Supplemental Tables & Figures

Pharmacological Actions of Carbamate Insecticides at Mammalian Melatonin Receptors

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18. SUPPLEMENTAL DATA

Supplemental Table 1. Binding affinity values and cooperativity factors for melatonin, luzindole, and carbaryl at hMT_1 and hMT_2 melatonin receptors expressed in CHO cells.

Ligand	Ligand Competition for 2-[^{125}I]-Iodomelatonin Binding					
	Human MT_1 (A)		Human MT_1 (R)		Human MT_2 (A)	
	pK_B	α	pK_B	α	pK_B	α
Melatonin	9.71 (9.09 - 10.3)	< 0.001	N.D.	N.D.	9.91 (9.68 - 10.2)	< 0.001
Luzindole	6.41 (6.27 - 6.56)	0.037 (0.003 - 0.093)	7.27 (7.15 - 7.39)	< 0.001	7.87 (7.65 - 8.08)	< 0.001
Carbaryl	5.43 (5.15 - 5.71)	0.017 (0.012 - 0.022)	5.36 (5.19 - 5.53)	< 0.001	6.40 (5.76 - 7.04)	< 0.001

Equilibrium binding constants (K_B) and cooperativity factors (α) for melatonin, luzindole, and carbaryl at hMT_1 and hMT_2 melatonin receptors stably expressed in CHO cells in active (A) or resting (R) buffer (+ 100 µM GTP, 1 mM EDTA.Na_2, 150 mM NaCl), were determined by ligand competition for various concentrations (30 -1400 pM) of 2-[^{125}I]-iodomelatonin. Radioligand concentration, IC_{50}, and maximal fractional inhibition obtained from binding curves (see Figs. 4 and S2) were used to calculate pK_B and α values (Cheng and Prusoff 1973, Lazareno and Birdsall 1995). Shown are mean pK_B and α values with 95% confidence intervals from 3 independent experiments. α < 0.01 reveals an orthosteric binding mechanism while α ≥ 0.01 indicates allosteric binding.

Supplemental Table 2. 2-[^{125}I]-Iodomelatonin binding dissociation rates from hMT_1 and hMT_2 melatonin receptors in the absence and presence of luzindole or cluster 1-carbamate insecticides.

Test Compound	2-[^{125}I]-Iodomelatonin Dissociation Binding			
	Human MT_1 (R)		Human MT_2 (R)	
	K_{off} (min^{-1})	$\Delta K_{off\ (Veh-Test)}$ (min^{-1})	K_{off} (min^{-1})	$\Delta K_{off\ (Veh-Test)}$ (min^{-1})
Vehicle	0.437 (0.366 - 0.507)	N/A	0.0827 (0.0551 - 0.110)	N/A
Luzindole	0.402 (0.358 - 0.446)	0.0345 (-0.0179 - 0.0869)	0.0890 (0.0420 - 0.136)	-0.00635 (-0.0337 - 0.0210)
Carbaryl	0.421 (0.316 - 0.527)	0.0152 (-0.0115 - 0.145)	0.0777 (0.0444 - 0.111)	0.00503 (-0.00317 - 0.132)
Fenobucarb	0.437 (0.366 - .509)	-0.0008 (-0.130 - 0.128)	0.0791 (0.0411 - 0.117)	0.00359 (-0.0120 - 0.0191)
Bendiocarb	0.447 (0.339 - 0.555)	0.0104 (-0.157 - 0.136)	0.0814 (0.0495 - 0.113)	0.00133 (-0.0109 - 0.0136)
Carbofuran	0.470 (0.356 - 0.584)	-0.0335 (-0.166 - 0.0992)	0.0796 (0.0543 - 0.105)	0.00310 (-0.0234 - 0.0296)

Dissociation rates (K_{off}) of 2-[^{125}I]-iodomelatonin (100 pM) binding to CHO-hMT_1 or CHO-hMT_2 membranes in resting buffer (R), initiated by challenge with 10 μM melatonin in the absence (vehicle) or the presence of 100 μM luzindole, carbaryl, fenobucarb, bendiocarb, or carbofuran (see Fig. S3). Shown are mean K_{off} values and 95% confidence intervals of 3 independent experiments run in duplicate. Mean difference in dissociation rates (with 95% CI) between vehicle and test compounds (ΔK_{off}) are also reported for comparison by Friedman test (MT_1: $P = 0.043$; MT_2: $P = 0.74$) with Dunn's post-test for multiple comparisons ($P > 0.05$ for all comparisons).

Supplemental Table 3. Additional information regarding descriptive statistics and analyses for Fig. 7, Fig. 8, & Fig. 9.

