} Nonetheless, the pK_i of 8.39 obtained for BIBP3226 in DPBS was in agreement with reported binding data ($\Delta pK_i \leq 0.5$, Table 3). For the sodium-free conditions, there is only one reported pK_i for BIBP3226, which is higher than the one determined in this study ($\Delta pK_i = 1.46$). A comparison of the binding data of pNPY, hNPY, hPYY, hPP and BIBP3226 determined in DPBS and *buffer II* showed no significant differences (Table S3, Supporting Information), indicating that buffer components other than sodium have no or only little impact on Y₁R binding.

Y₂R Binding

For all binding studies at Y₂R, the recently reported radioligand [³H]-[K⁴-[³H]propionyl]hPYY was used.²⁶ Y₂R binding of this radioligand was initially studied in *buffer II* ($K_d = 0.16$ nM²⁶) and in the hypotonic sodium-free *buffer Ia* ($K_d = 0.067$ nM²⁶). For the present study, Y₂R binding assays were performed in *buffer II*, DPBS, and in the isotonic sodium-free *buffer Ib*. As earlier mentioned, DPBS was used as it contains magnesium in contrast to *buffer II*. For the determination of Y₂R binding affinities under sodium-free conditions, the reported procedure was slightly modified by supplementing *buffer Ia* with 0.3 M glycine (*buffer Ib*) to obtain an isosmotic solution.⁹ Consequently, the used radioligand [K⁴-[³H]-propionyl]hPYY was first characterized by saturation binding in DPBS and *buffer Ib*, yielding K_d values of 0.17 nM and 0.078 nM, respectively (Figure S6C,D, Table S2; Supporting Information) before it was used in competition binding experiments. These K_d values were in good agreement with

the K_d values of [K⁴-[³H]propionyl]hPYY reported for *buffer II* ($K_d = 0.16$ nM²⁶) and *buffer Ib* ($K_d = 0.067$ nM²⁶). The results obtained for the sodium free buffers (*buffer Ia* vs *buffer Ib*) showed that hypotonicity is not affecting Y₂R binding of the used radioligand.

For all peptides under study, the differences in Y₂R binding affinities between DPBS and *buffer Ib* were low and statistically insignificant ($\Delta pK_i \leq 0.31$, $P > 0.05$, Table 2, Figure 2C,D). Whereas pNPY, hNPY and hPYY showed high Y₂R binding affinities in both buffers ($pK_i = 9.89$ – 10.34), hPP displayed considerably lower Y₂R binding (pK_i , DPBS = 5.55; pK_i , *buffer Ib* = 5.83) suggesting that hPP has no relevance for Y₂R activation in vivo. All pK_i values determined in DPBS and *buffer Ib* were in accordance with those reported in the literature (Table 2).

Regarding the binding of the Y₂R antagonists, i.e. JNJ31020028⁵² and BIIE0246³⁸ (structures shown in Figure S2, Supporting Information), both compounds showed similar binding affinities in DPBS and *buffer Ib* (Table 3). This result indicates that these Y₂R antagonists do not discriminate between the active receptor conformation (stabilized in sodium-free buffer) and the inactive receptor conformation (stabilized in sodium-containing buffer) unlike the Y₁R antagonist BIBP3226 (discussed above) and the Y₅R antagonist CGP71683A (see below), which apparently prefer the inactive receptor conformation (indicated by decreased binding affinities in the absence of sodium). The pK_i values of JNJ31020028 and BIIE0246 obtained in sodium-containing buffer were in line with reported data. Regarding the sodium-free conditions, the reported Y₂R binding affinity of BIIE0246 determined with fluorescently labeled pNPY ($pK_i = 8.59$, flow cytometric binding assay)⁵³ was in good agreement with the pK_i value determined in this study ($pK_i = 8.76$). However, the reported Y₂R binding affinity of BIIE0246 determined in a radiochemical competition binding assay is approximately 10-fold lower ($pK_i = 7.77$)⁵⁴ than the herein determined Y₂R binding affinity. This discrepancy could be explained by the unfavorable binding characteristics of the radioligand used by Pluym et al. showing a high K_d of 67 nM and incomplete dissociation.⁵⁴ For JNJ31020028, there are no reported Y₂R binding data determined in sodium-free buffer.

A comparison of the binding data of pNPY, hNPY, hPYY, hPP, JNJ31020028, and BIIE0246 determined in DPBS and *buffer II* reveals low differences ($\Delta pK_i \leq 0.61$, Table S3, Supporting Information). These differences were significant for pNPY, hPP and the antagonists ($P < 0.05$), indicating that the composition of the buffer may have had some impact on Y₂R binding of these ligands.

Y₄R Binding

Binding studies at Y₄R were performed with [³H]UR-JG102 using reported procedures for the sodium-free and sodium-containing buffer (*buffer Ia* and DPBS, respectively).¹⁰ To allow for a better comparison with literature data, Y₄R binding studies were conducted in the hypotonic sodium-free *buffer Ia* in contrast to Y₁R, Y₂R and Y₅R binding studies, performed in isotonic *buffer Ib* (sodium-free conditions). With respect to the buffers which have been used for YR binding studies, the Y₄R stands out since *buffer Ia* has been predominantly used in contrast to the other subtypes (for a summary of reported Y₄R binding assay buffer conditions see Table S4). To further test if hypotonicity affects Y₄R ligand binding, saturation binding experiments with [³H]UR-JG102 were conducted in *buffer Ib*

(see Figure S7A, Supporting Information). The obtained K_d value ($K_d = 0.017$ nM) was in good agreement with the reported K_d of [^{3} H]UR-JG102 determined in *buffer Ia* ($K_d = 0.012$ nM), being consistent with a previously reported tritiated hPP derivative, which showed similar Y_4R binding affinities in *buffer Ia* and in *buffer Ib*.⁹

The peptides pNPY, hNPY, and hPYY showed similar Y_4R binding affinities in sodium-containing buffer ($pK_i = 6.66$ – 7.02), and in sodium-free buffer ($pK_i = 7.56$ – 7.89) (Table 2, Figure 2E,F). For each of these peptides, the difference in Y_4R binding between the two buffers was significant (pK_i in DPBS < pK_i in *buffer Ia*; $P < 0.01$). A similar effect of sodium was recently observed for Y_4R binding of small oligopeptidic Y_4R agonists, showing a pronounced increase in Y_4R binding affinity in the absence of sodium (up to 25-fold).¹⁰ The Y_4R binding affinities of hNPY, pNPY, and hPYY determined in DPBS in the present study were markedly lower than reported binding affinities determined in sodium-containing buffer (up to 100-fold, Table 2). For sodium-free conditions, the determined pK_i of pNPY falls within the range of the reported data, whereas for hNPY and hPYY, the determined binding affinities were 3 to 10-fold lower compared to reported data (Table 2).

Unlike pNPY, hNPY, and hPYY, the Y_4R binding affinities of hPP, determined in sodium-free and sodium-containing buffer, were not significantly different. For both conditions, the pK_i values of hPP were in accordance with reported binding data.

Notably, hPP compared to the other peptides showed a considerably higher Y_4R binding affinity in both buffers (ca. 1000-fold, cf. Table 2), confirming that PP is the primary endogenous activator of Y_4R .^{55,56} On the other hand, the markedly lower Y_4R binding affinities of NPY and PYY ($pK_i < 7$) compared to PP ($pK_i = 10.06$) questions the suggested physiological relevance of NPY and PYY for Y_4R activation^{57–59} as peptide concentrations above 0.5 nM are rather unlikely to occur under physiological conditions.^{60,61}

In contrast to Y_1R , Y_2R , and Y_5R , only a few putative Y_4R antagonists have been reported. In this study, Y_4R binding of the Y_4R antagonist UR-MK188, which was derived from arginamide-type Y_1R antagonists, was investigated.⁶² The results showed that the difference between Y_4R binding of UR-MK188 in DPBS and *buffer Ia* was insignificant. Furthermore, the obtained pK_i values of UR-MK188 were similar to reported data (Table 3).

