Pharmacological Actions of Carbamate Insecticides at Mammalian Melatonin Receptors[§]

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ABSTRACT

Integrated *in silico* chemical clustering and melatonin receptor molecular modeling combined with *in vitro* 2-[¹²⁵I]-iodomelatonin competition binding were used to identify carbamate insecticides with affinity for human melatonin receptor 1 (hMT₁) and human melatonin receptor 2 (hMT₂). Saturation and kinetic binding studies with 2-[¹²⁵I]-iodomelatonin revealed lead carbamates (carbaryl, fenobucarb, bendiocarb, carbofuran) to be orthosteric ligands with antagonist apparent efficacy at hMT₁ and agonist apparent efficacy at hMT₂. Furthermore, using quantitative receptor autoradiography in coronal brain slices from C3H/HeN mice, carbaryl, fenobucarb, and bendiocarb competed for 2-[¹²⁵I]-iodomelatonin binding in the suprachiasmatic nucleus (SCN), paraventricular nucleus of the thalamus (PVT), and pars tuberalis (PT) with affinities similar to those determined for the hMT₁ receptor. Carbaryl (10 mg/kg *i.p.*) administered *in vivo* also competed *ex vivo* for 2-[¹²⁵I]-iodomelatonin binding to the SCN, PVT, and PT, demonstrating the ability to reach brain melatonin receptors in C3H/HeN mice. Furthermore, the same dose of carbaryl given to C3H/HeN mice in constant dark for three consecutive days at subjective dusk (circadian time 10) phase-advanced circadian activity rhythms

(mean = 0.91 hours) similar to melatonin (mean = 1.12 hours) when compared with vehicle (mean = 0.04 hours). Carbaryl-mediated phase shift of overt circadian activity rhythm onset is likely mediated via interactions with SCN melatonin receptors. Based on the pharmacological actions of carbaryl and other carbamate insecticides at melatonin receptors, exposure may modulate time-of-day information conveyed to the master biologic clock relevant to adverse health outcomes.

SIGNIFICANCE STATEMENT

In silico chemical clustering and molecular modeling in conjunction with *in vitro* bioassays identified several carbamate insecticides (i.e., carbaryl, carbofuran, fenobucarb, bendiocarb) as pharmacologically active orthosteric melatonin receptor 1 and 2 ligands. This work further demonstrated that carbaryl competes for melatonin receptor binding in the master biological clock (suprachiasmatic nucleus) and phase-advances overt circadian activity rhythms in C3H/HeN mice, supporting the relevance of circadian effects when interpreting toxicological findings related to carbamate insecticide exposure.

Introduction

Melatonin modulates melatonin receptor 1 (MT₁) and melatonin receptor 2 (MT₂) G protein-coupled receptor (GPCR) signaling to regulate circadian phase and amplitude of physiological processes through action at brain (suprachiasmatic nucleus or

SCN) and peripheral (pancreas) receptors (Bothorel et al., 2002; Dubocovich and Markowska, 2005; Dubocovich et al., 2010; Peschke et al., 2013; Jockers et al., 2016; Liu et al., 2016; Karamitri and Jockers, 2019). Notably, the “hormone of darkness” and its receptors regulate circadian rhythms, metabolism, mood as well as behavior, the cardiovascular and the immune systems, and

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other key physiological functions (Dubocovich et al., 2010; Jockers et al., 2016; Liu et al., 2016). Circadian misalignments, or out-of-phase rhythms, are linked to increased risk for obesity, diabetes, cancer, cardiovascular disease, as well as sleep and psychiatric disorders (Baron and Reid, 2014). It follows that exposure to environmental melatonin mimics, like recently reported carbamate insecticides (Popovska-Gorevski et al., 2017), could disrupt melatonin system signaling, resulting in disturbed physiological functions and exacerbation of disease etiologies.

Current initiatives aiming to identify risks of environmental chemical exposure (Tox21; Kavlock et al., 2009; Schmidt, 2009; Tice et al., 2013) do not have measures to assess the ability of target compounds to interact with melatonin receptors and alter associated circadian or other biologic functions. Our team is optimizing an integrated *in silico* to *in vivo* pipeline approach to identify environmental circadian disruptors, specifically those that target melatonin receptors. In collaboration with other teams, our group recently used a similar strategy that was successful in identifying novel MT₁ and MT₂ leads (Stein et al., 2020). Furthermore, we also demonstrated that two carbamate insecticides, structurally similar to melatonin, inhibited 2-[¹²⁵I]-iodomelatonin binding to recombinant human MT₁ (hMT₁) and human MT₂ (hMT₂) melatonin receptors (Popovska-Gorevski et al., 2017), thus prompting further investigations into the pharmacological actions of these compounds at melatonin receptors mediating time-of-day messages in mammals (Dubocovich, 2007; Liu et al., 2016).

Carbamate insecticides, used industrially and domestically, are acutely toxic to insects and mammals due to reversible inhibition of acetylcholinesterase (AChE) and other esterases (Casida, 1963; Ecobichon, 2001; Moser et al., 2015b; Casida and Bryant, 2017). Use of carbaryl (1-naphthyl methylcarbamate) and other carbamates (e.g., aldicarb, bendiocarb, carbofuran, fenobucarb, fenoxycarb, methomyl, oxamyl) in agriculture as well as at home results in exposure for humans and other mammals, evidenced by trace amounts in foods, soil, as well as surface water and groundwater (Gunasekara et al., 2008; Clark-Reyna et al., 2016). Environmental and occupational exposure to carbamate insecticides has been associated with various symptoms (Whorton et al., 1979; Wyrobek et al., 1981; Meeker et al., 2004; Xia et al., 2005; Ali et al., 2015; Manyilizu et al., 2017; Bini Dhoub et al., 2016; Meyer et al., 2017) and disease pathologies (Zheng et al., 2001; Mahajan et al., 2007; Saldana et al., 2007; Montgomery et al., 2008; Slager et al., 2010; Lebov et al., 2015; Baumert et al., 2018; Patel et al., 2018; Patel and Sangeeta, 2019) related to toxicity, thought to be caused by canonical actions of carbamate insecticides on the cholinergic system. However, recent epidemiologic evidence in US farmers showed that out of 63 pesticides tested (most of which act primarily on AChE), only exposure to carbamate insecticides (carbofuran and carbaryl) directly associated with sleep apnea, suggesting an ancillary mechanism of disease pathology that could involve interaction with melatonin receptors (Zirlik et al., 2013; Baumert et al., 2018). Carbamate insecticides could, therefore, influence the pathology of sleep as well as oncological, metabolic, and psychiatric disorders through

pharmacological actions at melatonin receptors independent of or in addition to canonical actions at AChE.

Both increased (Tuomi et al., 2016) and decreased (Sulkava et al., 2017, 2018) melatonin receptor signaling, presumably at inappropriate times of day, is associated with increased circadian disruption-related disease risk (Schroeder and Colwell, 2013). Disruption of GPCR signaling through allosteric binding of endogenous ligands such as ions, amino acids, peptides, lipids, and autoantibodies have also been implicated in disease pathology (van der Westhuizen et al., 2015). Most, if not all, GPCRs possess allosteric binding pockets, and although functional extracellular allosteric pockets on melatonin receptors have not yet been described, recent structural data support this possibility (Stauch et al., 2020).

Our overall hypothesis was that carbamate insecticides with high structural similarity to melatonin would interact with melatonin receptors at orthosteric sites, leading to alterations of circadian phase. We first used large library pharmacoinformatic screening tools and chemical similarity cluster analyses to identify carbamate insecticides with the highest likelihood of interacting with melatonin receptors. Pharmacological interactions between common carbamate insecticides with hMT₁ and hMT₂ melatonin receptors, structure-activity relationships, binding mechanism(s) via docking to *in silico* receptor models, and *in vitro* competition for 2-[¹²⁵I]-iodomelatonin binding were then used to identify binding mechanisms via orthosteric and/or allosteric sites as well as apparent efficacy. We next tested the ability of the most potent carbamates (carbaryl, bendiocarb, fenobucarb) to compete for 2-[¹²⁵I]-iodomelatonin binding to brain melatonin receptors in the SCN, paraventricular nucleus of the thalamus (PVT), and pars tuberalis (PT) of C3H/HeN mice *in vitro* as well as the ability of carbaryl to reach brain melatonin receptors *in vivo* via *ex vivo* quantitative receptor autoradiography. Lastly, we tested the ability of carbaryl to alter circadian phase in C3H/HeN mice when given at subjective dusk to determine whether carbamate exposure could modulate circadian biology in a translational mouse model.