	Post-hoc Test Comparisons (all α = 0.05)						
Figure	Control	mean ± SD	*n*	Treatment	mean ± SD	*n*	*P*-value *P* =
7a	Total + Vehicle	97.03 ± 9.54	4	1 μM	74.69 ± 7.83	7	**0.0011**
SCN				10 μM	61.95 ± 8.30	7	**< 0.0001**
WT	1 μM Melatonin	28.92 ± 10.47	7	100 μM	40.16 ± 8.83	7	**< 0.0001**
7b	Total + Vehicle	96.12 ± 7.37	4	1 μM	81.76 ± 11.90	7	**0.0821**
PVT				10 μM	64.41 ± 12.00	7	**0.0002**
WT	1 μM Melatonin	35.48 ± 9.69	7	100 μM	45.53 ± 7.25	7	**< 0.0001**
7c	Total + Vehicle	87.77 ± 12.25	4	1 μM	93.58 ± 8.94	4	0.7498
PT				10 μM	63.55 ± 7.83	4	**0.0127**
WT	1 μM Melatonin	15.10 ± 6.51	4	100 μM	29.21 ± 10.21	4	**< 0.0001**
7d	Total + Vehicle	94.05 ± 1.75	2	1 μM	83.91 ± 5.97	7	0.5403
SCN				10 μM	62.32 ± 16.02	7	**0.0123**
MT_2KO	1 μM Melatonin	29.87 ± 4.88	7	100 μM	34.00 ± 14.68	7	**< 0.0001**
7e	Total + Vehicle	100.4 ± 6.58	2	1 μM	91.19 ± 11.74	7	0.5630
PVT				10 μM	71.46 ± 15.91	7	**0.0151**
MT_2KO	1 μM Melatonin	36.05 ± 8.01	7	100 μM	43.62 ± 4.06	7	**< 0.0001**
7f	Total + Vehicle	105.3 ± 4.97	4	1 μM	73.19 ± 12.15	4	**0.0009**
PT				10 μM	65.46 ± 10.51	4	**0.0001**
MT_2KO	1 μM Melatonin	21.71 ± 1.94	4	100 μM	40.86 ± 6.97	4	**< 0.0001**
8a	Vehicle	2.33 ± 0.28	10	30 mins	1.77 ± 0.27	5	**0.0021**
SCN				60 mins	1.54 ± 0.25	6	**< 0.0001**
				120 mins	1.88 ± 0.14	4	**0.0236**
				240 mins	2.51 ± 0.28	3	0.7196
8b	Vehicle	1.89 ± 0.36	10	30 mins	1.38 ± 0.44	5	0.0943
PVT				60 mins	1.46 ± 0.18	6	0.1545
				120 mins	1.71 ± 0.32	4	0.8763
				240 mins	2.59 ± 0.74	3	**0.0403**
8c	Vehicle	2.36 ± 0.48	3	30 mins	1.20 ± 0.51	2	0.0908
PT				60 mins	0.95 ± 0.27	2	**0.0439**
				120 mins	1.64 ± 0.42	2	0.3434
				240 mins	2.17 ± 0.51	2	0.9700
9d	Vehicle	0.04 ± 0.12	7	Carbaryl	0.91 ± 0.34	12	**< 0.0001**
				Melatonin	1.15 ± 0.18	4	**< 0.0001**

Summary table describing details of statistical analyses and descriptive statistics. Alpha set *at P* < 0.05 for all analyses using Dunnett's post-test compared to total + vehicle (7A-F) or vehicle (8A-C, 9D). Bolded *P*-values represent significant results. Further details on statistical testing and experimental details for each figure (Fig 7, Fig. 8, Fig.9) found in corresponding figure legends or **Materials & Methods** section. Main effects and other statistical details as appropriate for each data set are reported in corresponding figure legends (Fig 7, Fig. 8, Fig.9) or **Results**.

Supplemental Table 4. Additional information regarding descriptive statistics and analyses for Supplemental Fig. 5 quantitative receptor autoradiography results.