To note, the Y_4R antagonism of UR-MK188 was concluded from the results of a functional assay with a proximal readout (Ca^{2+} aequorin assay).⁶² These data are shown in Figure 3A (blue curve, reported data) together with a concentration–response curve of hPP (green curve, determined in this study). The Y_4R potency of hPP ($pEC_{50} = 7.67$) was lower compared to reported potencies determined in other functional assays (see Table S1, Supporting Information). Given that the calcium aequorin assay is performed under nonequilibrium conditions, the measured signal occurs immediately after the addition of the agonist, allowing an association of the agonist to the receptor only to some extent compared to the receptor occupancy that would be reached in equilibrium. This must be compensated by higher agonist concentrations leading to higher EC_{50} values.

In the present study, UR-MK188 was further investigated in a recently established impedance-based functional Y_4R assay⁶³ representing a very distal readout to verify its antagonistic effect at Y_4R . In the impedance-based assay, UR-MK188 rather

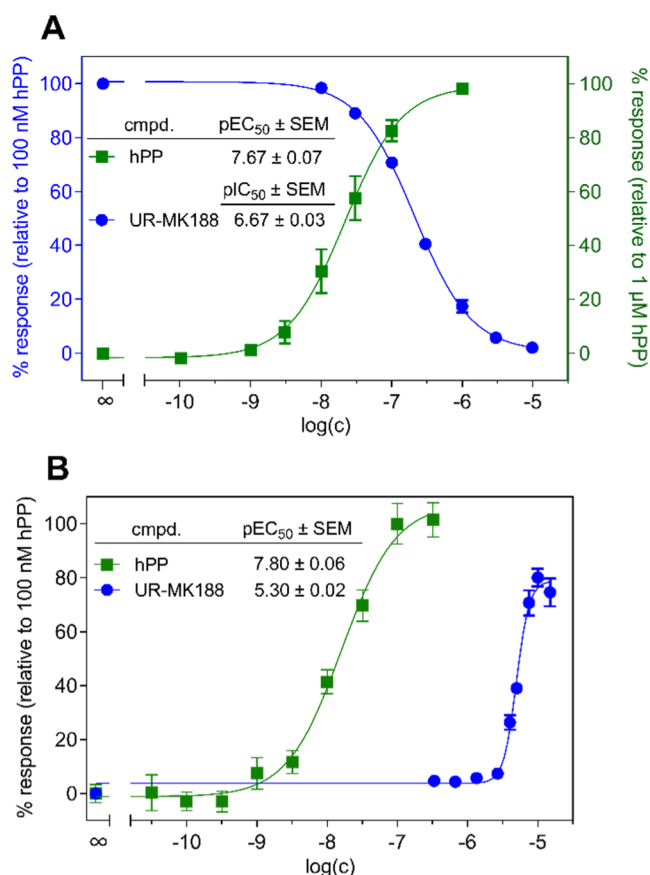


Figure 3. hPP and UR-MK188 studied in different functional Y_4R assays performed with CHO-h Y_4 -G_{q15}-mtAEQ cells. (A) Ca^{2+} aequorin assay measuring the proximal cellular response to Y_4R activation performed in agonist mode (right Y axis) and antagonist mode (left Y axis). UR-MK188 acts as an antagonist capable of inhibiting the Y_4R -mediated Ca^{2+} response elicited by 100 nM hPP (corresponds to the EC_{80} of hPP). (B) Impedance-based assay measuring a distal cellular response associated with morphological changes of the cells. hPP and UR-MK188 act as agonists. The assay vehicle for UR-MK188, containing $\leq 0.1\%$ DMSO, had no effect (data not shown). Presented are mean values $\pm$ SEM from three independent experiments (performed in triplicate). In A, data of UR-MK188 were taken from Keller et al.⁶² In B, data of hPP were taken from Wirth et al.⁶³ Data of hPP in A and data of UR-MK188 in B were determined in this study applying the previously used assay conditions.

behaved as a partial Y_4R agonist with an efficacy higher than 70% (Figure 3). This indicates that the signal transduction mirrored in the impedance assay time course is not dominated directly by the Ca^{2+} release, a prime source of morphological changes. Moreover, this result shows that functional assays with a distal readout also capture the downstream effect of signal amplifications, potentially unmasking partial receptor agonism that remains undetected in canonical second messenger assays. A similar observation was made for a peptidic Y_4R ligand that acted as an antagonist in the Ca^{2+} aequorin assay, but showed partial agonism in a Y_4R reporter gene assay also representing a distal readout.⁶⁴ Prompted by the results of the impedance-based assay, UR-MK188 was further examined in the Ca^{2+} aequorin assay performed in agonist mode. Under these conditions, this compound elicited no response (see Figure S9, Supporting Information).

Notably, the pEC_{50} value obtained for hPP in the impedance assay was similar to the pEC_{50} of hPP derived from the aequorin assay (cf. Figure 3). For GPCRs other than hY_4R , higher agonist potencies for impedance-based readouts were observed compared to other functional assays such as Ca^{2+} assays⁶⁵ even though this has not been broadly studied. This observation is in line with the common notion that the impedance time course integrates all changes across the entire cell body and accumulates various amplification steps during signal transduction, typical for a distal readout. Given that higher potencies of hPP are obtained in Y_4R cAMP assays (cf. Table S1, Supporting Information) compared to the lower potency from the impedance assay, the morphological effect (impedance signal) may also have not originated directly from increased cAMP levels. Instead, the impedance signal could be a result of a concerted action of several different signaling molecules or pathways. Irrespective of these considerations, the observed effects of hPP and UR-MK188 could be specific for the used cell line expressing a chimeric G protein (CHO- hY_4 -Gq β γ -mtAEQ cells).

To conclude, these findings suggest a need for further studies involving other Y_4R -expressing cells to shed more light on the mode of action of UR-MK188 at Y_4R , which is beyond the scope of this work.

Y_5R Binding

Like Y_1R and Y_2R binding studies, Y_5R binding of the endogenous YR agonists and the Y_5R antagonist CGP71683A, was investigated in the sodium-containing buffers DPBS and *buffer II*, and in the isotonic sodium-free *buffer Ib*. For binding experiments in the sodium-containing buffers, $[K^4\text{-}^3H\text{-}propionyl]hPYY^{26}$ was used as probe. To note, this radioligand was recently introduced for Y_2R binding studies in sodium-containing buffer (*buffer II*), but has not been used for Y_5R binding studies.²⁶ In the present study, $[K^4\text{-}^3H\text{-}propionyl]hPYY$ was used for Y_5R binding experiments (DPBS, *buffer II*) since it was readily available in the lab and its cold analogue $[K^4\text{-}propionyl]hPYY$ showed Y_5R binding affinity in *buffer II* similar to that of the formerly used radioligand $[K^4\text{-}^3H\text{-}propionyl]pNPY$.²⁶ Prior to the use of $[K^4\text{-}propionyl]hPYY$ for the investigation of ligand binding at Y_5R , it was characterized by saturation binding yielding K_d values of 14 nM (DPBS) and 9.5 nM (*buffer II*) (Figure S7B,C, Table S2; Supporting Information). As a control and validation of the assay in *buffer II*, the Y_5R binding affinity of the nontritiated analogue $[K^4\text{-}propionyl]hPYY$ was determined using $[K^4\text{-}^3H\text{-}propionyl]hPYY$ as probe. This experiment afforded a pK_i value of 8.09 ± 0.05 (mean value $\pm$ SEM from three individual experiments performed in triplicate; expressed as K_i : 8.3 nM) being in excellent agreement with the K_d of 9.5 nM determined for $[K^4\text{-}^3H\text{-}propionyl]hPYY$.

