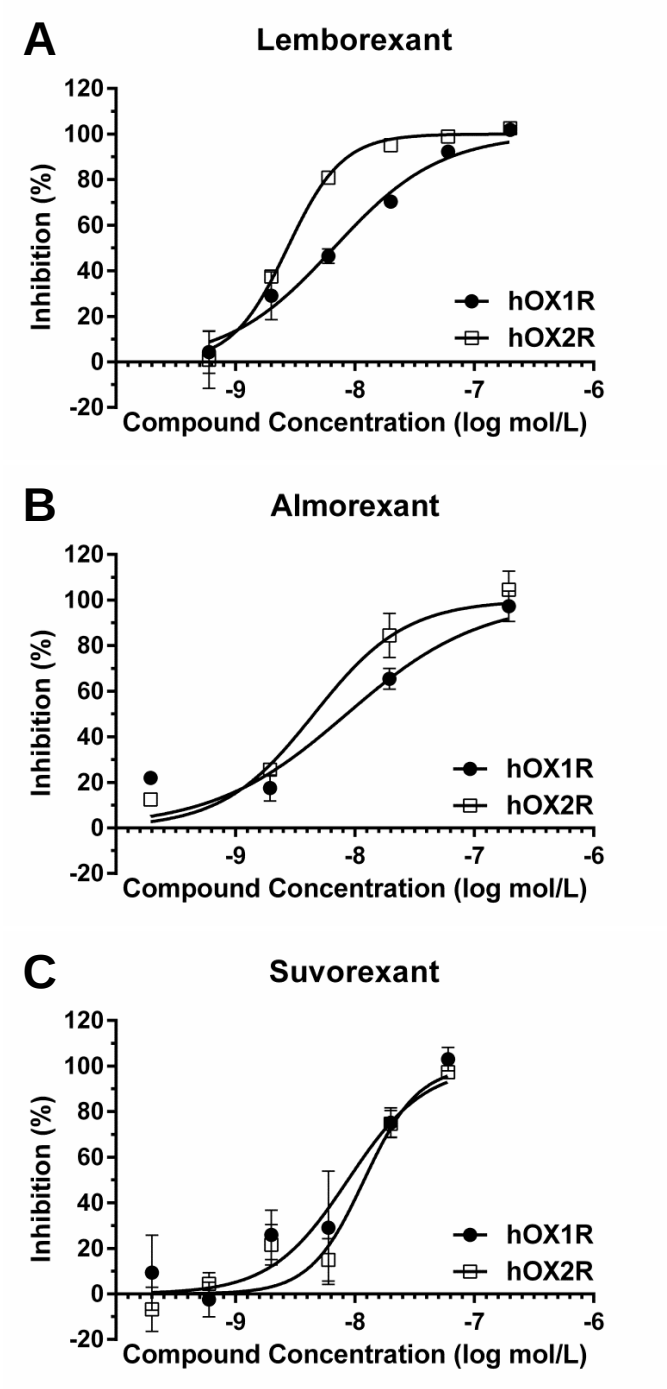
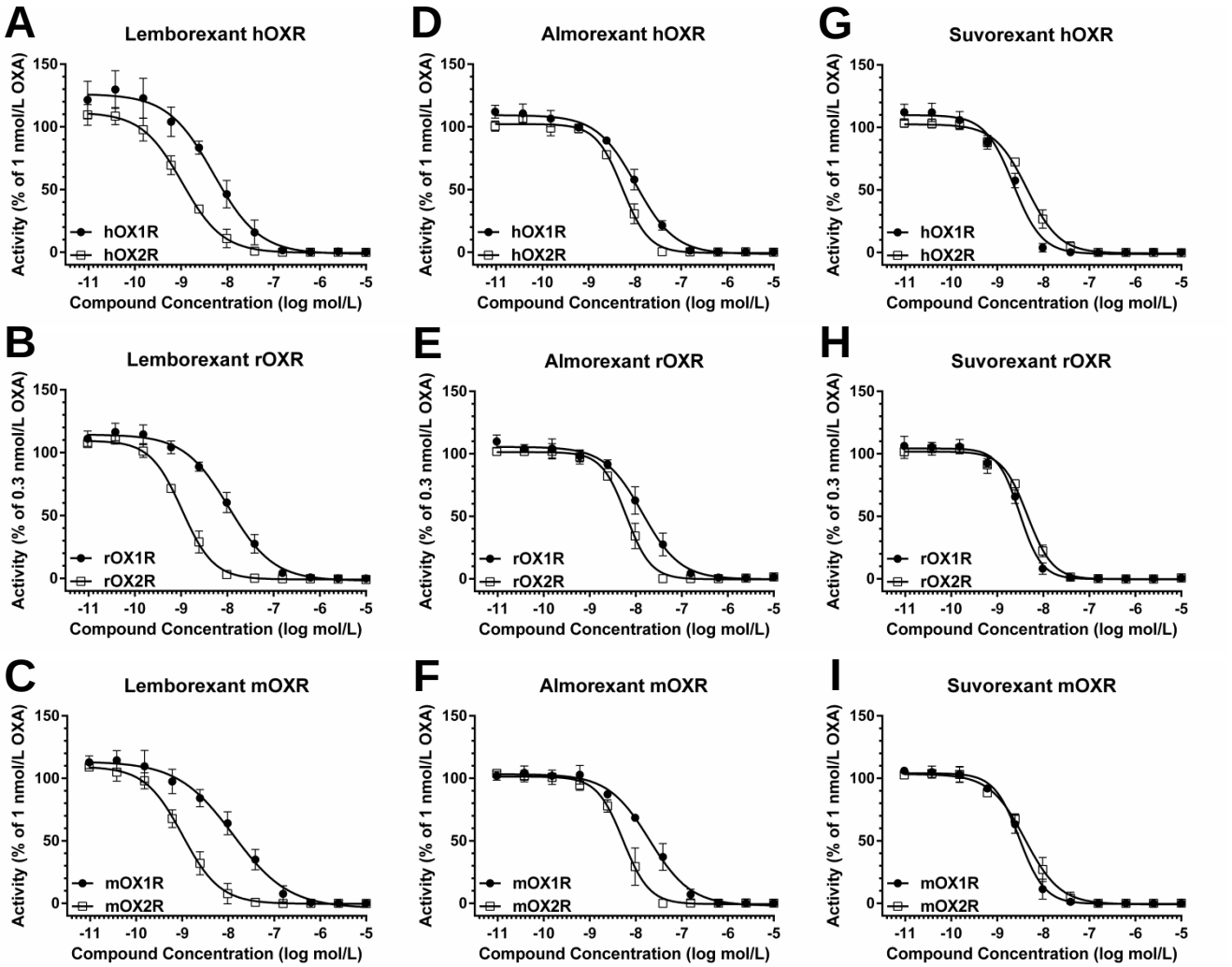
 Carsten Theodor Beuckmann, Michiyuki Suzuki, Takashi Ueno, Kazuya Nagaoka, Tohru Arai, Hiroyuki Higashiyama: In Vitro and *In Silico* Characterization of Lemborexant (E2006), a Novel Dual Orexin Receptor Antagonist. The Journal of Pharmacology and Experimental Therapeutics