Materials and Methods

Pharmacoinformatics and Chemical Similarity Clustering.

A series of carbamate-like structures were identified using a fragment-based query on Chem2Risk, a large pharmacoinformatics knowledge base containing more than 4 million environmental chemicals (Popovska-Gorevski et al., 2017). Chemical clustering was performed using computed two-dimensional and three-dimensional Tanimoto chemical similarity indices as described earlier (Popovska-Gorevski et al., 2017).

Molecular Docking of Environmental Melatonin Ligands. Two- and three-dimensional chemical structures of melatonin and all insecticides used in this study were generated using Marvin Sketch (ChemAxon). Protein structures of MT₁ and MT₂ melatonin receptors were generated as described in Popovska-Gorevski et al. (2017). The SYBYL X software (Cerata, Inc., Princeton, NJ) package was used to prepare the protein and ligands for molecular docking experiments. The putative binding pockets for MT₁ (inclusive of residues H195, S110, and S112) and for MT₂ (inclusive of residues N175, H208, N268,

ABBREVIATIONS: AChE, acetylcholinesterase; CT, circadian time; GPCR, G protein-coupled receptor; hMT₁, human melatonin receptor 1; hMT₂, human melatonin receptor 2; mMT₁, mouse melatonin receptor 1; mMT₂, mouse melatonin receptor 2; MT₁, melatonin receptor 1; MT₂, melatonin receptor 2; MT₂KO, homozygous for the MT₂ melatonin receptor gene deletion; OD, optical density; PT, pars tuberalis; PVT, paraventricular nucleus of the thalamus; S_{2D}, Tanimoto structural similarity index in two dimensions; S_{3D}, Tanimoto structural similarity index in three dimensions; SCN, suprachiasmatic nucleus; WT, wild type; ZT, Zeitgeber time.

and Y298) were inferred from mutagenesis data (See Tables 2 and 3 (Dubocovich et al., 2010). Surflex-Dock (SYBYL; Cerata, Inc.) and VINA (Autodock, Molecular Docking; The Scripps Research Institute) were employed to dock select environmental carbamates against human melatonin receptor models. Ligands were rendered flexible in surflex docking routine; multiple conformations (docked poses) were generated and scored using surflex score (CScore; arbitrary units), which includes a combination of Dock-score, Gold-score, PMF-score, and CHEM-score. Twenty conformations for each protein-ligand docking experiment were retrieved, and top docked poses were selected based on the orientation of the test ligand with respect to the reference ligand and its docking score. Recently, high-resolution crystal structures of MT₁ in complex with agomelatine (6ME5), ramelteon (6ME2), 2-phenylmelatonin (6ME3), and 2-iodomelatonin (6ME4) and MT₂ melatonin receptor in complex with ramelteon (6ME9) and 2-phenylmelatonin (6ME6) were released (Johansson et al., 2019; Stauch et al., 2019). Carbamate insecticides were docked into the binding pockets of these newly released melatonin receptors 6ME2–9 using the protocol as described above.

Cell Culture and Harvesting. The derivation of CHO cells stably expressing FLAG-tagged recombinant human MT₁ or MT₂ melatonin receptors (CHO-hMT₁ and CHO-hMT₂) was described previously (Gerdin et al., 2003). CHO cells were cultured in Ham's F12 media supplemented with 10% fetal calf serum, 1% HEPES, and 1% penicillin (10,000 IU/ml)/streptomycin (10,000 µg/ml) in 5% CO₂ at 37°C and harvested as described previously (Popovska-Gorevski et al., 2017). Cell lines were determined to be mycoplasma-free using the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, MO). Products for cell culture were obtained from VWR International (Pittsburgh, PA). All other chemicals were reagent grade.

2-[¹²⁵I]-Iodomelatonin Binding Assays. CHO-hMT₁ and CHO-hMT₂ cell pellets were suspended, homogenized, and washed twice by centrifugation (12,000 rpm) in Tris-HCl buffer. Competition binding studies for selected compounds were conducted as previously described (Popovska-Gorevski et al., 2017) in active conformation buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4 at 25°C) or in resting conformation buffer (50 mM Tris-HCl, 10 mM MgCl₂, 100 µM GTP, 1 mM EDTA-Na₂, 150 mM NaCl, pH 7.4 at 25°C). Briefly, CHO-hMT₁ or CHO-hMT₂ cell membrane suspensions [9 (7–11) and 14 (11–17) µg protein/assay (95% CI), respectively] were incubated with 2-[¹²⁵I]-iodomelatonin [75 (66–83) pM] in the absence and presence of carbamate insecticides (10 nM–10 mM) or vehicle at 25°C (1 hour for CHO-hMT₁; 1.5 hours for CHO-hMT₂). Final concentrations of ethanol in assays for concentrations of 1 or 0.1 mM did not exceed 4%. Additional equilibrium binding assays were performed using CHO-hMT₁ or CHO-hMT₂ cell membranes in active conformation buffer with titrated concentrations of 2-[¹²⁵I]-iodomelatonin (50–1400 pM) in the absence and presence of melatonin, luzindole, or carbaryl (10 pM–1 mM) at 25°C (1 hour for CHO-hMT₁; 1.5 hours for CHO-hMT₂). Further titration assays for luzindole and carbaryl binding to CHO-hMT₁ membranes were conducted in resting buffer to prevent high-affinity 2-[¹²⁵I]-iodomelatonin binding to the G protein-coupled form of the receptor. For allosteric screening dissociation assays, 2-[¹²⁵I]-iodomelatonin (75 ± 4 pM) binding to membranes in resting buffer was allowed to reach equilibrium at 25°C (1 hour for CHO-hMT₁; 1.5 hours for CHO-hMT₂); then dissociation was initiated using 10 µM melatonin in the absence or presence of vehicle, luzindole, or carbamate insecticide (100 µM). After 1 hour for competition binding studies or at various time points (1–40 minutes) for dissociation assays, incubation was terminated by vacuum filtration through glass microfiber filters presoaked in 0.5% polyethyleneimine. Filters were then washed twice, and counts per minute were measured by a gamma-counter.

Animals. Male C3H/HeN wild-type (WT) mice from our colony and C3H/HeN mice homozygous for the MT₂ melatonin receptor gene deletion (MT₂KO) originally donated by Dr. Steven Reppert (Worcester, MA) were bred and maintained in the Laboratory of Animal Facility at University at Buffalo as previously described (Hutchinson et al., 2012). Mice were housed in a 14/10 light/dark cycle [Zeitgeber

time (ZT); ZT 0: lights on 5 AM] in temperature (22 ± 1°C)– and humidity-controlled environments with ad libitum access to food (Harlan Teklad 2018sx) and water. Light levels were 200–300 lux at the level of the cage, and mice were housed with corn cob bedding in polycarbonate translucent cages without running wheels (30 × 19 cm) or with running wheels (33 × 15 cm; Phenome Technologies). All procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines. All mice for experiments in this manuscript were randomly assigned to respective treatment conditions.

Coronal Brain Slice Preparation. Preparation of coronal brain slices was adapted from previously described methods (Siuciak et al., 1990; Benlucif et al., 1997). WT and MT₂KO C3H/HeN mice were euthanized by decapitation between ZT 8 and ZT 10. Mouse brains were dissected, flash-frozen using 2-methylbutane, and stored at –80°C until sectioning. Adjacent coronal brain sections (20 µm) were cut at –20°C using a cryostat (CM3050S; Leica) encompassing regions of interest (i.e., SCN, PVT, PT). Sections were immediately thaw mounted onto silane-coated slides (Azer Scientific) and stored at –20°C until further use.