For Y_5R binding studies in sodium-free *buffer Ib*, $[K^4\text{-}^3H\text{-}propionyl]hPYY$ was considered to be used as radioligand. However, its binding affinity was lower ($K_d > 20$ nM) compared to that obtained in *buffer II* ($K_d = 9.5$ nM), implying high amounts of radioligand to be used for the experiment and high nonspecific binding relative to specific binding. Hence, its *N*-terminally extended histidyl analogue $[K^5\text{-}^3H\text{-}propionyl]His\text{-}hPYY$ ($[^3H]1$), available in our lab, was also tested. $[^3H]1$ was synthesized due to an unintended purchase of His- $hPYY$ instead of $hPYY$ as the labeling precursor (the lapse was noticed after the radiosynthesis of $[^3H]1$). Saturation binding experiments with $[K^4\text{-}^3H\text{-}propionyl]hPYY$ and $[^3H]1$ at

HEC-1B- hY_5R cells in *buffer Ib* yielded similar results (data of $[K^4\text{-}^3H\text{-}propionyl]hPYY$ not shown), however, $[^3H]1$ appeared to be slightly more advantageous with respect to saturability (specific binding) and the extent of nonspecific binding although a significant difference was not found based on the acquired data (data not shown). It should be kept in mind that the K_i values from competition binding studies should be independent of the used radioligand as the calculation of the K_i values involves the K_d of the respective radioligand, determined by saturation binding using the same assay conditions as for the competition experiments. Thus, the histidyl analogue $[^3H]1$ was used for Y_5R binding studies in *buffer Ib* to reduce the consumption of $[K^4\text{-}^3H\text{-}propionyl]hPYY$.

The K_d value obtained from saturation binding studies with $[^3H]1$ in *buffer Ib* was 20 nM (for a representative saturation binding curve see Figure S7D, Supporting Information). The synthesis of $[^3H]1$ and its nontritiated analogue $[K^5\text{-}propionyl]His\text{-}hPYY$ (**1**) is shown in Scheme S1 (Supporting Information). Chromatograms of the RP-HPLC analysis of crude $[^3H]1$ (reaction control), the micropreparative workup of $[^3H]1$ by RP-HPLC, and the determination of the radiochemical purity of $[^3H]1$ are shown in Figures S3–S5 (Supporting Information). The suitability of $[^3H]1$ for Y_5R competition binding studies in *buffer Ib* was evaluated by determining the pK_i value of **1** using $[^3H]1$ as a probe. This control experiment gave a pK_i value of 7.88 ± 0.11 (mean value $\pm$ SEM from three individual experiments performed in triplicate; expressed as K_i : 14 nM), which confirmed the Y_5R binding affinity determined by saturation binding with $[^3H]1$ ($K_d = 20$ nM).

In sodium-containing DPBS, all studied peptides displayed similar Y_5R binding affinities ($pK_i = 7.76\text{--}8.19$, Table 2, Figure 2G). The pK_i values obtained for pNPY, hNPY, and $hPYY$ in sodium-free *buffer Ib* were moderately higher ($P < 0.05$) compared to the pK_i values determined in DPBS (Table 2, Figure 2H). For hPP, similar Y_5R binding affinities were observed for DPBS and *buffer Ib*. All Y_5R binding affinities were largely in agreement with reported data (≤ 12 -fold difference).

For the investigated Y_5R antagonist CGP71683A,⁶⁶ the pK_i determined in DPBS was markedly higher than the pK_i determined in *buffer Ib* ($\Delta pK_i = 1.18$), indicating that this antagonist prefers the inactive receptor conformation as hypothesized above for the reported Y_1R antagonist BIBP3226. For both buffers, the pK_i values of CGP71683A were in agreement with reported data (Table 3).

Comparing the binding data determined in DPBS and *buffer II*, the pK_i values obtained in *buffer II* were slightly higher for all studied ligands ($\Delta pK_i \leq 0.52$, Table S3, Supporting Information). These differences were significant for pNPY, hNPY and CGP71683A ($P < 0.05$), suggesting that the buffer composition may have slightly influenced Y_5R binding of these ligands.

Summary of the Effect of Sodium on YR Binding of the Ligands under Study. Taken together, the results of this study indicate that the effect of sodium on YR binding (summarized in Table 4) is dependent on the receptor subtype and type of ligand. For example, hPP showed an increased binding affinity in the absence of sodium only at the Y_1R , while binding of this peptide to the other subtypes remained largely unaffected. Regarding a possible effect of potassium (absent in sodium-free *buffer Ia* and *Ib*, 4.15 mM in DPBS, and 5 mM in

Table 4. Summary of the Effects of Sodium on YR Binding Affinity (pK_i) of Agonists and Antagonists^a

YR	Compd.	Signif. change in pK_i with $Na^+ \rightarrow Na^+$ -free
agonists		
Y ₁ R	pNPY	—
	hNPY	↗
	hPYY	↗
	hPP	↗
Y ₂ R	pNPY	—
	hNPY	—
	hPYY	—
	hPP	—
Y ₄ R	pNPY	↗
	hNPY	↗
	hPYY	↗
	hPP	—
Y ₅ R	pNPY	↗
	hNPY	↗
	hPYY	↗
	hPP	—
antagonists		
Y ₁ R	BIBP3226	↘
Y ₂ R	JNJ31020028	—
	BIIE0246	—
Y ₄ R	UR-MK188	—
Y ₅ R	CGP71683A	↘

^aArrows up and down indicate a significant increase and decrease, respectively ($P < 0.05$), in binding affinity for the comparison of the pK_i values determined in sodium-containing buffer (DPBS) with the pK_i values determined in sodium-free buffer (cf. Tables 2 and 3). The dash indicates that pK_i values were not significantly different ($P > 0.05$).

buffer II) on YR binding of the studied ligands, a study performed by Parker et al.⁶⁷ suggests that the effect of potassium on YR binding at potassium concentrations ≤ 5 mM is insignificant compared to potassium-free buffers. Therefore, the differences in YR binding observed for sodium-containing and sodium-free buffers in this study, can rather not be attributed to the absence or presence of potassium in these buffers. As all buffers contained similar calcium concentrations (1.8–2.5 mM), this study allows no conclusions about the effect of calcium on YR ligand binding.

It should be noted that none of the reported X-ray/cryo-EM structures of Y₁R, Y₂R, and Y₄R (to date, no Y₅R structure has been reported) shows the presence of sodium ion(s) in the receptor, as reported in the PDB, although the receptor preparations used for crystallization or vitrification contained sodium in most cases.^{68–72} Consequently, the influence of sodium on receptor conformation is not addressed in the respective publications.

CONCLUSION

The neuropeptide Y receptor family and their endogenous ligands play several roles in numerous physiological processes, e.g., the regulation of anxiety, hormone release, stress, and food intake. This makes these receptors potential drug targets. Accordingly, in vitro studies of these receptors, such as the determination of ligand–receptor binding affinities, should be conducted in buffers containing sodium at a physiological concentration. However, for Y₁R, Y₂R, and Y₄R, receptor–ligand binding studies have been frequently performed in sodium-free buffers or buffers containing low sodium concentrations (≤ 30 mM). Since the binding of receptor agonists to class A GPCRs is often influenced by the absence or presence of sodium, a determination of binding affinities under sodium-free conditions might lead to a distorted picture of the binding profiles. To gain a comprehensive view of the YR binding profiles of the endogenous agonists NPY, PYY, and PP, the Y₁R, Y₂R, Y₄R, and Y₅R binding affinities of these peptides were determined in radiochemical competition binding assays in both sodium-free and sodium-containing buffers. For these studies, well-characterized tritium-labeled radioligands were used, and binding data were obtained in the same lab using well-established procedures. To the best of our knowledge, this study is the first of its kind. A major finding from this study is that for the different YR subtypes, the binding of agonists was influenced differently by the absence or presence of sodium, known to allosterically modulate the activation of GPCRs. However, the effect of sodium depends not only on the individual receptor subtype but also on the type of agonist binding to the receptor. Notably, the previously reported YR binding profiles of NPY, PYY, and PP were generally confirmed. Nonetheless, the relevance of these peptides for activating the individual YR subtypes in vivo may need to be re-evaluated based on the YR binding affinities determined in the present study. Beyond the YR family, the present study reinforces that GPCR–ligand binding studies should consistently be conducted in buffers containing sodium at a physiological concentration to avoid the generation of biased ligand binding profiles, unless the effect of sodium on receptor binding is of interest.