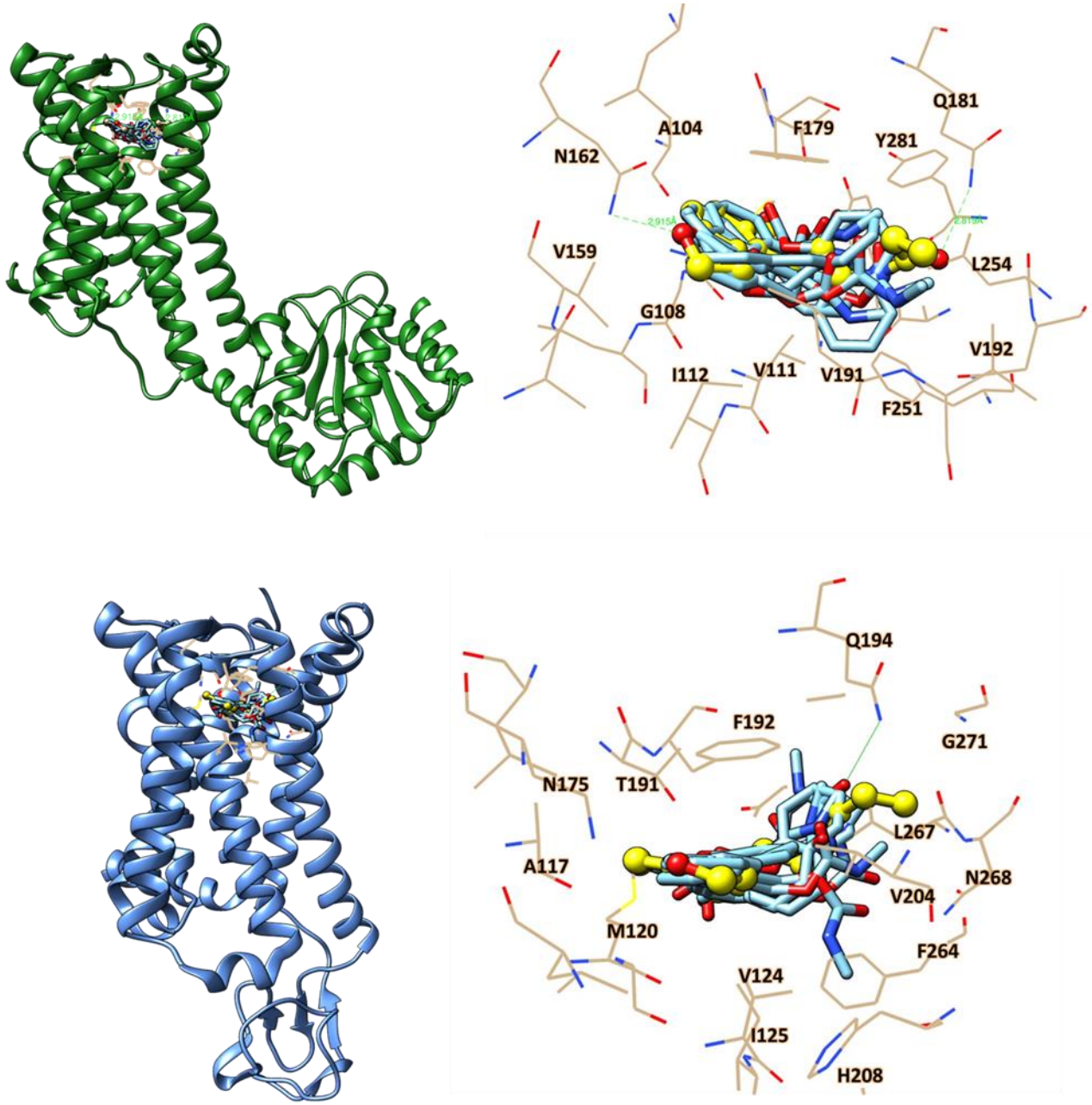
				Post-hoc Test Comparisons (all α = 0.05)				
Figure	Control	mean ± SD	*n*	Treatment	Concentration	mean ± SD	*n*	*P*-value *P* =
Supp. 5a	Total + Vehicle	101.7 ± 2.93	3		1 μM	99.65 ± 6.49	3	0.9799
SCN				Bendiocarb	10 μM	89.73 ± 4.57	2	0.3245
WT	1 μM Melatonin	36.98 ± 2.49	3		100 μM	54.95 ± 13.00	3	**0.0005**
Supp. 5b	Total + Vehicle	102.3 ± 3.99	3		1 μM	109.4 ± 7.49	3	0.3274
PVT				Bendiocarb	10 μM	94.84 ± 0.16	2	0.3639
WT	1 μM Melatonin	45.14 ± 4.46	3		100 μM	68.52 ± 5.36	3	**0.0003**
Supp. 5c	Total + Vehicle	91.67 ± 6.92	2		1 μM	84.37 ± 14.57	2	0.8540
PT				Bendiocarb	10 μM	82.32 ± 11.49	2	0.7556
WT	1 μM Melatonin	16.34 ± 3.29	2		100 μM	40.32 ± 10.62	2	**0.0233**
Supp. 5d	Total + Vehicle	111.8 ± 16.89	3		1 μM	86.66 ± 18.62	3	0.9857
SCN				Fenobucarb	10 μM	62.32 ± 16.02	3	0.1486
WT	1 μM Melatonin	32.70 ± 0.88	3		100 μM	36.14 ± 4.95	3	**0.0005**
Supp. 5e	Total + Vehicle	99.86 ± 14.84	3		1 μM	95.22 ± 5.59	3	0.9114
PVT				Fenobucarb	10 μM	72.15 ± 11.06	3	**0.0337**
WT	1 μM Melatonin	31.85 ± 4.22	3		100 μM	31.82 ± 9.53	3	**0.0002**
Supp. 5f	Total + Vehicle	97.03 ± 15.91	2		1 μM	59.95 ± 12.43	2	0.0598
PT				Fenobucarb	10 μM	45.92 ± 2.39	2	**0.0210**
WT	1 μM Melatonin	22.86 ± 4.01	2		100 μM	23.61 ± 7.63	2	**0.0057**

Summary table describing details of statistical analyses and descriptive statistics. Alpha set *at P* < 0.05 for all analyses using Dunnett's post-test compared to total + vehicle. Non-specific binding by melatonin is shown in each data set for reference. Bolded *P*-values represent significant results. Further details on statistical testing and experimental details for each figure (Supplemental Fig. 5A, B, C for bendiocarb and D, E, F for fenobucarb) found in corresponding figure legends or **Materials & Methods**. Main effects and other details as appropriate for each data set are reported in corresponding figure legends or **Results**.