In Vitro Quantitative Receptor Autoradiography. Receptor autoradiography experiments were conducted as previously described (Siuciak et al., 1990; Dubocovich et al., 1998). Each C3H/HeN mouse brain provided two sets of six separate slides with six to eight adjacent sections containing SCN and PVT or PT brain regions. Slide-mounted sections stored at –20°C were air-dried for 15 minutes at room temperature before incubation with various treatments during a 1-hour incubation period. Treatments all contained 2-[¹²⁵I]-iodomelatonin (75 pM) prepared in Tris-Ca buffer (50 mM Tris-HCl and 4 mM CaCl₂, pH 7.4) in the absence (total binding) or presence of varying concentrations (1, 10, 100 µM) of carbaryl, bendiocarb, fenobucarb, melatonin (1 µM, positive control, nonspecific binding), or vehicle (Tris-Ca Buffer 7.7% ethanol). After incubation, slides were rinsed twice with Tris-Ca buffer (10 minutes) and rapidly rinsed in deionized water (all solutions ice cold) before being air-dried at room temperature under dim light. Slides were then pressed to x-ray film (Kodak) for 14 days before being developed (D19; Kodak). Optical densities (ODs) from autoradiograms in brain regions of interest were then measured with Image J analysis software (National Institutes of Health), transformed using ¹⁴C standard slides calibrated for use with ¹²⁵I (Miller and Zahniser, 1987), and used to determine competition for 2-[¹²⁵I]-iodomelatonin binding across treatments (Miller and Zahniser, 1987; Masana et al., 2000). Data points are composed of averages of raw OD values obtained in two to three adjacent sections per treatment per brain region divided by the total density (treated OD value/total nontreated OD value × 100) to yield percent total binding for each set of adjacent sections for individual mouse brains. Vehicle-treated control groups contained a minimum of *n* = 2–4, whereas experimental treatment groups contained *n* = 4–7, depending on the number of viable sections for each brain.

Ex Vivo Quantitative Receptor Autoradiography. Based on methods described in Beresford et al. (1998), C3H/HeN mice were treated in vivo with vehicle (corn oil, intraperitoneal) or carbaryl (10 mg/kg i.p.) at ZT 8. Treatments were administered at ZT 8 to avoid influence of endogenous melatonin produced in C3H/HeN mice (Masana et al., 2000). Carbaryl dose (10 mg/kg) and route (intraperitoneal) of administration were selected based on lack of known AChE inhibition-mediated toxic and/or behavioral effects at this dose as well as reported brain biodistribution (Declume and Benard, 1977; Albright and Simmel, 1979; Ruppert et al., 1983; Moser et al., 1988, 2012, 2015a,b; Moser, 1995; Krolski et al., 2003; Wang et al., 2014). Mice were euthanized by decapitation at 0, 30, 60, 120, or 240 minutes postinjection. Brains were dissected immediately and prepared for quantitative autoradiography as described (Siuciak et al., 1990; Masana et al., 2000). Brain sections from mice treated in vivo with vehicle or carbaryl were then labeled in vitro and processed for quantitative receptor autoradiography with 2-[¹²⁵I]-iodomelatonin (50 pM) prepared in Tris-Ca buffer (50 mM Tris-HCl and 4 mM CaCl₂, pH 7.4) in

the absence (total binding) or presence of melatonin (1 μ M) (non-specific binding). Specific binding was defined by subtracting non-specific binding values from total binding for each brain slice. Each value represents data from two to three adjacent slices from a single mouse brain. Final n values for carbaryl-treated mice ranged from three to six (SCN, PVT) or two to three (PT) due to the number of viable slices in each brain assessed. Values for vehicle controls euthanized 0 ($n = 6$) or 240 ($n = 4$) postinjection were pooled for comparisons to carbaryl treatment across time (total $n = 10$). Data represent two individual experiments used to compare competition for radioligand binding ex vivo across treatment time points.

Carbaryl-Mediated Phase Shift of Circadian Running Wheel Activity Onset. Methods are previously described in Benloucif and Dubocovich (1996) and Dubocovich et al. (1998), (2005). Briefly, male C3H/HeN mice were housed in constant darkness for 2 weeks before treatment to establish stable free-running circadian rhythms of running wheel activity. Vehicle (saline/15% ethanol), carbaryl (10 mg/kg i.p.), or melatonin (3 mg/kg, s.c.) treatments were given for three consecutive days at approximately circadian time (CT) 10 (CT 12 = onset of running wheel activity; CT 10 = 2 hours onset of circadian rhythm of wheel-running activity or subjective dusk). Times of treatments were determined from actograms by predicted onset of running wheel activity for each mouse based on stable free-running activity rhythm onsets for 7–12 days before treatments and were centered around CT 10, occurring from CT 9 to CT 11 (average time of injections was CT 10.1–10.2, respectively, for days 1–3). Injections and animal care in constant dark were done by dim red light (<5 lux) to avoid influence of light on circadian running wheel activity rhythms (Benloucif and Dubocovich, 1996; Benloucif et al., 1999). The shift in time of post-treatment activity rhythm onset fits of 7–12 days were compared with pretreatment onset fits on the first day after treatment to determine phase shift values. An earlier onset in the post-treatment onsets relative to pretreatment onsets is considered a phase-advance, whereas the opposite would be a phase-delay in activity rhythms. Actograms were analyzed blind to treatment and were excluded before analyses if mice displayed tau changes greater than 0.3 or low activity or if two thirds of the injections occurred outside the target window. Only one mouse was excluded based on the aforementioned criteria from the carbaryl treatment group for having two thirds of the injections fall outside the acceptable time window. Negative (vehicle: $n = 7$) and positive (melatonin: $n = 4$) controls were compared with carbaryl-treated mice ($n = 12$) to assess effects of treatment on magnitude of phase changes post-treatment. Data were replicated in two separate experiments (experiment 1: $n = 3$ vehicle, $n = 5$ carbaryl; experiment 2: $n = 4$ vehicle, $n = 8$ carbaryl, $n = 4$ melatonin) pooled together for statistical analysis (total $n = 24$ individual mice).

Reagents. 2-[125 I]-iodomelatonin (Specific Activity: 2200 ci, 81.4 TBq/mmol) was purchased from Perkin Elmer (Shelton, CT). Guanosine 5'-triphosphate sodium salt hydrate (GTP), melatonin, aldicarb, bendiocarb, carbaryl, carbofuran, fenobucarb, fenoxycarb, methomyl, oxamyl, and corn oil were obtained from Sigma-Aldrich. Luzindole was purchased from Tocris (Minneapolis, MN).

Compound and Drug Preparation. For in vitro experiments, melatonin and luzindole (13 mM stock solutions) as well as aldicarb, bendiocarb, carbaryl, and fenobucarb (130 mM stock solutions) were prepared in ethanol and subsequently diluted 1/10 in 50% ethanol/50% Tris-HCl buffer (50, 10 mM MgCl₂, pH 7.4 at 25°C). Fenoxycarb (130 and 13 mM) was made in 100% ethanol and next diluted 1/10 in 50% ethanol/50% Tris-HCl buffer. Methomyl and oxamyl (130 mM stock solutions) were dissolved in water. All subsequent dilutions were performed in Tris-HCl buffer.

For ex vivo binding studies, carbaryl was dissolved in corn oil (1 mg/ml) at 37°C and administered at 0.01 ml/g mouse body weight for a dose of 10 mg/kg (i.p.). For phase shift experiments, carbaryl was dissolved in 100% ethanol and diluted in sterile saline to 1 mg/ml in 15% ethanol under continuous sonication and administered at 0.01 ml/g mouse body weight for a dose of 10 mg/kg (i.p.). Melatonin (0.9 mg/ml) was dissolved in vehicle and administered at 0.1 ml/30 g mouse body weight for a dose of 3 mg/kg s.c. similar to as previously described (Dubocovich et al., 2005).