EXPERIMENTAL SECTION

Materials

Acetonitrile (MeCN) (HPLC gradient grade) was from VWR (Ismaning, Germany). Leibovitz's L-15 (L-15) without phenol red was purchased from Thermo Fisher Scientific (Nidderau, Germany). The peptides hPP, pNPY, hNPY, hPYY, and His-hPYY were purchased from Synpeptide (Shanghai, China). Bacitracin and bovine serum albumin (BSA) were obtained from Serva (Heidelberg, Germany). Glycine, dimethyl sulfoxide (DMSO), streptomycin, Geneticin (G418), L-glutamine, forskolin, trypsin, and Ham's F-12 medium were obtained from Sigma-Aldrich (Taufkirchen, Germany). Zeocin and hygromycin B were obtained from InvivoGen (Toulouse, France). The Y₂R antagonists BIIE0246 and JNJ31020028 were obtained from Boehringer Ingelheim (Ingelheim, Germany) and Cayman Chemical (Ann Arbor, USA), respectively. HATU and the Y₅R antagonist CGP71683A were obtained from BLD Pharmatech (Reinbek, Germany). Succinimidyl [³H]propionate (molar activity: 3.44 TBq/mmol) was purchased from Novandi (Södertälje, Sweden). The syntheses of the Y₁R antagonist BIBP3226,⁷³ the Y₁R antagonist BIBO3304,⁴⁹ [³H]UR-JG102,¹⁸ (molar activity: 3.44 TBq/mmol), [³H]UR-MK299²⁶ (molar activity: 3.885 TBq/mmol), and [³H]-[³H]propionyl]hPYY²⁶ (molar activity: 3.44 TBq/mmol) were described previously. Succinimidyl propionate was synthesized

according to a reported procedure.³⁰ The Y₄R antagonist UR-MK188 was synthesized as previously described⁶² with the following modifications: for the coupling of two equivalents of the argininamide moiety (amine (R)-24 in Keller et al.⁶²) to one equivalent of the central linker building block (dicarboxylic acid 15 in Keller et al.⁶²), HATU was used as coupling reagent together with DIPEA instead of TBTU/HOBt/triethylamine. The dicarboxylic acid (1.0 equiv) was preactivated with HATU (2.0 equiv) in the presence of DIPEA (3 equiv) at rt for 15 min. This was followed by the addition of the amine (2.0 equiv) and stirring at rt for 3 h instead of 60 °C (microwave) for 50 min.

Ultrapure water was used to prepare stock solutions, buffers, and eluents for HPLC. Polypropylene reaction vessels (1.5 and 2 mL) from Sarstedt (Nümbrecht, Germany) were used to keep stock solutions. For the preparation of dilution series of the peptides, siliconized (Sigmacote, Sigma) polypropylene reaction vessels were used.

Mass Spectrometry

High-resolution mass spectrometry (HRMS) was performed with an Agilent 6540 UHD accurate-mass Q-TOF LC/MS system coupled to an Agilent 1290 analytical HPLC system (Agilent Technologies, Santa Clara, CA) using an ESI source and the following LC method: column: Luna Omega C18, 1.6 μ m, 50 $\times$ 2.1 mm (Phenomenex, Aschaffenburg, Germany), column temperature: 40 °C, solvent/linear gradient: 0–4 min: 0.1% aqueous HCOOH/MeCN supplemented with 0.1% HCOOH 95:5–2:98, 4–5 min: 2:98, flow: 0.6 mL/min.

Preparative HPLC

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Gemini NX-C18, 5 μ m, 250 mm $\times$ 21 mm (Phenomenex) was used as reversed-phase (RP) column at a flow rate of 20 mL/min using mixtures of 0.1% aqueous TFA and MeCN as the mobile phase. A detection wavelength of 220 nm was used throughout. Collected fractions were lyophilized using a Scanvac CoolSafe 100-9 freeze-dryer (Labogene, Allerød, Denmark) equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany).

Analytical HPLC

Analytical HPLC analysis was performed with an 1100 series system from Agilent Technologies composed of a degasser (G1379A), a binary pump (G1312A), a variable wavelength detector (G1314A), a thermostated column compartment (G1316A), and an autosampler (G1329A). Detection was performed at 220 nm, and the oven temperature was 30 °C.

For the analysis of reaction mixtures and purity controls, a Kinetex XB C18 100A, 5 μ m, 250 $\times$ 4.6 mm (Phenomenex) served as stationary phase at a flow rate of 0.8 mL/min. Mixtures of 0.05% aqueous TFA (A) and MeCN (B) were used as the mobile phase. The following linear gradient was applied: 0–30 min: A/B 90:10–5:95, 30–40 min: 5:95 (isocratic). The injection volume was 80 μ L. Retention (capacity) factors *k* were calculated from the retention times *t_R* according to $k = (t_R - t_0)/t_0$ (*t₀* = dead time, 2.6 min for the system, column and flow rate mentioned above).

Synthesis of His-Tyr-Pro-Ile

-N^ε-Propionyl-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-Ala-Ser-Pro-Glu-Glu-Leu-Asn-Arg-Tyr-Tyr-Ala-Ser-Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gln-Arg-Tyr-Amide ([K⁵-Propionyl]His-hPYY) Heptakis(hydrotrifluoroacetate) (1)

The reaction was carried out in a 2 mL polypropylene reaction vessel. A solution of *N*-succinimidyl propionate (0.31 mg, 1.8 μ mol) in anhydrous DMF (7.6 μ L) was added in two portions (3.8 μ L + 3.8 μ L with a time lag of 2 min) to a stirred solution of His-hPYY (7.9 mg, 1.5 μ mol) and DIPEA (3.6 μ L, 21.1 μ mol) in DMF/NMP/H₂O 60:25:15 (200 μ L). The mixture was stirred at rt for 2 h. 10% aqueous TFA (17 μ L) was added, followed by isolation of the product by preparative HPLC (gradient: 0–30 min: 0.1% aqueous TFA/MeCN 90:10–60:40, *t_R* = 23 min) yielding 1 as a white fluffy solid (1.3 mg,

17%) (note: the number of seven TFA[−] counterions corresponds to the number of seven basic groups in the peptide; the correlation of the number of basic groups with the number of TFA[−] counterions was recently verified for [K⁴-propionyl]hPYY hexakis-(hydrotrifluoroacetate) by ¹⁹F NMR spectroscopy²⁶). HRMS (ESI): *m/z* [M+4H]⁴⁺ calcd. for [C₂₀₃H₃₁₀N₅₈O₅₉]⁴⁺ 1126.5771, found: 1126.5781. RP-HPLC (220 nm): 98% (*t_R* = 12.7 min, *k* = 3.9). C₂₀₃H₃₀₆N₅₈O₅₉•C₁₄H₇F₂₁O₁₄ (4503.03 + 798.14).