# Supplemental Figure 1



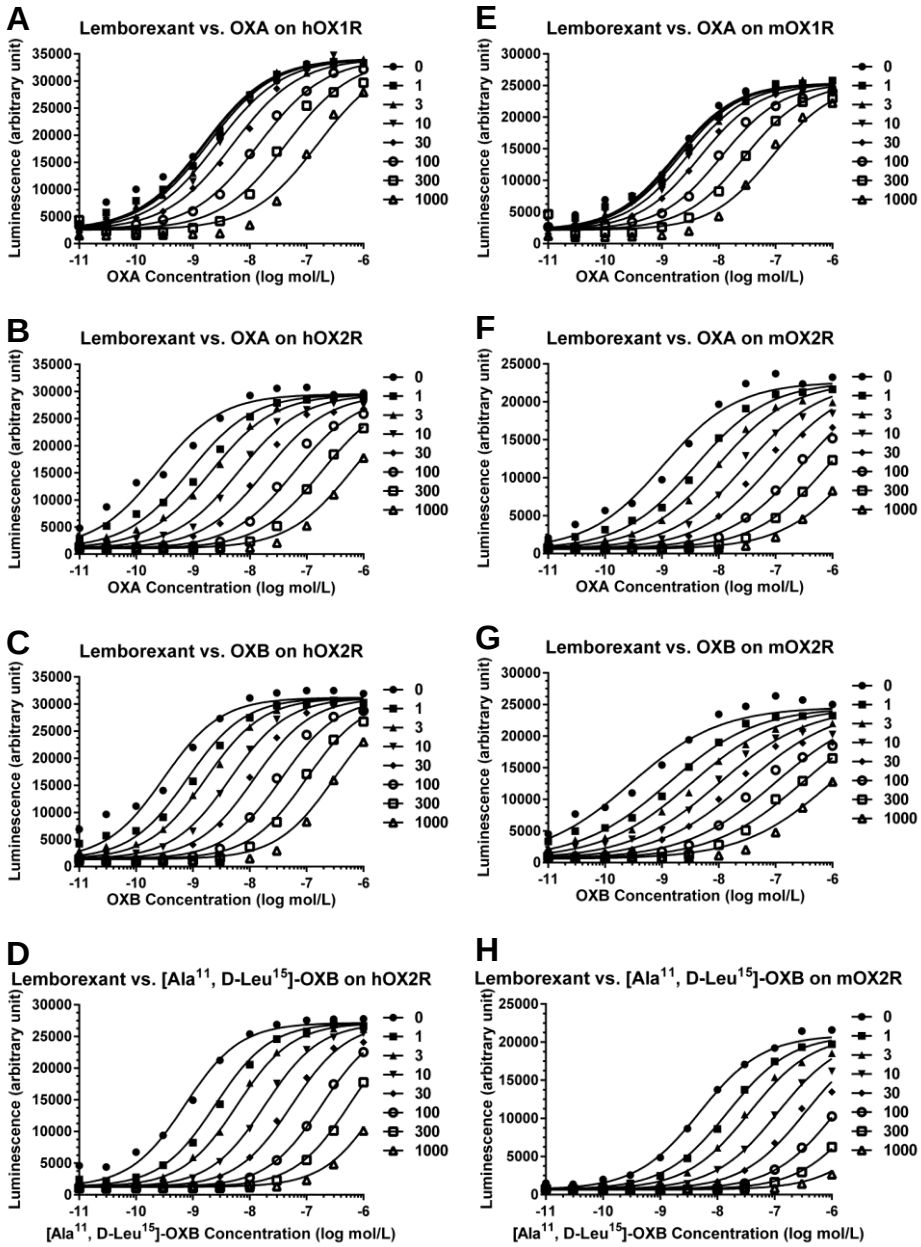

**Supplemental Figure 1: Inhibition curves of radiolabelled OXA binding by OXR antagonists lemborexant (A), almorexant (B), and suvorexant (C).** Cell membrane-bound radioactivity without antagonist addition was defined as 0% inhibition, while membrane-bound radioactivity after addition of 10 μmol/L unlabeled OXA was defined as non-specific binding and 100% inhibition.

## Supplemental Figure 2




**Supplemental Figure 2: Inhibition curves** of lemborexant (A-C), almorexant (D-F), and suvorexant (G-I) from direct $Ca^{2+}$-imaging assay of OXR antagonists on OX1R and OX2R of human (A, D, G), rat (B, E, H), and mouse (C, F, I). Activity in the absence of OXA is defined as 0%, activity of 1 nmol/L OXA (for human and mouse receptors) or 0.3 nmol/L (for rat receptors) is defined as 100%. OXR, orexin receptor.