Data Analysis and Statistics. All data analyses were done using GraphPad Prism 8 software (La Jolla, CA). For in vitro binding studies, counts per minute were converted to percent total binding, with 100% defined as uninhibited 2-[125 I]-iodomelatonin binding and with 0% being nonspecific binding (NS) for each experiment. Concentration-response curves were fit to competition binding data using the equation " $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(X - \text{LogEC}_{50})})$ " (Slope = 1). Dissociation curves were fit to kinetic binding data using the equation " $Y = (Y_0 - \text{NS}) \times e^{(-K_{\text{off}} \times X)} + \text{NS}$." Top constraints were set to "100%" for all in vitro binding experiments. For structure-activity relationship and GTP shift competition binding studies, curve bottoms were constrained to "0%." Bottoms of curves were constrained to be "greater than 0" for binding titration and dissociation assays. For in vitro quantitative receptor autoradiography experiments curve bottoms were constrained to nonspecific binding values determined for each set of adjacent sections analyzed. Individual K_i , K_B , and α values were calculated using commercial software (GraphPad Prism) according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973; Lazareno and Birdsall, 1995). K_D values used in calculations correspond to specific receptors and conformation in buffer (hMT₁ active: 116 pM; hMT₁ resting: 280 pM; hMT₂ active: 119 pM; hMT₂ resting: 215 pM). 2-[125 I]-iodomelatonin dissociation rates (K_{off}) in the presence of test compounds are compared with vehicle using a Friedman test ($\alpha = 0.05$) with Dunn's post-test for multiple comparisons ($\alpha = 0.05$). Significant differences between $pK_i(\text{GTP})$ and $pK_i(\text{Control})$ were determined by two-tailed paired t tests ($\alpha = 0.05$). Apparent efficacy at melatonin receptors of compounds was assessed by subtracting pK_i values obtained in active buffer from resting buffer ($pK_i(\text{GTP}) - pK_i(\text{Control}) = \Delta pK_i$) and comparison melatonin ΔpK_i (CHO-hMT₁: 1.19; CHO-hMT₂: 0.41). Affinity shifts or lack thereof between active and resting buffers indicate apparent efficacy (Lefkowitz et al., 1976; Nonno et al., 1998); thus, ligands were characterized as agonists ($\Delta pK_i > 20\%$ melatonin), antagonists ($\Delta pK_i < 20\%$ melatonin, $> -20\%$ melatonin), or inverse agonists ($\Delta pK_i < -20\%$ melatonin) accordingly. K_D used to determine IC_{50} for in vitro quantitative receptor autoradiography experiments was 87.3 pM (unpublished data). For in vitro and ex vivo quantitative receptor autoradiography data, average values for each treatment condition were compared with the control group (vehicle-treated) using one-way ANOVA ($\alpha = 0.05$) with a Dunnett's post-test ($\alpha = 0.05$) to assess differences between groups. For in vivo circadian rhythm experiments, circadian phase changes were compared via one-way ANOVA ($\alpha = 0.05$) with a Dunnett's post-test ($\alpha = 0.05$) comparing carbaryl and melatonin to vehicle-treated mice.

Results

Chemical Clustering of Carbamate Insecticides. A search for a carbamate functional group in the Chem2Risk knowledge base resulted in the identification of eight carbamate insecticides (Fig. 1A). In addition to carbaryl and carbofuran, which were previously identified as structurally similar to melatonin (Popovska-Gorevski et al., 2017), carbamate insecticides fenobucarb, fenoxycarb, and bendiocarb cluster together with normalized Tanimoto structural similarity indices in two dimensions (S_{2D}) and three dimensions (S_{3D}) in the range 0.4–0.6 (cluster 1; Fig. 1B). Interestingly, all the carbamate insecticides in cluster 1 contain at least one aromatic ring system and a carbamate ($-\text{N}-\text{C}(=\text{O})-$) moiety, which aligns well with the melatonin pharmacophore. The carbamate insecticides aldicarb, methomyl, and oxamyl clustered together with very low structural similarity with S_{2D} and $S_{3D} < 0.4$ (cluster 2; Fig. 1B).

Molecular Docking of Carbamate Insecticides to hMT₁ and hMT₂ Receptors. Melatonin and the carbamate insecticides were docked into the putative melatonin binding pockets in hMT₁ and hMT₂ receptor models. Cluster 1 carbamate insecticides (carbaryl, carbofuran, bendiocarb, fenobucarb, and

Fig. 1. Structural similarity clustering of melatonin and carbamates. (A) Structures of melatonin and carbamates. (B) The carbamate insecticides carbaryl, carbofuran, bendiocarb, fenoxycarb, and fenobucarb cluster together and are structurally similar to melatonin. Normalized Tanimoto index of chemical similarity in two and three dimensions are indicated as S_{2D} and S_{3D} , respectively.

fenoxycarb) dock into the putative melatonin binding site with H195 (in hMT₁) or H208 (in hMT₂) within 2 to 3 Å, similar to melatonin (Fig. 2). Surflex docking experiments with cluster 1 carbamates yielding at least five docked poses per receptor-ligand complex, where the ligand was positioned into the putative binding pocket, were chosen for further analysis. Surflex docking scores of top ligand-hMT₂ receptor complexes were melatonin (11.6; Fig. 2G) > carbaryl (9.2; Fig. 2H) > fenobucarb (8.9; Fig. 2K) > bendiocarb (8.5; Fig. 2I) > carbofuran (8.1; Fig. 2L) > fenoxycarb (7.8; Fig. 2J), whereas the corresponding scores for the ligand-hMT₁ complexes were melatonin (11.9; Fig. 2A) > fenobucarb (7.1; Fig. 2E) > carbaryl (7.0; Fig. 2B) > bendiocarb (5.6; Fig. 2C) > carbofuran (5.1; Fig. 2F) > fenoxycarb (4.7; Fig. 2D). Root mean square deviation of the top five binding poses for each ligand-receptor complexes was <2.0 Å. Autodock

binding affinities of cluster 1 carbamate-MT₂ complexes were in the range -4 to -8 kcal/mol compared with -11.5 kcal/mol for melatonin-MT₂ complex consistent with the results of surflex docking scores. Based on these results, cluster 1 carbamates were predicted to mimic melatonin actions at MT₁ and MT₂ receptors and were propagated for further validation using receptor binding in vitro.

The recent availability of high-resolution x-ray crystal structures of melatonin receptors in complex with agomelatine, ramelteon, 2-iodomelatonin, and 2-phenylmelatonin (Johansson et al., 2019; Stauch et al., 2019), after completion of the entire in silico to in vivo Chem2Risk pipeline, warranted a comparative analysis of the protein structures. A closer look at the ramelteon bound complexes reveals that it makes hydrogen binding contact with the amino acid residue Q181 and various nonbonding contacts with A104, M107, G108, N162, L168, T178, F179, T188, V191, and L254 in the MT₁ melatonin receptor complex (Protein Data Bank identifier: 6ME2; MT₁-CC-ramelteon; Stauch et al., 2019) and that in MT₂ melatonin receptor complex it makes hydrogen binding contact with N175 and various nonbonding contacts with A117, M120, G121, V124, I125, L181, T191, F192, Q194, Y200, V204, V205, L267, N268, and G271 (Protein Data Bank identifier: 6ME9; MT₂-CC-ramelteon; Johansson et al., 2019). Docking of the top-scoring carbamate, carbaryl, into ramelteon binding pockets in hMT₁ and hMT₂ models derived from these crystal structures show considerable overlap of binding pocket residues between the models used in our computational pipeline and the carbamate-melatonin receptor complexes generated using the x-ray crystal structures 6ME2-9. Top carbaryl-MT₁ docked poses indicate potential hydrogen bonding interactions at Q181 of hMT₁, like ramelteon (Supplemental Fig. 1A), and at N175 or Q194 of hMT₂ (Supplemental Fig. 1B). The residues N175 and H208 were within the 4 Å zone of the bound ligand carbaryl in MT₂ complexes, consistent with our previously published model (Popovska-Gorevski et al., 2017).