Synthesis of [K⁵-³H]Propionyl]His-hPYY Heptakis(hydrotrifluoroacetate) ([³H]1)

The radioligand [K⁵-³H]propionyl]His-hPYY ([³H]1) was prepared following a reported procedure for synthesizing [³H]propionylated neurotensin receptor ligands and was modified as required.⁷⁴ A solution of succinimidyl [³H]propionate (molar activity: 3.44 TBq/mmol; Novandi, Södertälje, Sweden) (77.7 MBq, 1.05 mL, 22.6 nmol) in *n*-heptane/EtOAc 3:2 v/v was transferred from the delivered ampule into a 1.5 mL polypropylene reaction vessel with screw cap, and the solvent was removed in a vacuum concentrator (rt, ca. 45 min). A solution of the precursor His-hPYY (0.29 mg, 56.8 nmol) and DIPEA (1.0 μ L, 5.73 μ mol) in DMF/NMP/H₂O 60:25:15 v/v/v (35 μ L) was added, immediately followed by vortexing. Subsequently, the vessel was shaken at rt for 2.5 h. The mixture was acidified by the addition of 2% aqueous TFA (30 μ L), followed by the addition of 0.05% aqueous TFA (200 μ L) and MeCN/0.05% aqueous TFA 1:9 v/v (150 μ L). The resulting solution was directly injected into the HPLC system (no filtration). [³H]1 was isolated using an HPLC system from Waters (Eschborn, Germany) consisting of two pumps 510, a pump control module, a manual injector (loop size: 200 μ L), a 486 UV/vis detector, and a Flow-one Beta series A-500 radiodetector (Packard, Meriden, USA) (the latter was disconnected during the purification process, i.e. fractions containing [³H]1 were collected based on UV detection). A Luna C18(2) column (3 μ m, 150 mm $\times$ 4.6 mm, Phenomenex, Aschaffenburg, Germany) was used as the stationary phase at a flow rate of 0.8 mL/min. Mixtures of MeCN supplemented with 0.04% TFA (A) and 0.05% aqueous TFA (B) were used as mobile phase. The following linear gradient was applied: 0–20 min: A/B 20:80–34:66, 20–22 min: 34:66–95:5, 22–30 min: 95:5 (isocratic). Four runs were conducted (for a representative chromatogram see Figure S4, Supporting Information). All fractions containing [³H]1 (*t_R* = 20.2 min) were collected and combined in a 2 mL polypropylene reaction vessel with a screw cap. The volume of the combined eluates was reduced by evaporation in a vacuum concentrator to approximately 350 μ L. EtOH (35 μ L) and a mixture of EtOH/water 1:9 v/v (420 μ L) were added, resulting in a mixing ratio of EtOH/aqueous solvent 1:9 v/v and a total volume of 800 μ L (preliminary stock solution). To determine the radiochemical purity and to prove the identity of [³H]1, 5 μ L of the preliminary stock solution were added to 100 μ L of a 20 μ M solution of [K⁵-propionyl]His-hPYY (1) in MeCN/0.05% aqueous TFA 1.5:8.5 v/v, affording the sample to be analyzed using the aforementioned HPLC system, column and solvents (injection volume: 100 μ L). The following linear gradient was applied: 0–20 min: A/B 15:85–42:58, 20–30 min: 42:58–95:5, 30–38 min: 95:5 (isocratic). The radiochemical purity was >99% (*t_R* = 17.2 min). To quantify the activity of [³H]1 and to determine the molar concentration, 2 $\times$ 2 μ L of the preliminary stock solution were added to 998 μ L of DMSO/H₂O 3:7 v/v and 4 $\times$ 10 μ L of these dilutions were counted in 3 mL of liquid scintillator (Rotiscint Eco Plus, Carl Roth, Karlsruhe, Germany) with a Tri-Carb 3100TR liquid scintillation counter (PerkinElmer). The activity concentration was adjusted to 11.10 MBq/mL by adding EtOH/H₂O 1:9 v/v (56.2 μ L), resulting in a final concentration of 3.23 μ M and a total volume of 847.2 μ L. Radiochemical yield: 9.40 MBq (0.25 mCi), 12.7%. Molar activity: As the supplier of the labeling reagent succinimidyl [³H]propionate (Novandi, Södertälje, Sweden) provides a precisely determined molar activity and as [³H]1 bears exactly one tritiated propionyl residue originating from the labeling reagent, the molar activity of [³H]1 was defined to be equal to the molar activity of the labeling reagent, amounting to 3.44 TBq/mmol (it is assumed that under the mild

reaction conditions the carbon-tritium bonds remained intact). The molar activity of the labeling reagent succinimidyl [^3H]propionate was determined by LC–MS analysis. Co-injection of “cold” succinimidyl propionate allowed the quantification of the incorporated tritium (Novandi, Södertälje, Sweden).

Cell Culture

Cells were cultured in T75 or T175 flasks (Sarstedt, Nümbrecht, Germany) in a humidified atmosphere (95% air, 5% CO_2) at 37 °C. SK-N-MC neuroblastoma cells (obtained from the American Type Culture Collection, ATCC HTB-10) were maintained in EMEM supplemented with 7.5% FBS (Pan Biotech; Aidenbach, Germany). CHO-hY₂R cells (obtained from PerkinElmer, Rodgau, Germany) were cultured in Ham's F-12 supplemented with 7.5% FBS and G418 (400 $\mu\text{g/mL}$). CHO-hY₄-Gq α -mtAEQ cells³² were cultured in Ham's F-12 supplemented with 7.5% FBS, hygromycin B (400 $\mu\text{g/mL}$), Zeocin (250 $\mu\text{g/mL}$), and G418 (400 $\mu\text{g/mL}$). HEC-1B-hY₅ cells³³ were maintained in EMEM supplemented with 10% FBS and G418 (400 $\mu\text{g/mL}$).

Buffers and Media used for Binding Assays

Buffer Ia (used for binding experiments at the Y₄R, sodium-free conditions): a hypotonic sodium-free 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (25 mM HEPES, 2.5 mM CaCl_2 , 1 mM MgCl_2 , pH 7.4 (adjusted using 25 mM aqueous ammonium hydroxide solution)) supplemented with 1% BSA (Serva, Heidelberg, Germany) and 0.1 mg/mL bacitracin (Serva).

Buffer Ib (used for binding experiments at the Y₁R, Y₂R, and Y₅R, sodium-free conditions): *buffer Ia* additionally supplemented with 0.3 M glycine (Sigma-Aldrich, Steinheim, Germany).

Buffer II (used for binding experiments at the Y₁R, Y₂R and Y₅R in the presence of sodium): an isotonic sodium-containing HEPES buffer (10 mM HEPES, 150 mM NaCl, 25 mM NaHCO_3 , 2.5 mM CaCl_2 , 5 mM KCl, pH 7.4) supplemented with 1% BSA and 0.1 mg/mL bacitracin.

DPBS (used for binding studies at the Y₄R in the presence of sodium): Dulbecco's phosphate-buffered saline with calcium and magnesium (1.8 mM CaCl_2 , 2.68 mM KCl, 1.47 mM KH_2PO_4 , 3.98 mM MgSO_4 , 137 mM NaCl, 8.06 mM Na_2HPO_4 , pH 7.4) supplemented with 1% BSA and 0.1 mg/mL bacitracin.

L-15 (used for the impedance assay): Leibovitz's L-15 medium without phenol-red (140 mM NaCl, 1.3 mM CaCl_2 , 1 mM MgCl_2 , various amino acids and vitamins, pH 7.4, Thermo Fischer Scientific, Nidderau, Germany).

Radioligand Binding Assays

All radioligand binding assays were performed with whole Y receptor-expressing cells at 23 ± 2 °C. The radioligands used for Y₁, Y₂, and Y₄ receptor binding assays were previously characterized (for K_d values, see Table S2, Supporting Information), except for Y₁R binding of the tritiated Y₁R ligand [^3H]UR-MK299 in DPBS and sodium-free buffer, and Y₂R binding of [^3H]propionylhPYY in DPBS and *buffer Ib*. For Y₅R binding studies, a new radioligand ([^3H]1), which was characterized by saturation binding prior to the competition binding assays, was used. All experiments were performed in triplicate.