Supplemental Table 5. Affinity constants of carbaryl for 2-[^{125}I]-iodomelatonin binding to SCN, PVT, and PT in brain slices from C3H/HeN WT & MT_2KO mice.

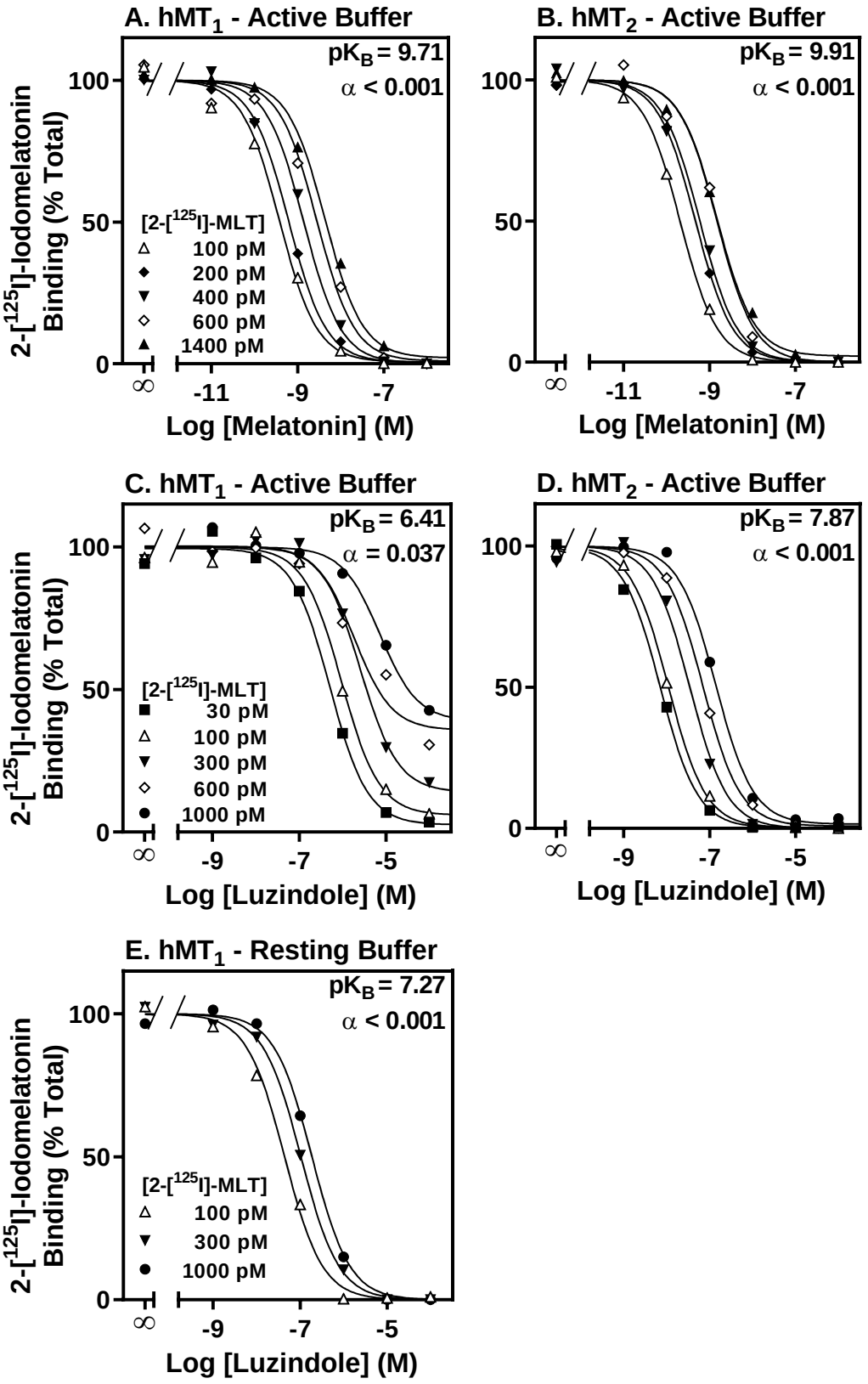
Genotype	Competition for 2-[^{125}I]-Iodomelatonin Binding		
	Mouse SCN	Mouse PVT	Mouse PT
	pK_i	pK_i	pK_i
Carbaryl WT	5.50 (5.30 - 5.71)	5.51 (5.03 - 5.98)	5.10 (4.76 - 5.44)
Carbaryl MT_2KO	5.38 (4.99 - 5.77)	5.23 (4.93 - 5.52)	5.29 (4.42 - 6.12)

Carbaryl (1-100 μM) competed for 2-[^{125}I]-iodomelatonin (75 pM) binding to melatonin receptors in SCN, PVT, and PT C3H/HeN mouse brain slices as determined by quantitative receptor autoradiography. pK_i values were calculated from K_i values determined by the method of Cheng and Prusoff (1973). Shown are mean pK_i values and 95% confidence intervals from *n* = 4-7 independent determinations. Values for WT mice are also appear in Table 2.