Competition of Carbamate Insecticides for 2-[¹²⁵I]-Iodomelatonin Binding to hMT₁ and hMT₂ Melatonin Receptors. Affinities for carbaryl, carbofuran, fenobucarb, fenoxycarb, bendiocarb, aldicarb, methomyl, and oxamyl (Fig. 3) were determined by assessing competition for 2-[¹²⁵I]-iodomelatonin binding (75 pM) at hMT₁ and hMT₂ stably expressed in CHO cell membranes. All eight carbamates competed for 2-[¹²⁵I]-iodomelatonin binding at hMT₁ (K_i range = 3.34–1070 μM) and hMT₂ (K_i range = 0.163–438 μM; Fig. 3; Table 1). Affinity constants (K_i values) for all compounds tested derived from competition with 2-[¹²⁵I]-iodomelatonin are listed in Table 1. Aldicarb, carbofuran, carbaryl, fenobucarb, bendiocarb, oxamyl, methomyl, and fenoxycarb displayed 55-, 26-, 20-, 18-, 14-, 2.6-, 2.4-, and 1.1-fold selectivity for competition binding at hMT₂ compared with hMT₁ (Table 1), respectively. Rank order of affinities for competition binding of carbamate insecticides at hMT₁ is carbaryl > fenobucarb > fenoxycarb > bendiocarb > carbofuran > oxamyl > aldicarb > methomyl and at hMT₂ is carbaryl > fenobucarb > bendiocarb > carbofuran > aldicarb > fenoxycarb > oxamyl > methomyl (Table 1).

Binding Mechanism(s) of Carbamate Insecticide for hMT₁ and hMT₂ Melatonin Receptors. Next, we determined the mode of binding (i.e., orthosteric vs. allosteric) of carbamate insecticides to the hMT₁ and hMT₂ melatonin receptors. Binding cooperativity factors (α) of melatonin,

hMT₁ Melatonin Receptor hMT₂ Melatonin Receptor

Fig. 2. Molecular docking of melatonin and carbamate insecticides with human MT₁ and MT₂ melatonin receptors. Top docking poses of melatonin (rendered in blue) (A), carbaryl (gray) (B), bendiocarb (yellow) (C), fenoxycarb (green) (D), fenobucarb (magenta) (E), and carbofuran (orange) (F) with human MT₁ melatonin receptor. Cluster 1 carbamate insecticides (B–F) were able to bind to the putative melatonin binding pocket, similar to the cognate ligand melatonin (A). (G–L) Molecular docking of melatonin and carbamate insecticides with human MT₂ melatonin receptor. Top docking poses of melatonin (rendered in blue) (G), carbaryl (gray) (H), bendiocarb (yellow) (I), fenoxycarb (green) (J), fenobucarb (magenta) (K), and carbofuran (orange) (L) with human MT₂ melatonin receptor. Cluster 1 carbamate insecticides (H–L) were able to bind to the putative melatonin binding pocket, similar to the cognate ligand melatonin (G).

Fig. 3. Carbamate insecticides compete for 2-[¹²⁵I]-iodomelatonin binding to hMT₁ and hMT₂ melatonin receptors. The ordinate represents 2-[¹²⁵I]-iodomelatonin (2-[¹²⁵I]-MLT) binding expressed as percent total binding. Membranes from CHO cells stably expressing hMT₁ (A and B) or hMT₂ (C and D) melatonin receptors were incubated with 2-[¹²⁵I]-iodomelatonin (75 pM) in the absence (○) and the presence of various concentrations of ligands: carbaryl (◆), fenoxycarb (□), and carbofuran (▲) (A and C); fenobucarb (◇), bendiocarb (■), and aldicarb (△) (B and D). Symbols shown are the mean from representative experiments independently repeated three to eight times. See Table 1 for derived affinity constants.

luzindole (a nonselective MT₁/MT₂ competitive receptor antagonist/inverse agonist), and carbaryl were derived from determining the maximal fractional inhibition (MFI) of 2-[¹²⁵I]-iodomelatonin binding at five different radioligand concentrations (30–1400 pM) to CHO-hMT₁ and CHO-hMT₂ membranes (Supplemental Table 1). For both hMT₁ and hMT₂ receptors, melatonin (hMT₁ K_B = 0.220 nM, Supplemental Fig. 2A; hMT₂ K_B = 0.124 nM, Supplemental

Fig. 2B) completely inhibited binding (hMT₁ and hMT₂ α < 0.001, Supplemental Fig. 2, A and B) of saturating concentrations of radioligand confirming both melatonin and 2-[¹²⁵I]-iodomelatonin bind to the same site. Luzindole (K_B = 13.8 nM, α < 0.001, Supplemental Fig. 2D) and carbaryl (K_B = 453 nM, α < 0.001, Fig. 4B), also completely inhibited over 1000 pM 2-[¹²⁵I]-iodomelatonin binding at hMT₂ indicating that they bind to the orthosteric site. Interestingly, for luzindole (K_B = 387 nM,

TABLE 1
Competition of carbamate insecticides for 2-[¹²⁵I]-iodomelatonin binding to hMT₁ or hMT₂ melatonin receptors expressed in CHO cells
Competition of various carbamates insecticides (10 nM–10 mM) for 2-[¹²⁵I]-iodomelatonin (75 pM) binding to hMT₁ or hMT₂ melatonin receptors stably expressed in CHO cells. pK_i values were calculated from IC₅₀ values obtained from competition curves (see Fig. 3) by the method of Cheng and Prusoff (1973). Shown are mean pK_i values and 95% confidence intervals of at least three independent determinations. K_i ratios represent fold difference (K_{i(hMT1)}/K_{i(hMT2)}}) in affinity of each carbamate insecticide for hMT₁ and hMT₂ melatonin receptors. pK_i values for melatonin are 9.84 (95% CI = 9.55–10.14; n = 5) at the hMT₁ and 9.65 (95% CI = 9.53–9.76; n = 5) at the hMT₂ (K_{i(hMT1)}/K_{i(hMT2)}} = 0.70).}}

Ligand	Ligand competition for 2-[¹²⁵ I]-iodomelatonin binding					
	Human MT ₁		Human MT ₂		Ratio K _{i hMT1} /K _{i hMT2}	
	pK _i	n	pK _i	n		
Carbaryl	5.52 (5.29–5.75)	6	6.81 (6.65–6.96)	6	20	
Fenobucarb	5.01 (4.89–5.13)	6	6.27 (6.16–6.38)	6	18	
Fenoxycarb	4.53 (4.41–4.66)	8	4.55 (4.40–4.70)	5	1.1	
Bendiocarb	4.38 (4.30–4.45)	5	5.53 (5.40–5.67)	6	14	
Carbofuran	4.03 (3.91–4.14)	3	5.46 (5.12–5.80)	3	26	
Oxamyl	3.59 (3.04–4.15)	3	3.99 (3.60–4.48)	3	2.6	
Aldicarb	3.29 (2.88–3.70)	3	5.01 (4.90–5.12)	3	55	
Methomyl	3.01 (2.43–3.60)	3	3.38 (2.96–3.81)	3	2.4

Fig. 4. Carbaryl binds to melatonin receptor orthosteric sites. The ordinates represent 2-[¹²⁵I]-iodomelatonin (2-[¹²⁵I]-MLT) binding expressed as percent total 2-[¹²⁵I]-iodomelatonin binding to membranes from CHO cells stably expressing hMT₁ (A and C) or hMT₂ (B) melatonin receptors. Membranes were incubated with 2-[¹²⁵I]-iodomelatonin (■ = 30 pM, △ = 100 pM,