For Y₁R binding experiments in *buffer Ib*, Y₂R binding experiments (DPBS, *buffer II*, *buffer Ib*), and Y₄R binding experiments (DPBS, *buffer Ia*, *buffer Ib*), the same filtration procedure for separating free radioligand from cell-bound radioligand and for measuring the activity of the latter was used: after completed incubation, cells were collected on GF/C filter mats (0.26 mm; Whatman, Maidstone, UK) (pretreated with 0.3% polyethylenimine for 30 min) using an in-house manufactured harvester for 96-well plates (precision mechanic workshop of the University of Regensburg, Regensburg, Germany), and the wells of the plate and the cells on the filter mat were immediately washed twice with ice-cold PBS (200 μL). Filter pieces for each well were punched out and transferred into flexible 96-well sample plates (part no. 1450-401; PerkinElmer, Rodgau, Germany) followed by the addition of Rotiscint Routine (Carl Roth, Karlsruhe, Germany) (200 μL). The plates were sealed with a transparent sealing tape (Greiner Bio-One EASYseal, part no. 676001; Greiner Bio-One,

Frickenhausen, Germany) and shaken in the dark for at least 3 h before measurement. Radioactivity (dpm) was measured with a MicroBeta2 plate counter equipped with six pairs of photomultiplier tubes (PerkinElmer, Rodgau, Germany).

Y₁R Binding

Competition binding assays at Y₁R-expressing SK-N-MC neuroblastoma cells in sodium-containing *buffer II*, using [^3H]UR-MK299 as radioligand, were performed as previously described with the minor modification of using a radioligand concentration of 0.075 nM instead of 0.15 nM.³⁰ Data were processed as described below (competition binding with [^3H]UR-MK299 in *buffer Ib*). Y₁R saturation and competition binding experiments with [^3H]UR-MK299 in DPBS were performed at intact SK-N-MC cells using the procedures reported for saturation and competition binding experiments with this radioligand in *buffer II*, but a radioligand concentration of 0.1 nM instead of 0.15 nM was used in the competition binding assay.³⁰ Data were processed as described below (saturation and competition binding with [^3H]UR-MK299 in *buffer Ib*). The obtained K_d value amounted to 0.084 nM (cf. Table S2, Supporting Information; for a representative saturation binding curve, see Figure S6A, Supporting Information).

Y₁R binding assays with [^3H]UR-MK299 in sodium-free *buffer Ib* were performed with suspended SK-N-MC cells. Cell suspensions were prepared as follows: cells were grown in T175 flasks to reach a confluency of 80–90% on the day of the experiment. The culture medium was removed, and the adherent cells were washed twice with *buffer Ib* (in this case, the buffer was not supplemented with BSA and bacitracin). Subsequently, the cells were covered with BSA- and bacitracin-free *buffer Ib* and scraped off the flask, followed by centrifugation at 300g at rt for 5 min. The supernatant was discarded and the cells were resuspended in *buffer Ib* at a density of 200,000–500,000 cells/mL. To determine the K_d value of [^3H]UR-MK299 for these conditions, saturation binding experiments were performed prior to competition binding assays.

Saturation Binding with [^3H]UR-MK299 in *Buffer Ib*. The wells of a 1450-401 96-well sample plates (PerkinElmer) were prefilled with freshly prepared cell suspension (160 μL) followed by the addition of 20 μL of *buffer Ib* (determination of total binding) or 20 μL of a 10-fold concentrated solution (compared to the final concentration) of BIBO3304 in *buffer Ib* (determination of nonspecific binding; the final concentration of BIBO3304 was 1000-fold higher than the radioligand concentration), and the addition of 20 μL of a 10-fold concentrated (compared to the final concentration) solution of [^3H]UR-MK299 in *buffer Ib*. Samples were incubated under shaking for 2 h followed by cell harvesting and further processing as described afore. Specific binding data, obtained by subtracting triplicate radioactivity mean values (dpm) of nonspecific binding from triplicate dpm mean values of total binding, were plotted against the free radioligand concentration and analyzed by a two-parameter equation describing hyperbolic single site binding (one-site, specific binding, GraphPad Prism 5) to obtain K_d values (cf. Table S2, Supporting Information; for a representative saturation binding curve, see Figure S6B, Supporting Information). The free concentration of [^3H]UR-MK299 (nM) was calculated by subtracting the amount of specifically bound [^3H]UR-MK299 (nM) (calculated from specifically bound radioligand in dpm, the molar activity and the volume per well) from the total concentration of [^3H]UR-MK299.

Competition Binding Assay with [^3H]UR-MK299 in *Buffer Ib*. The wells of a 1450-401 96-well sample plates (PerkinElmer) were prefilled with freshly prepared cell suspension (160 μL). Twenty μL of a 10-fold concentrated (relative to the final concentration) solution of the compound of interest in *buffer Ib*, and 20 μL of a 5 nM solution of [^3H]UR-MK299 in *buffer Ib* were added (final radioligand concentration: 0.5 nM). To determine total binding in the absence of a competitor, 20 μL of *buffer Ib* were added, followed by the addition of 20 μL of the aforementioned radioligand solution. To determine nonspecific binding, 20 μL of a 5 μM solution of BIBO3304 in *buffer Ib* (final concentration: 0.5 μM) and 20 μL of the aforementioned radioligand solution were added. Samples were

incubated under shaking for 2 h followed by cell harvesting and further processing as described afore.

Total binding data from competition binding experiments (dpm, including total binding in the absence of competitor) were plotted against $\log(\text{concentration competitor})$ and analyzed by a four-parameter logistic equation ($\log(\text{inhibitor})$ vs response-variable slope, GraphPad Prism 5, GraphPad Software, San Diego, CA, USA) followed by normalization (100% = "top" of the four-parameter logistic fit, 0% = nonspecifically bound radioligand) and analysis of the normalized data by a four-parameter logistic equation. Individual IC_{50} values were converted to K_i values according to the Cheng-Prusoff equation⁷⁵ followed by the conversion of individual K_i values to pK_i values and calculation of mean pK_i values.

Y₂R Binding

Competition binding experiments in sodium-containing *buffer II* were performed as reported using suspended CHO-hY₂R cells and the radioligand [K^4 -³H]propionyl]hPYY ($K_d = 0.16$ nM, $c = 0.4$ nM).²⁶ Nonspecific binding was determined in the presence of BIIE0246 and JNJ31020028 each 800 nM. Data were processed as described under *Y₁R binding*. Y₂R saturation and competition binding experiments with [K^4 -³H]propionyl]hPYY in DPBS were performed at intact CHO-hY₂R cells using the procedures reported for saturation and competition binding experiments with this radioligand in *buffer II*, but the buffer was replaced by DPBS.²⁶ Data were processed as described under *Y₁R binding*. The obtained K_d value amounted to 0.17 nM (cf. Table S2, Supporting Information; for a representative saturation binding curve, see Figure S6C, Supporting Information).

Competition binding experiments in sodium-free *buffer Ib* were performed at suspended CHO-hY₂R cells with the radioligand [K^4 -³H]propionyl]hPYY ($K_d = 0.078$ nM $c = 0.12$ nM) according to a reported procedure, but the formerly used buffer (*buffer Ia*) was replaced by *buffer Ib*.²⁶ Nonspecific binding was determined in the presence of BIIE0246 and JNJ31020028 each 240 nM. Prior to the competition binding studies, the K_d value of [K^4 -³H]propionyl]hPYY was determined by saturation binding at CHO-hY₂R cells in *buffer Ib* following the reported procedure for saturation binding experiments with [K^4 -³H]propionyl]hPYY²⁶ with the following modifications: *buffer Ib* was used instead of *buffer Ia* and nonspecific binding was determined in the presence of BIIE0246 and JNJ31020028 each 800 nM. Data were processed as described under *Y₁R binding* yielding an average K_d of 0.078 nM (cf. Table S2, Supporting Information; for a representative saturation binding curve, see Figure S6D, Supporting Information).