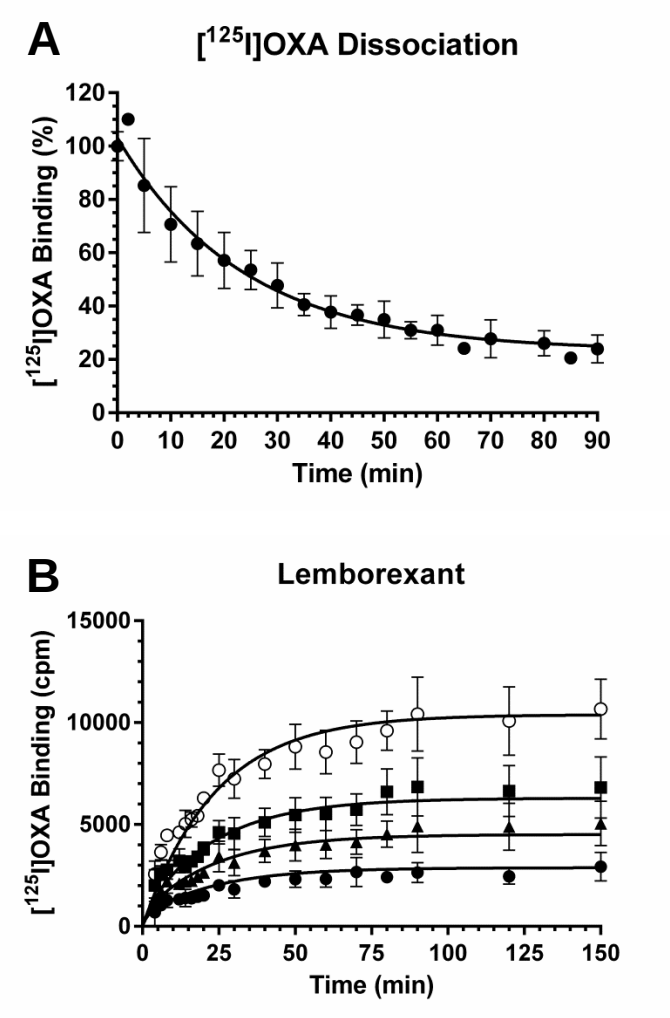
# Supplemental Figure 3




**Supplemental Figure 3: Dextral shift of three distinct orexin peptides' activity dose-response curves by titration with lemborexant.**

Orexin peptides used were orexin-A (OXA; A, B, E, F), orexin-B (OXB; C, G) and [Ala$^{11}$, D-Leu$^{15}$]OXB (D, H) on human orexin-1 receptor (hOX1R; A), human orexin-2 receptor (hOX2R; B-D), mouse orexin-1 receptor (mOX1R; E), and mouse orexin-2 receptor (mOX2R; F-H). Each data point represents the mean of quadruplicate measurements. Legends indicate concentration of lemborexant in nmol/L.