α = 0.037, Supplemental Fig. 2C) and carbaryl ($K_B = 3790$ nM, α = 0.017, Fig. 4A) at hMT₁, the levels of maximal fractional inhibition of binding decreased with increasing concentrations of radioligand. Because 2-[¹²⁵I]-iodomelatonin is an agonist radioligand, additional titration tests for luzindole and carbaryl binding to CHO-hMT₁ membranes were conducted in resting buffer to minimize confounding effects associated with G protein coupling. Luzindole ($K_B = 62.6$ nM, α < 0.001, Supplemental Fig. 2E) and carbaryl ($K_B = 4400$ nM, α < 0.001, Fig. 4C) completely inhibited higher than 1000 pM 2-[¹²⁵I]-iodomelatonin binding at hMT₁ in resting buffer, thus providing supportive evidence of an orthosteric binding mechanism. To corroborate the likely orthosteric binding modes of the other cluster 1 carbamate insecticides, we tested their ability to alter the dissociation rate of 2-[¹²⁵I]-iodomelatonin from hMT₁ and hMT₂ bound in resting buffer. Alterations in radioligand dissociation rate by ligands, at concentrations lower than those that would significantly compete for binding with cold orthosteric dissociation initiators, are indicative of an allosteric binding mode. There were no rate differences detected in 2-[¹²⁵I]-iodomelatonin (100 pM) dissociation from hMT₁ and hMT₂ initiated by melatonin (10 μM) when tested in the absence (MT₁ vehicle: 0.437 minute⁻¹; MT₂ vehicle: 0.0827 minute⁻¹) or presence of test compounds (100 μM luzindole, carbaryl, fenobucarb, bendiocarb, or bendiocarb; Supplemental Fig. 3; Supplemental Table 2).

Apparent Efficacy of Carbamate Insecticides in 2-[¹²⁵I]-Iodomelatonin GTP Shift Assays. Affinity shifts for 2-[¹²⁵I]-iodomelatonin competition binding (75 pM) by carbamates with or without G protein inactivation by 100 μM GTP, 1 mM EDTA.Na₂, and 150 mM NaCl, were used to define apparent efficacy (affinity decrease/rightward shift for agonists, no change for antagonists, affinity increase/leftward shift for inverse agonists) (Lefkowitz et al., 1976; Nonno et al., 1998). For reference, in our system, competition binding in resting buffer decreased the affinity of full agonist, melatonin, for both hMT₁ and hMT₂ receptors (hMT₁ $\Delta K_{i(GTP-control)} = -1.19$; hMT₂ $\Delta K_{i(GTP-control)} = -0.41$; Supplemental Fig. 4, A and B; Table 3). Resting buffer did not change affinity for carbaryl, fenobucarb, bendiocarb, or carbofuran at hMT₁ suggesting antagonist efficacy (Figs. 5A; Supplemental Fig. 4, E and G; Table 3; (Popovska-Gorevski et al., 2017), whereas at hMT₂, affinities are decreased displaying differences consistent with an agonist (Figs. 5B; Supplemental Fig. 4, F and H; Table 3; (Popovska-Gorevski et al., 2017)).

In Vitro Competition of Carbamate Insecticides for 2-[¹²⁵I]-Iodomelatonin Binding to Melatonin Receptors in C3H/HeN Mouse Brain Slices. Carbaryl, fenobucarb, and bendiocarb were selected for experiments in brain slices from mice based on their affinity at hMT₁ in CHO cells (Table 1). Of note, quantitative receptor autoradiography in mouse brain tissue utilizing 2-[¹²⁵I]-iodomelatonin cannot detect quantifiable MT₂ receptor binding, as indicated by lack of specific radiolabeled sites in brain slices from MT₁ global

▼ = 300 pM, ◇ = 600 pM, ● = 1000 pM) and control or carbaryl (10 nM - 1 mM) in active (A and B) and resting buffer (C) (+100 μM GTP, 1 mM EDTA.Na₂, 150 mM NaCl). Symbols shown are the mean from representative experiments independently repeated three times. See Supplemental Table 1 for derived binding constants (K_B) and cooperativity factors (α; α < 0.01, orthosteric; α ≥ 0.01, allosteric).

Fig. 5. Carbamate insecticides compete for 2-[¹²⁵I]-iodomelatonin binding to hMT₁ and hMT₂ melatonin receptors without and with G protein inactivation. Membranes from CHO cells stably expressing hMT₁ (A) or hMT₂ (B) melatonin receptors were incubated with 2-[¹²⁵I]-iodomelatonin (75 pM) in the absence and the presence of various concentrations of melatonin (MLT), luzindole (LUZ), carbaryl[†] (CBRL), fenobucarb (FNBC), bendiocarb (BNDc), or carbofuran[†] (CBFN). [†] Denotes K_i values transformed from data obtained from Popovska-Gorevski et al. (2017). See Supplemental Fig. 4 for binding curves and Table 3 for derived affinity constants. (Left panels) The ordinate represents binding affinity in the absence (solid bars) and presence (dotted bars) of GTP expressed as pK_i. Connected points indicate values from simultaneously run experiments with the same tissue. (Right panels) The ordinate represents the mean differences in pK_i (pK_iGTP - pK_iControl = ΔpK_i) and 95% confidence intervals (95% CI) produced by G protein inactivation. Ligands are classified as agonists (mean ΔpK_i below 20% MLT dashed line), antagonists (mean ΔpK_i between 20% and -20% MLT dashed lines), or inverse agonists (mean above -20% MLT dashed line).

knock out mice (Liu et al., 1997; Dubocovich et al., 1998). However, MT₂ melatonin receptor mRNA has been detected by in situ hybridization (Dubocovich et al., 1998; Hunt et al., 2001) and protein has been identified via immunohistochemistry in rodents (Lacoste et al., 2015). Furthermore, attempts to label melatonin receptors with an MT₂ specific radioligand (Legros et al., 2016) failed to display specific binding in the SCN, PVNT, or PT, further supporting that MT₂ levels in rodent brain are too low to detect via available melatonin receptor radioligands by quantitative receptor autoradiography. Representative autoradiograms of adjacent (20 μm) brain

sections from a single WT mouse treated with vehicle, melatonin (1 μM), or carbaryl (1–100 μM) display a concentration-dependent decrease in visible 2-[¹²⁵I]-iodomelatonin labeling in the SCN, PVT, and PT (Fig. 6). These images also reveal the expected competition by positive control melatonin (1 μM; nonspecific binding; (Liu et al., 1997; Masana et al., 2000) for 2-[¹²⁵I]-iodomelatonin binding sites in the SCN, PVT, and PT (Fig 6, A and B). Quantification of optical density in autoradiograms revealed that carbaryl competed in a concentration-dependent manner for 2-[¹²⁵I]-iodomelatonin (75 pM) binding in the SCN (F_{3, 21} = 42.07, *P* < 0.001), the PVT

Fig. 6. Representative autoradiograms show carbaryl competes in vitro for 2-[¹²⁵I]-iodomelatonin binding at melatonin receptors in slices containing the SCN, PVT, and PT from C3H/HeN WT mice. (A and B) Vehicle, melatonin (1 μM), or carbaryl (1, 10, 100 μM) treatments in representative magnified images taken from autoradiograms containing adjacent coronal sections containing the SCN and PVT (A) or PT (B) treated with 2-[¹²⁵I]-iodomelatonin (75 pM). Additional treatments are listed below in each section.