Y₄R Binding

Competition binding experiments were performed in *buffer Ia* and DPBS as reported using CHO-hY₄-G_{q15}-mtAEQ cells³² and the radioligand [³H]UR-JG102 (*buffer Ia*: $K_d = 0.012$ nM, concentration: 0.030 nM; DPBS: $K_d = 0.11$ nM, concentration: 0.25 nM).¹⁰ Data were processed as described under *Y₁R binding*. Saturation binding experiments with [³H]UR-JG102 at CHO-hY₄-G_{q15}-mtAEQ cells in isotonic *buffer Ib* (performed to study a potential effect of the hypotonicity of *buffer Ia* on Y₄R binding) were conducted as reported saturation binding experiments with [³H]UR-JG102 at CHO-hY₄-G_{q15}-mtAEQ cells in *buffer Ia*, but *buffer Ia* was replaced by *buffer Ib*. Data were processed as described under *Y₁R binding* yielding an average K_d of 0.017 nM (cf. Table S2, Supporting Information; for a representative saturation binding curve, see Figure S7A, Supporting Information).

Y₅R Binding

Binding studies at Y₅R were performed with adherent HEC-1B-hY₅R cells.³³ Competition binding experiments in the sodium-containing buffers DPBS and *buffer II* were performed in white 96-well plates with transparent flat bottom (catalog no. 732–3740, VWR, Ismaning, Germany) as previously described, but [K^4 -³H]propionyl]hPYY was used as radioligand instead of [K^4 -³H]propionyl]pNPY and Ultima Gold (PerkinElmer) was used as scintillator instead of Optiphase Supermix (PerkinElmer).⁴⁶ The previously used incubation time of 2 h was considered appropriate since Y₅R binding of a ¹²⁵I-labeled NPY

derivate was shown to reach equilibrium within ca. 90 min.²⁵ Prior to the conduction of competition binding experiments, the K_d values of [K^4 -³H]propionyl]hPYY were determined by saturation binding under the same assay conditions (nonspecific binding was determined in the presence of 4 μ M CGP71683A, the incubation time was 2 h). Data were processed as described above for saturation binding experiments with [³H]UR-MK299 at SK-N-MC cells in *buffer Ib*. The obtained K_d values amounted to 14 nM (DPBS) and 9.5 nM (*buffer II*) (cf. Table S2, Supporting Information; for representative saturation binding curves, see Figure S7B,C, Supporting Information).

For competition binding experiments, a final concentration of 10 nM of [K^4 -³H]propionyl]hPYY was used. Nonspecific binding was determined in the presence of 2 μ M CGP71683A. Data were processed as described under *Y₁R binding*.

For Y₅R binding studies in sodium-free *buffer Ib*, the radioligand [³H]1 was used. The binding experiments were performed in transparent 24-well plates (catalog no. 662160, Greiner Bio-One, Frickenhausen, Germany) because this gave a markedly higher ratio of total over nonspecific binding compared to experiments performed in white 96-well plates with transparent bottom (data of the latter are not shown). Cells were seeded in the plates 1 day prior to the experiment. On the day of the experiment, with cells at a confluence of 80–100%, the culture medium was aspirated, and the cells were washed with PBS (500 μ L), followed by prefilling the wells with 160 μ L of *buffer Ib*. For saturation binding experiments, 20 μ L of *buffer Ib* (determination of total binding) or 20 μ L of an 80 μ M solution of CGP71683A (final concentration: 8 μ M) in *buffer Ib* (determination of nonspecific binding), and 20 μ L of a 10-fold concentrated (compared to the final concentration) solution of [³H]1 in *buffer Ib* were added. In the case of competition binding experiments, 20 μ L of a 10-fold concentrated (compared to the final concentration) solution of the compound of interest in *buffer Ib*, and 20 μ L of a 150 nM solution of [³H]1 in *buffer Ib* (final concentration: 15 nM) were added. To determine total binding in the absence of a competitor, 20 μ L of *buffer Ib* were added followed by the addition of 20 μ L of the aforementioned 150 nM radioligand solution. To determine nonspecific binding, 20 μ L of a 30 μ M solution of CGP71683A in *buffer Ib* (final concentration: 3 μ M) and 20 μ L of the aforementioned 150 nM radioligand solution were added.

Samples were incubated under shaking for 2 h. The binding buffer was aspirated, the cells were washed twice with ice-cold PBS (2 $\times$ 200 μ L), and lysis solution (urea (8 M), acetic acid (3 M) and Triton-X-100 (1%) in water) (200 μ L) was added. The plates were shaken at rt for 30 min and the lysis solutions were transferred into 6 mL scintillation vials filled with 3 mL of liquid scintillator (Rotiszint Routine; Carl Roth, Karlsruhe, Germany). Radioactivity (dpm) was measured with a Beckman LS6500 liquid scintillation counter. Data from saturation and competition binding experiments were processed as described under *Y₁R binding*.

Ca²⁺ Aequorin Assay

The assay was performed in agonist mode using a modified procedure reported by Ziemek et al.³² CHO-hY₄-G_{q15}-mtAEQ cells of a T175 flask (80%–90% confluency) were trypsinized and detached with Ham's F12 supplemented with 7.5% FCS. After centrifugation at 300g for 5 min, the cells were resuspended in phenol red-free DMEM (Sigma) supplemented with 1% FBS at a density of 1 $\times$ 10⁷ cells/mL. Coelenterazine h (Biotrend, Köln, Germany) (1 mM stock solution in methanol) was added to a final concentration of 2 μ M and the cell suspension was gently stirred at rt in the dark for 2 h. Subsequently, the cell suspension was diluted with loading buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1.5 mM CaCl₂, and 10 mM glucose, pH 7.4) to a density of 5 $\times$ 10⁵ cells/mL followed by gentle stirring at rt in the dark for 3 h. After this incubation, the cells were immediately used for sample preparation and measurement. All experiments were performed in triplicate.

90 μ L of a 2-fold concentrated (relative to the final concentration) solution of the compound of interest in assay buffer (obtained by supplementing loading buffer with 1% BSA and 100 μ g/mL bacitracin) added to the empty wells of a white 96-well plate

(Brand 781605, Wertheim, Germany). To determine the maximal response and the basal effect in the absence of agonist (blank controls), wells were pre-filled with 90 μL of a 2 μM solution of hPP in assay buffer (final concentration: 1 μM) and with neat assay buffer (90 μL), respectively. After prefilling the plate, it was subjected to a preheated (27 $^{\circ}\text{C}$) LB 942 Tristar 3 multimode microplate reader (Berthold, Bad Wildbad, Germany) equipped with two JET injectors. Kinetic measurements were performed well by well using the following instrument settings (Light Compass software v6.20 basic addition): plate speed: medium, number of repeats: 1, cycle time: 10 s, temperature control: 27 $^{\circ}\text{C}$. The protocol was composed of three kinetics and two dispense steps (kinetic—dispense—kinetic—dispense—kinetic). The kinetic settings were as follows: technology: luminescence, delay before measurement: Off, counting time: 0.2 s, total measurement times for the individual kinetics: 1 s (first kinetic), 43 s (second kinetic), 22 s (third kinetic), delay time: Off, emission filter: no filter.