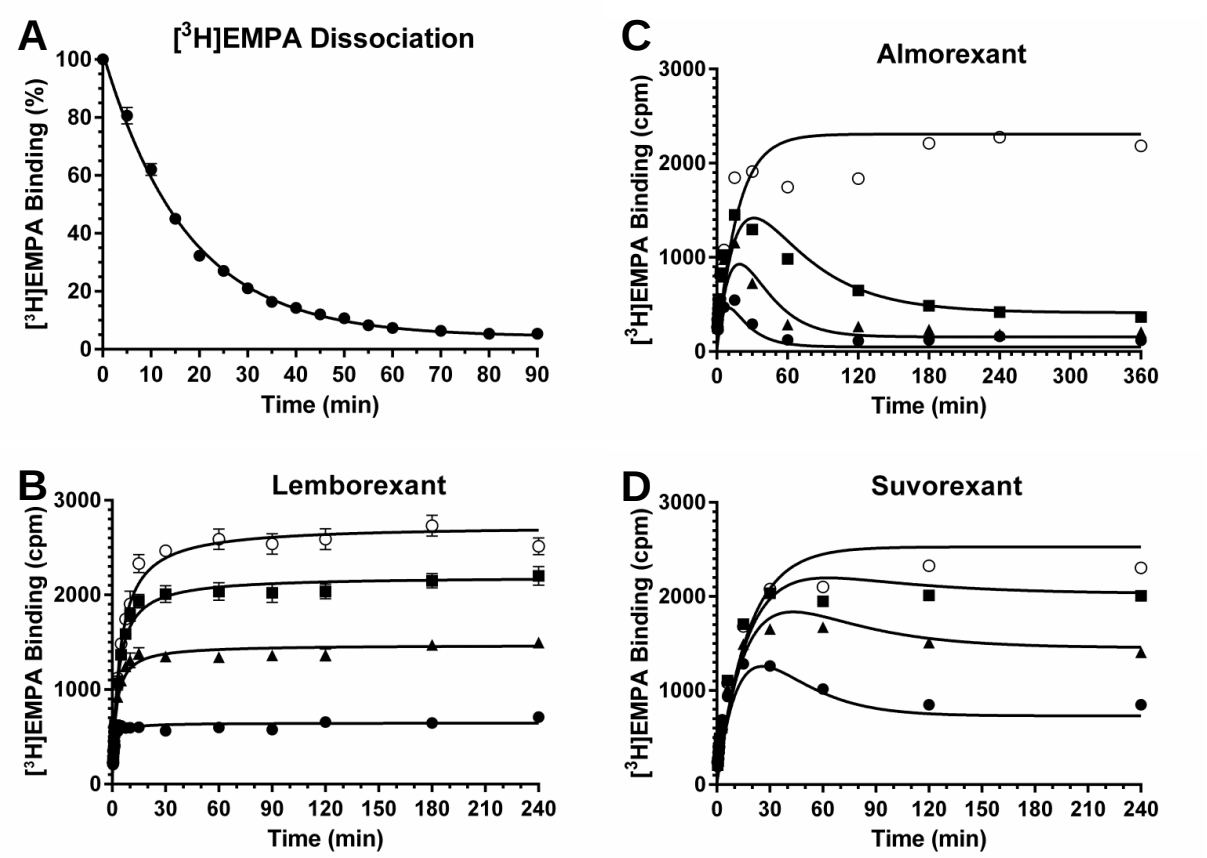
## Supplemental Figure 4




**Supplemental Figure 4: Dissociation of [$^{125}$I]OXA from and competition kinetics against lemborexant for [$^{125}$I]OXA association to the human OX1R.**

Data points represent the mean ± SEM from three independent experiments performed in triplicate values. A: Dissociation of [$^{125}$I]OXA expressed as percentage of maximum binding. B: Competition association curves of [$^{125}$I]OXA in the presence of increasing concentrations of lemborexant. (open circles: absence of lemborexant; closed squares 7 nmol/L; closed triangles: 14 nmol/L; closed circles 28 nmol/L lemborexant). cpm, counts per minute.

# Supplemental Figure 5




**Supplemental Figure 5: Dissociation of [$^3$H]EMPA from and competition kinetics against three DORAs for [$^3$H]EMPA association to the human OX2R.**

Data points represent the mean ± SEM from three independent experiments performed in triplicate values (A, B) and the mean from one experiment performed in triplicate values (C, D).

A: Dissociation of [$^3$H]EMPA expressed as percentage of maximum binding. B, C, and D: Competition association curves of [$^3$H]EMPA in the presence of increasing concentrations of lemborexant, almorexant, and suvorexant, respectively (open circles: absence of antagonist; closed squares 1 nmol/L; closed triangles: 3 nmol/L; closed circles 10 nmol/L antagonist). cpm, counts per minute.