($F_{3,21} = 25.83$, $P < 0.0001$), and the PT ($F_{3,12} = 34.63$, $P < 0.0001$) compared with vehicle-treated adjacent sections (Fig. 7, A–C; Supplemental Table 3). Similar competition was exhibited by carbaryl in brain slices from MT₂KO mice in the SCN ($F_{3,19} = 22.60$, $P < 0.001$), the PVT ($F_{3,16} = 22.19$, $P < 0.001$), and the PT ($F_{3,12} = 34.16$, $P < 0.001$; Fig. 7, D–F; Supplemental

Table 3). Fenobucarb and bendiocarb (1–100 μ M) also competed in a concentration-dependent manner for 2-[¹²⁵I]-iodomelatonin (75 pM) binding in the SCN ($F_{3,7} = 17.80$, $P < 0.001$; $F_{3,7} = 21.37$, $P < 0.001$), in the PVT ($F_{3,8} = 24.95$, $P < 0.001$; $F_{3,7} = 33.14$, $P < 0.001$), and in the PT ($F_{4,5} = 19.27$, $P < 0.01$; $F_{4,5} = 21.04$, $P < 0.01$) compared with vehicle-treated

Fig. 7. Quantitative receptor autoradiography demonstrates carbaryl competes in vitro for 2-[¹²⁵I]-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 percent total binding in the absence of drug treatment of each set of adjacent brain slices analyzed. Brain slices were treated with vehicle (VEH) or carbaryl (1, 10, 100 μ M in vehicle) for 1 hour to assess competition for 2-[¹²⁵I]-iodomelatonin binding (75 pM) at melatonin receptors in slices containing the SCN, PVT, and PT. Dotted lines in each panel represent nonspecific binding for adjacent slices treated with 1 μ M melatonin for each data set. Comparison of treatment with carbaryl vs. vehicle on percent total binding from slices obtained from C3H WT mice (A–C) and MT₂KO mice (D–F). Values ($n = 4–7$ WT, $n = 2–7$ MT₂KO) in each panel are compared with percent total binding of vehicle-treated slices using a one-way ANOVA with Dunnett's post-test ($P < 0.05$). * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. See Supplemental Table 5 and Table 2 for derived affinity constants. See Supplemental Table 3 for additional information regarding descriptive statistics and data comparisons.

TABLE 2

Affinity constants of carbamate insecticides compete for 2-[¹²⁵I]-iodomelatonin binding to SCN, PVT, and PT in brain slices from C3H/HeN mice

Carbaryl, fenobucarb, and bendiocarb (1–100 μ M) competed for 2-[¹²⁵I]-iodomelatonin (75 pM) binding to melatonin receptors in SCN, PVT, and PT C3H/HeN mouse brain slices as determined by quantitative receptor autoradiography. K_i values determined by the method of Cheng and Prusoff (1973) were used to calculate pK_i values. Shown are mean pK_i values and 95% confidence intervals from independent determinations: SCN ($n = 3-7$), PVT ($n = 3-7$), and PT ($n = 2-4$). pK_i for hMT₁ expressed in CHO cells is shown for comparison (Fig. 3; Table 1).

Ligand	Competition for 2-[¹²⁵ I]-iodomelatonin binding in vitro			
	hMT ₁ CHO pK_i	Mouse SCN pK_i	Mouse PVT pK_i	Mouse PT pK_i
Carbaryl	5.52 (5.29–5.75)	5.50 (5.30–5.71)	5.51 (5.03–5.98)	5.10 (4.76–5.44)
Fenobucarb	5.01 (4.89–5.13)	4.93 (4.27–5.58)	5.18 (4.59–5.78)	6.17 (2.36–9.97)
Bendiocarb	4.38 (4.30–4.45)	4.74 (3.70–5.77)	4.41 (4.07–4.76)	3.70 (3.39–4.00)

adjacent sections (Supplemental Fig. 5; Supplemental Table 4). Affinity (pK_i) values for carbaryl, fenobucarb, and bendiocarb regarding competition for 2-[¹²⁵I]-iodomelatonin binding in these brain regions are shown in Supplemental Table 5 and Table 2. Across all treatments and experiments, mean nonspecific binding determined by 1 μ M melatonin was 31.65% $\pm$ 8.22%, 36.87% $\pm$ 8.82%, and 17.35% $\pm$ 5.82% in the SCN, PVT, and PT, respectively.

Ex Vivo Time-Course for Competition of Carbaryl for 2-[¹²⁵I]-Iodomelatonin Binding in Brain Slices Containing the SCN. Carbaryl was chosen for in vivo administration experiments due to its superior affinity at hMT₁ recombinant receptors (Fig. 3; Table 1) and potency at native mouse MT₁ (Figs. 6 and 7; Table 2) compared with other carbamates tested. Of note, ex vivo binding using 2-[¹²⁵I]-iodomelatonin cannot detect quantifiable levels of specific binding to MT₂, allowing quantification of only MT₁ receptor affinity as previously mentioned. In vivo administration of carbaryl (10 mg/kg, i.p.) reduced specific 2-[¹²⁵I]-iodomelatonin binding (50 pM) in SCN brain slices processed ex vivo at 30, 60, and 120 minutes postadministration compared with vehicle-treated mice ($F_{4,23} = 13.21$, $P < 0.001$), which recovered by 240 minutes (Fig. 8A; Supplemental Table 3). Carbaryl also reduced specific 2-[¹²⁵I]-iodomelatonin binding (50 pM) in PVT brain slices processed ex vivo ($F_{4,23} = 5.79$, $P < 0.01$; Fig. 8B; Supplemental Table 3). Interestingly, specific binding in the PVT was found to be specifically increased at 240 minutes versus control sections (Fig. 8B; Supplemental Table 3). In the PT, specific 2-[¹²⁵I]-iodomelatonin binding was reduced by carbaryl only at 60 minutes postadministration ($P < 0.05$; Fig. 8C).

In Vivo Administration of Carbaryl at CT 10 Phase-Advances Onset of Circadian Running Wheel Activity Rhythms. Figure 9, A–C, shows representative actograms for single mice treated with vehicle (15% ethanol/saline), melatonin (3 mg/kg, s.c.), or carbaryl (10 mg/kg, i.p.) for three consecutive days at CT 10, 2 hours before onset of activity (CT 12). Quantification of phase shifts indicates that positive control melatonin and experimental drug carbaryl produced significant phase-advances of onset of activity rhythms compared with vehicle-treated controls ($F_{2,20} = 29.59$; $P < 0.05$; Dunnett's post-test; Fig. 9D; Supplemental Table 3). A single mouse was excluded from the carbaryl treatment group because it met exclusion criterion of two thirds of the injections falling outside of the acceptable range (CT 9–11).

Discussion

Carbamate insecticides sharing high structural similarity to melatonin bind competitively to the orthosteric site of the

human recombinant MT₁ and MT₂ melatonin receptors. Pharmacological data validated in silico and pharmacoinformatic predictions for carbamates, demonstrating binding to the orthosteric sites of melatonin receptors with antagonist apparent efficacy for hMT₁ and agonist apparent efficacy for hMT₂ receptors. Furthermore, carbaryl competed for 2-[¹²⁵I]-iodomelatonin binding in mouse brain areas with high expression of melatonin receptors involved in the modulation of circadian (i.e., SCN), neurochemical, behavioral (i.e., PVT), and endocrine (i.e., PT) functions. Lastly, carbaryl phase-advanced overt circadian activity rhythm onsets akin to melatonin when given to C3H/HeN wild-type mice 2 hours before (CT 10) onset of activity (CT 12) at subjective dusk. Here we discuss implications for these novel properties of carbamate insecticides in toxicological outcomes not explained by canonical actions at AChE as well as potential limitations of the present data.