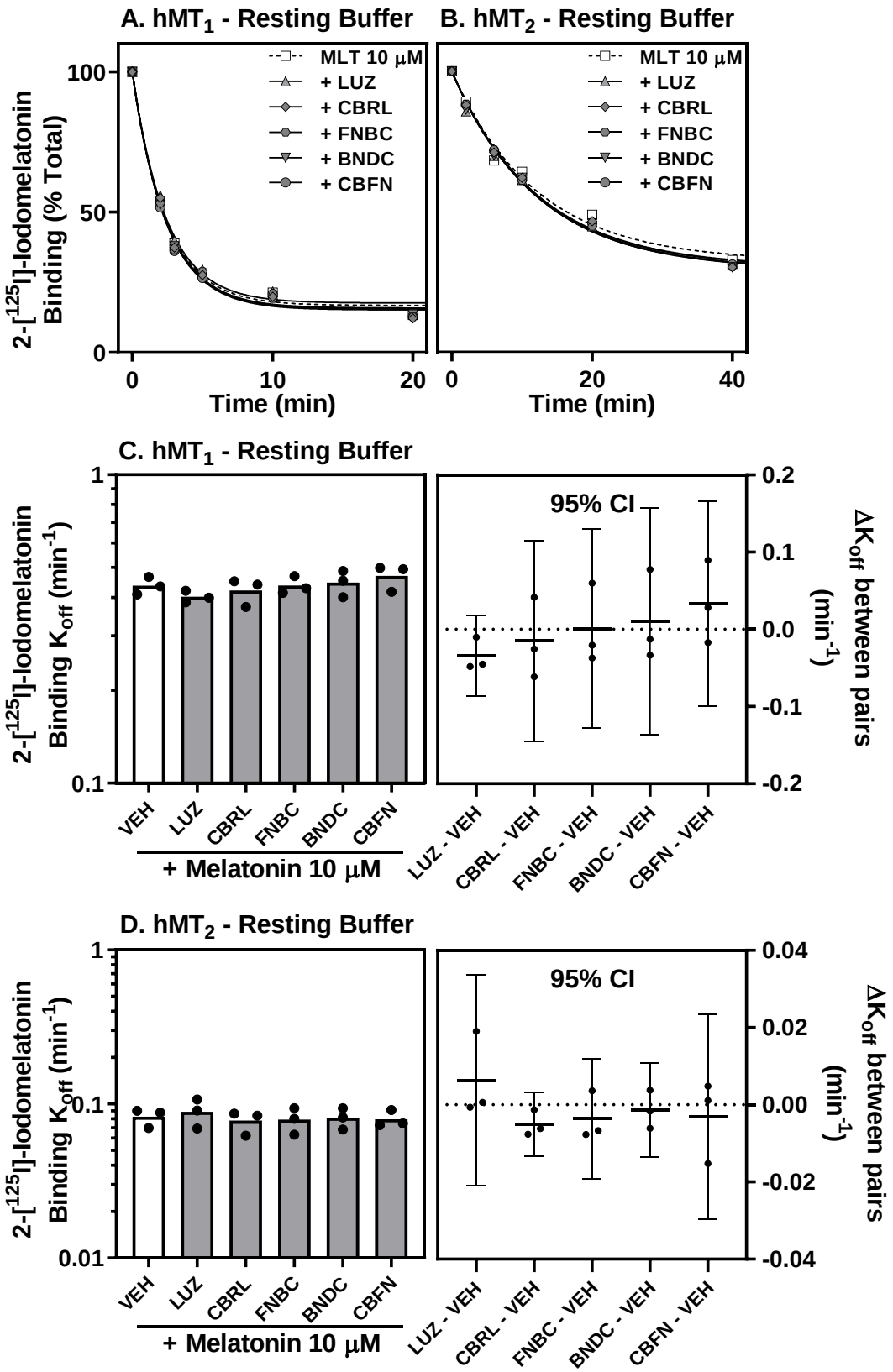


Supplemental Figure 1. Molecular docking of carbaryl with human MT_1 and MT_2 melatonin receptor models derived from X-ray crystal structures. MT_1 (A; rendered in green; PDB ID: 6ME2) and MT_2 (B; blue; PDB ID: 6ME9) melatonin receptors in complex with ramelteon (yellow) with top 5 docked poses of carbaryl (cyan). Carbaryl