**Supplemental Figure 6:** Three-dimensional homology model of lemborexant bound to human orexin-1 receptor (pdb format)

**Supplemental Figure 7:** Three-dimensional homology model of lemborexant bound to human orexin-2 receptor (pdb format)

**Supplemental Table 1:** summary results of panel binding assay of lemborexant (at 1 and 10 μmol/L)

| **Assay**<br>target (specification) (radioligand)<br>(h = human) | % inhibition of control specific binding at 1 μmol/L | % inhibition of control specific binding at 10 μmol/L |
|---|---|---|
| $A_1$ (h) (antagonist DPCPX) | -26 | 1 |
| $A_{2A}$ (h) (agonist NECA) | 8 | 17 |
| $A_3$ (h) (agonist IB-MECA) | -30 | -27 |
| $\alpha_1$ (non-selective) (antagonist prazosin) | 0 | 6 |
| $\alpha_2$ (non-selective) (antagonist yohimbine) | 9 | 13 |
| $\beta_1$ (h) (agonist atenolol) | -8 | 3 |
| $\beta_2$ (h) (agonist ICI 118551) | 2 | 6 |
| $AT_1$ (h) (antagonist saralasin) | -12 | -28 |
| $AT_2$ (h) (agonist angiotensin-II) | 1 | 13 |
| BZD (central) (agonist diazepam) | -1 | -13 |
| BZD (peripheral) (antagonistPK 11195) | -18 | 2 |
| BB (non-selective) (agonist bombesin) | -9 | 7 |
| $B_2$ (h) (agonist NPC 567) | 7 | 3 |
| CGRP (h) (agonist hCGRPα) | -13 | -11 |
| $CB_1$ (h) (agonist CP 55940) | -6 | 3 |
| $CCK_1$ ($CCK_A$) (h) (agonist CCK-8s) | -11 | -7 |
| $CCK_2$ ($CCK_B$) (h) (agonist CCK-8s) | -6 | -3 |
| $CRF_1$ (h) (agonist sauvagine) | -14 | -16 |
| $CRF_{2\alpha}$ (h) (agonist sauvagine) | -9 | -6 |
| $D_1$ (h) (antagonist SCH 23390) | 21 | 15 |
| $D_{2S}$ (h) (antagonist (+)butaclamol) | 6 | 6 |
| $D_3$ (h) (antagonist (+)butaclamol) | -4 | -2 |
| $D_{4,4}$ (h) (antagonist clozapine) | -3 | -1 |
| $D_5$ (h) (antagonist SCH 23390) | -11 | -14 |

**Supplemental Table 1 (continued)**

| | | |
|---|---|---|
| $ET_A$ (h) (agonist endothelin-1) | -9 | -8 |
| $ET_B$ (h) (agonist endothelin-3) | -19 | -16 |
| GABA (non-selective) (agonist GABA) | -7 | 12 |
| $GAL_1$ (h) (agonist galanin) | -10 | -26 |
| $GAL_2$ (h) (agonist galanin) | 7 | -9 |
| PDGF (agonist PDGF BB) | 1 | 9 |
| CXCR2 (IL-8B) (h) (agonist IL-8) | 6 | -8 |
| TNF-α (h) (agonist TNF-α) | 3 | -1 |
| CCR1 (h) (agonist MIP-1α) | 1 | -4 |
| $H_1$ (h) (antagonist pyrilamine) | -1 | -2 |
| $H_2$ (h) (antagonist cimetidine) | -15 | -21 |
| $MC_4$ (h) (agonist NDP-α-MSH) | -21 | -19 |
| $MT_1$ ($ML_{1A}$) (h) (agonist melatonin) | 21 | **74** |
| $M_1$ (h) (antagonist pirenzepine) | -4 | -7 |
| $M_2$ (h) (antagonist methoctramine) | -17 | -6 |
| $M_3$ (h) (antagonist 4-DAMP) | 0 | -17 |
| $M_4$ (h) (antagonist 4-DAMP) | -14 | -40 |
| $M_5$ (h) (antagonist 4-DAMP) | -10 | -8 |
| $NK_1$ (h) (agonist [$Sar^9$,$Met(O_2)^{11}$]-SP | 1 | 38 |
| $NK_2$ (h) (agonist [$Nleu^{10}$]-NKA (4-10)) | 7 | 22 |
| $NK_3$ (h) (antagonist SB 222200) | 7 | 8 |
| $Y_1$ (h) (agonist NPY) | 0 | -1 |
| $Y_2$ (h) (agonist NPY) | -3 | 2 |
| $NTS_1$ ($NT_1$) (h) (agonist neurotensin) | -7 | -25 |
| N neuronal α4β2 (h) (agonist nicotine) | 20 | 5 |
| opioid (non-selective) (antagonist naloxone) | -4 | 9 |
| $\delta_2$ (DOP) (h) (agonist DPDPE) | 6 | 21 |
| κ (KOP) (h) (agonist U 50488) | 4 | 35 |
| μ (MOP) (h) (agonist DAMGO) | 5 | 18 |
| NOP (ORL1) (h) (agonist nociceptin) | 11 | -2 |
| $PAC_1$ (PACAP) (h) (agonist $PACAP_{1-38}$) | -18 | -12 |
| $PPAR_\gamma$ (h) (agonist rosiglitazone) | -5 | -14 |
| PCP (antagonist MK 801) | -12 | -21 |
| $DP_1$ (h) (agonist BW245C) | -8 | 3 |
| $EP_1$ (h) (agonist $PGE_2$) | -9 | -11 |