In each sample well, 90 μL of the cell suspension (kept in the dark under gently stirring using a magnetic stirrer) were injected (first injection, injection speed: slow) immediately followed by measurement of luminescence for 43 s to record the agonist-induced signal (peak 1). Subsequently, 20 μL of a 1% Triton-X-100 solution in assay buffer were injected (second injection, injection speed: moderate) to permeabilize the cells and discharge remaining aequorin, and luminescence was measured for additional 22 s to record peak 2. Areas under the curves (peaks 1 and 2) were calculated using GraphPad Prism 5. Fractional luminescences, obtained by dividing the area of peak 1 by the total area (sum of the areas of peak 1 and peak 2), were normalized (100% = fractional luminescence obtained from 1 μM hPP, 0% = basal effect obtained in the absence of agonist) and normalized responses were plotted against log(agonist concentration). In the case of hPP, data of individual experiments were fitted according to a four-parameter logistic equation (log(agonist) vs response-variable slope, GraphPad Prism 5) to obtain pEC_{50} values. Mean pEC_{50} values $\pm$ SEM were calculated from the pEC_{50} values of individual experiments. As UR-MK188 elicited no effect, data of this compound were not fitted (cf. Figure S9).

Impedance Assay

To perform impedance measurements, 96-well electrode arrays with circular gold-film electrodes (SP-96 CardioExcyte Sensor Plates, electrode diameter 0.6 mm, growth area: 34 mm^2) were first sterilized in argon plasma (Argon Plasma Cleaner PDC 002, Harrick Plasma, Ithaca, US) for 30 s and subsequently preincubated with 50 μL Ham's F-12 medium for 2 h. The medium from a T75 flask with CHO-hY₄-G_{q15}-mtAEQ cells³² of confluency 80–90% was aspirated, and a cell suspension in Ham's F-12 of density 340,000 cells/mL was prepared. 100 μL of the cell suspension were added to each well, approximately resulting in a cell density of 100,000 cells per cm^2 . The cells were allowed to sediment for 15 min, after which the plates were placed in the incubator for 48 h, with a medium exchange after 24 h.

On the day of the experiment, the culture medium was aspirated, and the cells were washed with *L-15*. Each well was filled with 50 μL of *L-15* to record a stable impedance baseline at 12 kHz. The impedance was measured inside the CardioExcyte 96 tabletop incubator/measurement chamber (Nanon Technologies Inc., Munich, Germany), designed for 96-well electrode arrays. It was coupled to a heating and ventilation system as well as a gas mixer (ibidi, Gräfelfing, Germany) to maintain 37 $^{\circ}\text{C}$, 0% CO_2 , and 80% humidity.

Cells were then prestimulated with forskolin (50 μL /well, dissolved in *L-15*) at a final concentration of 0.4 μM . The test ligands (hPP and UR-MK188) were diluted to obtain two times the final concentrations in *L-15*, which contained 0.4 μM forskolin. 100 μL of this solution were added to each well after the forskolin prestimulation signal was stable. For the assay controls, 0.2 μM of hPP (yielding a final concentration of 0.1 μM) and a solution of 0.01% (v/v) DMSO were added as positive and vehicle control, respectively. All experiments were conducted in *L-15* medium and in triplicate. Data were collected using the software SpectraControl (Nanon Technologies Inc., Munich, Germany), which also allowed setting the measurement

frequency of the AC to 12 kHz with a noninvasive driving voltage of 40 mV. The data acquired was first baseline corrected by subtracting the baseline measurements from the agonist stimulation measurement. The area under the curves (AUC) of observation time between 1200 s - 2400 s of the baseline-corrected data were then calculated using GraphPad Prism 10. Resulting AUCs were normalized (100% = AUC obtained from 0.1 μM hPP, 0% = AUC obtained by vehicle addition (basal effect), GraphPad Prism 10). The normalized responses were then plotted against log (concentration of agonist), and the data were fitted using a four-parameter logistic equation (log(agonist) vs response—variable slope, GraphPad Prism 10) to obtain pEC_{50} values, which were converted to EC_{50} values.

Statistical Significance

Statistical tests (Welch *t*-test, GraphPad Prism 5) for the binding affinities were performed based on pK_i values. The significance level was set to $P \leq 0.05$.

Calculation of Propagated Errors

Propagated errors (applying to specifically bound radioligand calculated from saturation binding data) were calculated as described elsewhere.¹⁰

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspstsci.6c00127>.

Synthesis of [K^5 -propionyl]His-hPYY (1) and [K^5 - ^3H]propionyl]His-hPYY (^3H 1) (Scheme S1); structures of the radioligands used in the present study (Figure S1); structures of the studied YR antagonists (Figure S2); RP-HPLC chromatograms of crude ^3H 1 (UV detection at 220 nm and radiometric detection) (Figure S3); RP-HPLC chromatogram of the micro-preparative workup of ^3H 1 (Figure S4); radiochemical purity of ^3H 1 determined by RP-HPLC analysis directly and 24 months after synthesis (Figure S5); representative saturation binding isotherms from saturation binding experiments with ^3H UR-MK299 (Y₁R, DPBS, buffer Ib) and [K^4 - ^3H]propionyl]hPYY (Y₂R, DPBS, buffer Ib) (Figure S6); representative saturation binding isotherms from saturation binding experiments with ^3H UR-JG102 (Y₄R, buffer Ib), [K^4 - ^3H]propionyl]hPYY (Y₅R, DPBS, buffer II), and ^3H 1 (Y₅R, buffer Ib) (Figure S7); radioligand displacement curves from competition binding experiments at Y₁R, Y₂R and Y₅R performed in sodium-containing buffer II (Figure S8); Ca^{2+} aequorin assay data of UR-MK188 studied in agonist mode (Figure S9); overview of reported potencies of the endogenous YR agonists (Table S1); summary of the binding affinities (K_d values) of the radioligands used in the competition binding assays (Table S2); YR binding affinities of the endogenous agonists pNPY, hNPY, hPYY, and hPP as well as subtype-selective antagonists determined in DPBS and buffer II (Table S3); overview of buffer conditions of reported Y₄R binding studies (Table S4) (PDF)

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Author Contributions

A.O.G. and F.S. conducted the radioligand binding assays. M.K., F.S., and T.B. performed the syntheses. J.E. performed the impedance assays. M.K. initiated and planned the project. M.K., P.K., and J.W. supervised the research. A.O.G. and M.K. with the help of all coauthors wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Susanne Bollwein, Maria Beer-Krön, Susanne Deinert, and Brigitte Wenzl for excellent technical assistance, and Prof. Christina Ziegler and Gregor Madej for evaluating the data of YR x-ray structures with respect to the identification of sodium and its binding sites within these receptors. The project was supported by the Deutsche Forschungsgemeinschaft (DFG) (GRK1910, research grant KE 1857/1-3).

ABBREVIATIONS USED

BSA	bovine serum albumin
CHO	chinese hamster ovary
DIPEA	<i>N,N</i> -diisopropylethylamine
FBS	fetal bovine serum
HATU	1-[bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo-[4,5- <i>b</i>]pyridinium 3-oxid hexafluorophosphate
HEC	human endometrial cancer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hNPY	human neuropeptide Y
hPP	human pancreatic polypeptide
hPYY	human peptide YY
hY _x R	human NPY Y _x receptor

<i>k</i>	retention (or capacity) factor (HPLC)
PBS	phosphate-buffered saline
p <i>K</i> _i	negative decadic logarithm of the dissociation constant <i>K</i> _i (in M) obtained from a competition binding experiment
pIC ₅₀	negative logarithm of the inhibition concentration IC ₅₀ (in M) obtained from a competition binding experiment
<i>K</i> _d	equilibrium dissociation constant (in M) obtained from a saturation binding experiment
pNPY	porcine neuropeptide Y
pPYY	porcine peptide YY
RP	reversed phase
SEM	standard error of the mean
<i>t</i> _R	retention time
YR	NPY receptor.

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