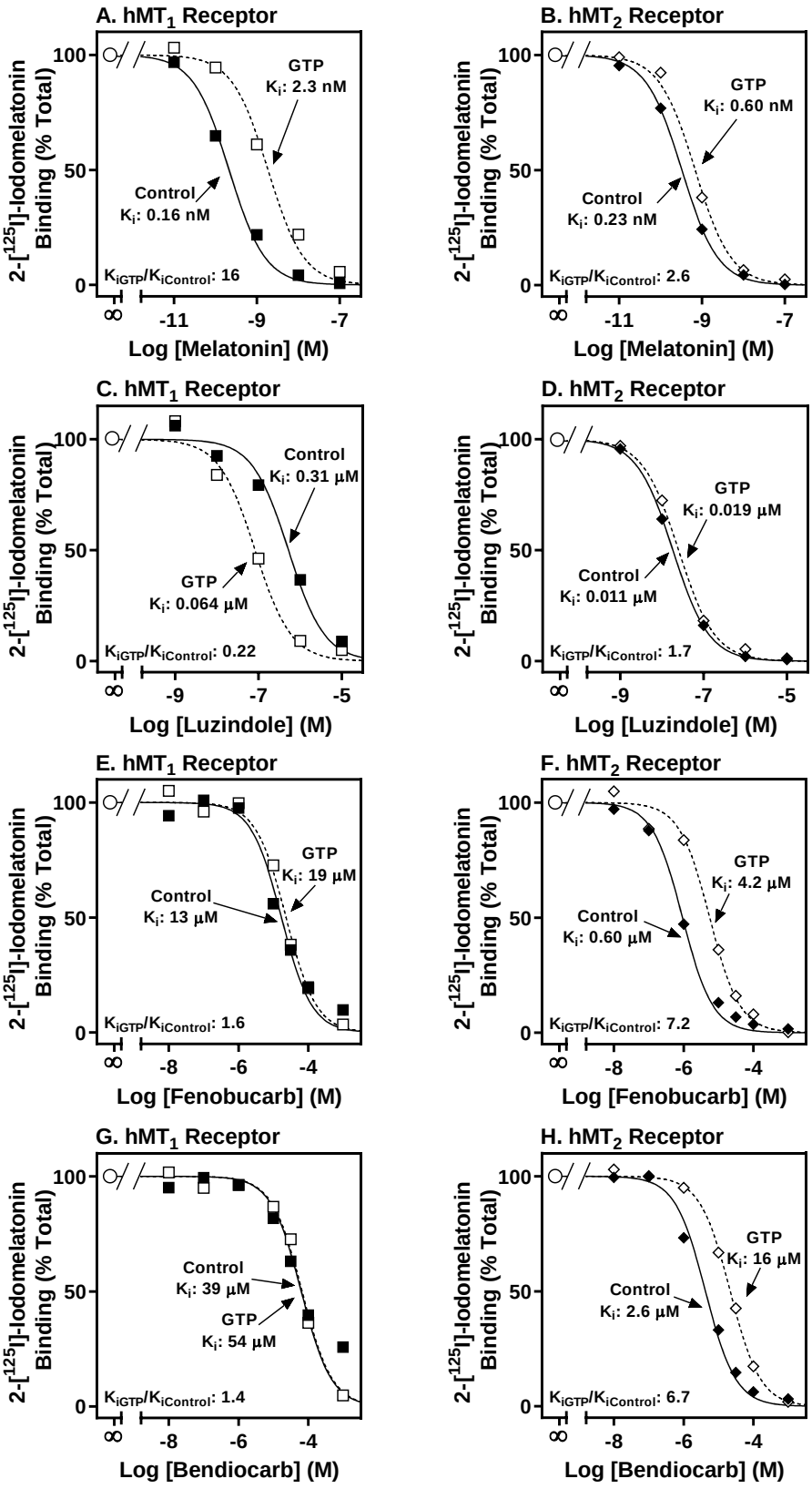
bound to the putative MT_1-melatonin binding pocket like the cognate ligand ramelteon, however at the MT_2-melatonin binding pocket, carbaryl and ramelteon displayed dissimilar interactions.


A. hMT1 - Active Buffer
pKB = 9.71
α < 0.001
[2-[125I]-MLT]
100 pM
200 pM
400 pM
600 pM
1400 pM
2-[125I]-Iodomelatonin Binding (% Total)
Log [Melatonin] (M)
B. hMT2 - Active Buffer
pKB = 9.91
α < 0.001
Log [Melatonin] (M)
C. hMT1 - Active Buffer
pKB = 6.41
α = 0.037
[2-[125I]-MLT]
30 pM
100 pM
300 pM
600 pM
1000 pM
2-[125I]-Iodomelatonin Binding (% Total)
Log [Luzindole] (M)
D. hMT2 - Active Buffer
pKB = 7.87
α < 0.001
Log [Luzindole] (M)
E. hMT1 - Resting Buffer
pKB = 7.27
α < 0.001
[2-[125I]-MLT]
100 pM
300 pM
1000 pM
2-[125I]-Iodomelatonin Binding (% Total)
Log [Luzindole] (M)