**Supplemental Table 1 (continued)**

| | | |
|---|---|---|
| $EP_2$ (h) (agonist $PGE_2$) | -4 | 6 |
| $EP_3$ (h) (agonist sulprostone) | -8 | -4 |
| $EP_4$ (h) (agonist $PGE_2$) | 2 | 1 |
| TP ($TXA_2$/$PGH_2$) (h) (antagonist U 44069) | -4 | -4 |
| IP ($PGI_2$) (h) (agonist iloprost) | -7 | -1 |
| P2X (agonist α,β-MeATP) | -10 | -17 |
| P2Y (agonist dATPαS) | 12 | 13 |
| 5-$HT_{1A}$ (h) (agonist 8-OH-DPAT) | 2 | 10 |
| 5-$HT_{1B}$ (h) (agonist serotonin) | -13 | -16 |
| 5-$HT_{2A}$ (h) (antagonist ketanserin) | 13 | -3 |
| 5-$HT_{2B}$ (h) (agonist (±)DOI) | -9 | -9 |
| 5-$HT_{2C}$ (h) (antagonist RS 102221) | -4 | -9 |
| 5-$HT_3$ (h) (antagonist MDL 72222) | -6 | 4 |
| 5-$HT_{5a}$ (h) (agonist serotonin) | -10 | 0 |
| 5-$HT_6$ (h) (agonist serotonin) | 8 | 6 |
| 5-$HT_7$ (h) (agonist serotonin) | -2 | 10 |
| σ (non-selective) (agonist haloperidol) | 5 | 4 |
| sst (non-selective) (agonist somatostatin-14) | -3 | -2 |
| GR (h) (agonist dexamethasone) | -10 | -10 |
| $VPAC_1$ ($VIP_1$) (h) (agonist VIP) | -9 | -6 |
| $V_{1a}$ (h) (agonist [d$(CH_2)_5^1$,Tyr(Me)$_2$]-AVP) | -2 | 12 |
| $Ca^{2+}$ channel (L, verapamil site) (phenylalkylamine) (antagonist D 600) | -22 | -9 |
| $K_V$ channel (antagonist α-dendrotoxin) | -16 | -16 |
| $SK_{Ca}$ channel (antagonist apamin) | -7 | -14 |
| $Na^+$ channel (site 2) (antagonist veratridine) | -8 | 31 |
| Cl channel (GABA-gated) (antagonist picrotoxinin) | 4 | 25 |
| norepinephrine transporter (h) (antagonist protriptyline) | -7 | -10 |
| dopamine transporter (h) (antagonist BTCP) | 14 | 23 |
| 5-HT transporter (h) (antagonist imipramine) | -6 | -2 |