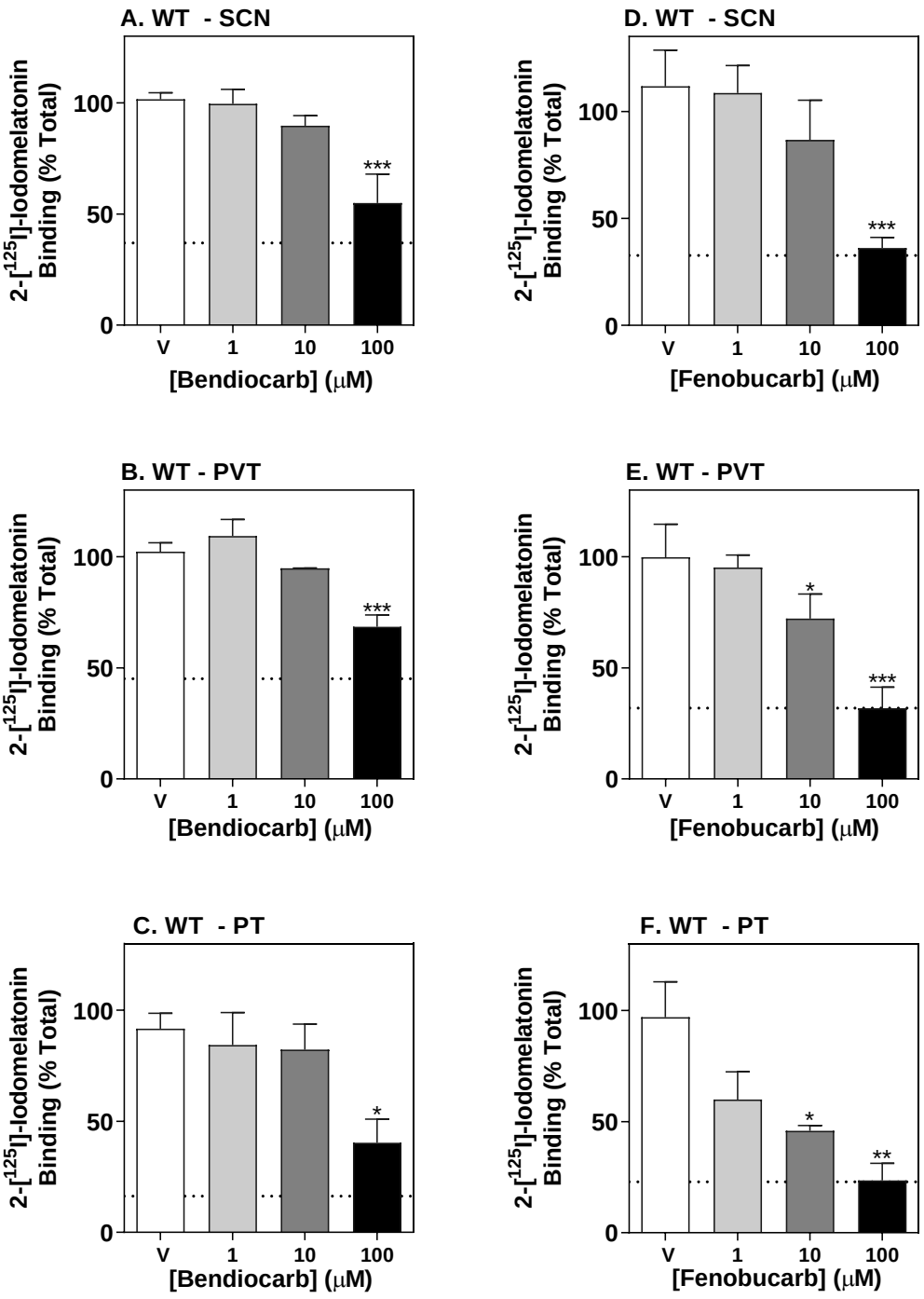
Supplemental Figure 2. Characterization of 2-[^{125}I]-iodomelatonin binding mechanism for melatonin and luzindole. The ordinates represent 2-[^{125}I]-iodomelatonin binding expressed as percent total 2-[^{125}I]-iodomelatonin binding to membranes from CHO cells stably expressing hMT_1 (A,C,E) or hMT_2 (B,D) melatonin receptors. Membranes were incubated with 2-[^{125}I]-iodomelatonin (■ = 30 pM, △ = 100 pM, ▼ = 300 pM, ◇ = 600 pM, ● = 1000 pM, ▲ = 1400) and control, melatonin (A,B; 10 pM – 1 μM) or luzindole (C-E; 1 nM-100 μM) in active (A-D) and resting buffer (E; + 100 μM GTP, 1 mM $EDTA.Na_2$, 150 mM NaCl). Points shown are the mean from representative experiments independently repeated three times. See Table S1 for derived binding constants (K_B) and cooperativity factors (α; α < 0.01, orthosteric; α ≥ 0.01, allosteric).


A. hMT1 - Resting Buffer
B. hMT2 - Resting Buffer
MLT 10 µM
+ LUZ
+ CBRL
+ FNBC
+ BNDC
+ CBFN
2-[125I]-Iodomelatonin Binding (% Total)
Time (min)
C. hMT1 - Resting Buffer
D. hMT2 - Resting Buffer
2-[125I]-Iodomelatonin Binding Koff (min-1)
ΔKoff between pairs (min-1)
95% CI
VEH
LUZ
CBRL
FNBC
BNDC
CBFN
+ Melatonin 10 µM
LUZ - VEH
CBRL - VEH
FNBC - VEH
BNDC - VEH
CBFN - VEH

Supplemental Figure 3. Cluster 1-carbamate insecticides likely bind to melatonin receptor orthosteric sites. 2-[^{125}I]-iodomelatonin (100 pM) binding to CHO-hMT$_1$ (A,C) or CHO-hMT$_2$ (B,D) membranes, in resting buffer (100 µM GTP, 1 mM EDTA.Na$_2$, 150 mM NaCl), is allowed to reach equilibrium and dissociation is initiated by challenge with 10 µM melatonin in the absence or the presence of 100 µM luzindole (LUZ), carbaryl (CBRL), fenobucarb (FNBC), bendiocarb (BNDC), or carbofuran (CBFN). (A,B) The ordinate represents 2-[^{125}I]-iodomelatonin binding expressed as percent total 2-[^{125}I]-iodomelatonin binding and the abscissa represents time after concurrent addition of melatonin and test compound. Symbols shown are the mean from three independent experiments, each run in duplicates. (C,D) Symbols on the left panels are means of K_{off} from technical replicates run in duplicate while bars represent the mean of these values (n = 3). Symbols on the right panels indicate the difference (Δ) in 2-[^{125}I]-iodomelatonin K_{off} for individually paired determinations with vehicle or test compounds. The mean ΔK_{off} and 95% confidence intervals are reported on the right as well. See Table S2 for dissociation rates. K_{off} values with test compounds are compared to vehicle using a Friedman test (A: $P = 0.043$; B: $P = 0.74$) with Dunn's post-test for multiple comparisons ($P > 0.05$ for all comparisons).


A. hMT1 Receptor
GTP
Ki: 2.3 nM
Control
Ki: 0.16 nM
KiGTP/KiControl: 16
2-[125I]-Iodomelatonin Binding (% Total)
Log [Melatonin] (M)
B. hMT2 Receptor
GTP
Ki: 0.60 nM
Control
Ki: 0.23 nM
KiGTP/KiControl: 2.6
Log [Melatonin] (M)
C. hMT1 Receptor
Control
Ki: 0.31 µM
GTP
Ki: 0.064 µM
KiGTP/KiControl: 0.22
Log [Luzindole] (M)
D. hMT2 Receptor
GTP
Ki: 0.019 µM
Control
Ki: 0.011 µM
KiGTP/KiControl: 1.7
Log [Luzindole] (M)
E. hMT1 Receptor
GTP
Ki: 19 µM
Control
Ki: 13 µM
KiGTP/KiControl: 1.6
Log [Fenobucarb] (M)
F. hMT2 Receptor
GTP
Ki: 4.2 µM
Control
Ki: 0.60 µM
KiGTP/KiControl: 7.2
Log [Fenobucarb] (M)
G. hMT1 Receptor
Control
Ki: 39 µM
GTP
Ki: 54 µM
KiGTP/KiControl: 1.4
Log [Bendiocarb] (M)
H. hMT2 Receptor
GTP
Ki: 16 µM
Control
Ki: 2.6 µM
KiGTP/KiControl: 6.7
Log [Bendiocarb] (M)

Supplemental Figure 4. Carbamate insecticides compete for 2-[^{125}I]-iodomelatonin binding to hMT_1 and hMT_2 melatonin receptors without and with G protein inactivation: Representative Curves. The ordinate represents 2-[^{125}I]-iodomelatonin binding expressed as percent total binding. Membranes from CHO cells stably expressing hMT_1 (A,C,E,G, ■ for control, □ for 100 μM GTP) or hMT_2 (B,D,F,H, ◆ for control, ◇ for 100 μM GTP) melatonin receptors were incubated with 2-[^{125}I]-iodomelatonin (75 pM) in the absence (○) and the presence of various concentrations of melatonin (A,B), luzindole (C,D), fenobucarb (E,F) or bendiocarb (G,H). Points shown are the mean from representative experiments independently repeated at least three times. See Table 3 for derived affinity constants.



Supplemental Figure 5. Quantitative receptor autoradiography demonstrates bendiocarb and fenobucarb competes *in-vitro* for 2-[^{125}I]-iodomelatonin binding at

melatonin receptors in slices containing the SCN, PVT, and PT from C3H/HeN mice. (A-F) Optical densities obtained for each treatment are normalized to proportion total binding in the absence of drug treatment (bendiocarb A-C, fenobucarb D-F) for each animal. Brain slices were treated with vehicle or drug (1, 10, 100 μM) in vehicle during a 1-hour incubation to determine competition for 2-[^{125}I]-iodomelatonin binding (75 pM) at melatonin receptors in slices containing the SCN, PVT, and PT. Dotted lines in each panel represent non-specific binding for adjacent slices treated with 1 μM melatonin for each data set. Values (n = 2-3) in each panel are compared to % total binding of vehicle using a one-way ANOVA with Dunnet's post-test ($P < 0.05$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. See Table 2 for derived affinity constants. See Supplemental Table 4 for additional information regarding descriptive statistics and data comparisons.