The Chem2Risk knowledge base pipeline was used to discover environmental melatonin receptor ligands. Cluster 1 carbamate insecticides (carbaryl, carbofuran, fenobucarb, bendiocarb, and fenoxycarb), with Tanimoto indices S_{2D} and $S_{3D} > 0.4$ possess typical melatonin pharmacophore fingerprints, which include an aromatic ring system and a carbonyl moiety in position to leverage interactions with the binding site residues H195 or Q181 in MT₁ and N175, Q194 or H208 in MT₂. Comparative molecular docking of top carbamates with the recently reported crystal structures (Johansson et al., 2019; Stauch et al., 2019) revealed a considerable overlap of binding pocket residues. Carbamate insecticides competitively bind to the MT₁ and MT₂ melatonin receptor orthosteric sites likely formed by the aforementioned residues (Lazareno and Birdsall, 1995; Kenakin, 2009). The higher selectivity of carbaryl for binding to the hMT₂ receptors over the hMT₁ (33-fold) (Popovska-Gorevski et al., 2017) is likely attributable to the ring stacking interactions with H208 and partial occupancy of the hydrophobic cavity formed by the residues V124, I125, P212, I213, and F260 similar to reported ligand binding modes of selective MT₂ ligands (Rivara et al., 2005; Pala et al., 2013a,b; Jockers et al., 2016; Johansson et al., 2019). Carbamates, carbaryl, bendiocarb, and fenobucarb competed for mMT₁ receptors expressed in the SCN, PVT, and PT in C3H/HeN mouse brain slices yielding pK_i values similar to the affinity constants for binding at recombinant hMT₁ receptors expressed in CHO cells. Altogether, our computational predictions were translatable interactions for recombinant human and endogenous mouse melatonin receptors and suggest these model systems may be useful in risk assessment of environmental compounds to human health

TABLE 3

Competition of melatonin, luzindole, and cluster 1 carbamate insecticides for 2-[¹²⁵I]-iodomelatonin binding to hMT₁ and hMT₂ expressed in CHO cells without and with G protein inactivation

Competition for 2-[¹²⁵I]-iodomelatonin (75 pM) binding of melatonin, luzindole, and cluster 1 carbamate insecticides were performed in the absence (active buffer) or presence (resting buffer) of 100 μM GTP, 1 mM EDTA, Na₂, and 150 mM NaCl at 25°C for 1 hour. pK_i values were calculated from IC₅₀ values obtained from competition curves (see Supplemental Fig. 4) by the method of Cheng and Prusoff (1973). Shown are mean pK_i values and 95% confidence intervals of at least three independent determinations. pK_is obtained in active and resting buffer were analyzed for differences using two-tailed paired *t* tests. Decreases (ΔpK_i) = pK_{i(GTP)} – pK_{i(Control)}; negative ΔpK_i and >20% melatonin effect) of affinity in resting buffer indicate agonist apparent efficacy, whereas no change or increases in affinity (<20% melatonin effect or positive ΔpK_i) indicate antagonist or inverse agonist apparent efficacy, respectively.

Ligand	Ligand competition for 2-[¹²⁵ I]-iodomelatonin binding				
	Human MT ₁		Human MT ₂		
	Control	GTP	Control	GTP	n
Melatonin	9.84 (9.55–10.1)	8.65 (8.50–8.80)	9.65 (9.53–9.76)	9.23 (9.10–9.36)	5
Luzindole	6.52 (6.33–6.71)	7.20 (6.96–7.44)	7.97 (7.67–8.28)	7.77 (7.35–8.18)	4
Carbaryl ^a	5.22 (4.94–5.51)	5.36 (5.20–5.45)	6.99 (6.76–7.21)	6.17 (5.97–6.36)	5
Fenobucarb	4.92 (4.55–5.29)	4.72 (4.58–4.86)	6.24 (5.89–6.59)	5.38 (5.18–5.59)	3
Bendiocarb	4.41 (4.24–4.58)	4.27 (4.05–4.50)	5.60 (5.25–5.94)	4.79 (4.75–4.83)	3
Carbofuran ^a	3.85 (3.62–4.08)	3.93 (3.71–4.15)	5.64 (5.42–5.86)	4.71 (4.40–5.02)	5

^aDenotes K_i values transformed from data obtained from Popowska-Gorevski et al. (2017).
^{*}*P* < 0.05; ^{**}*P* < 0.01; ^{***}*P* < 0.001.

Fig. 8. Carbaryl competes ex vivo for 2-[¹²⁵I]-iodomelatonin binding at melatonin receptors in slices containing the SCN, PVT, and PT from C3H/HeN WT mice. Specific binding of 2-[¹²⁵I]-iodomelatonin (50 pM) ex vivo in the SCN (A), PVT (B), or PT (C) of brain slices quantified at 0, 30, 60, 120, and 240 minutes after in vivo administration of vehicle (VEH) or carbaryl (10 mg/kg i.p. in vehicle). Values (*n* = 3–6) are compared with VEH (*n* = 10) using a one-way ANOVA with Dunnett's post-test (*P* < 0.05). Specific binding was determined by subtracting nonspecific values from adjacent slices treated with only the radioligand. **P* < 0.05; ***P* < 0.01; *****P* < 0.0001.

based on success here with predictions relevant to the melatonin system.

Ex vivo quantitative receptor autoradiography data from the current report are in line with previously reported findings for the temporal pharmacokinetic (Declume and Benard, 1977; Krolski et al., 2003), biochemical (Moser et al., 2012, 2015a,b; Wang et al., 2014), and behavioral effects of carbaryl (Albright and Simmel, 1979; Ruppert et al., 1983; Moser et al., 1988; Moser, 1995). Peak effects on behavior, brain cholinesterase inhibition, and radiolabeled drug recovery in brain tissue occur from 30 to 120 minutes after delivery of carbaryl depending on the route of administration. Therefore, our results from time course experiments showing ex vivo competition binding of

Fig. 9. Carbaryl phase-advances running wheel activity onset after 3 days of injections at CT 10, similar to melatonin. Representative running wheel activity actograms from C3H/HeN mice treated with vehicle (VEH; saline 15% EtOH i.p.; $n = 7$) (A), carbaryl (10 mg/kg i.p.; $n = 12$) (B), or melatonin (3 mg/kg, s.c.; $n = 4$) (C) for three consecutive days, indicated by the black dots. (D) Quantification of phase shift by vehicle, carbaryl, or melatonin treated mice. Phase shift values were compared to vehicle by one-way ANOVA followed by Dunnett's post-test $***P < 0.001$.

carbaryl at mouse melatonin receptors maximally at 60 minutes postadministration in brain areas expressing mMT₁ melatonin receptors (SCN, PT) are in agreement with reported temporal

presentation of effects on behavior and biodistribution to the brain related to interactions with cholinergic systems. This study was admittedly limited in that we did not explore exact brain concentrations of carbaryl when administered at 10 mg/kg i.p.; however, this dose of carbaryl competed *ex vivo* for binding to melatonin receptors in the SCN and maximally phase-advanced circadian phase compared to melatonin in a translational behavioral mouse model.

The present data demonstrate interactions of carbaryl and/or metabolites, and/or melatonin itself as carbaryl can increase its production (Attia et al., 1991a,b) at brain melatonin receptors with resulting behavioral effects on circadian phase. Maximal functional response for phase shift at CT 10 are obtained with 1 and 3 mg/kg melatonin acting on brain MT₁ melatonin receptors in the SCN (Benloucif and Dubocovich, 1996; Dubocovich et al., 1998; Stein et al., 2020). Acute carbaryl administration at subjective dusk (CT 10) maximally phase-shifted circadian activity rhythms similarly to melatonin receptor agonists (Dubocovich et al., 2005; Liu et al., 2016) in contrast to present results from GTP-shift assays, suggesting antagonist apparent efficacy. Behavioral efficacy of compounds acting at melatonin receptors has been shown to vary with time of administration as well as chronobiological context (Stein et al., 2020), suggesting the need to test carbaryl at other times in the circadian cycle sensitive to melatonin receptor stimulation (i.e., CT 1–3) (Benloucif and Dubocovich, 1996) or in the jet-lag paradigm (Dubocovich et al., 2005). Drug-mediated phase shifts directly translate from the C3H/HeN mouse to human models (Benloucif and Dubocovich, 1996; Burgess et al., 2008). Therefore, carbaryl could be a possible circadian modulator in humans via actions at SCN melatonin receptors. However, it is possible that carbaryl exposure may also influence circadian physiology through reported modulation of pineal and blood melatonin levels, levels of pineal serotonin as well as its precursors, and hypothalamic uptake of norepinephrine (Jabloniska and Brzeziński, 1990; Attia et al., 1991a,b). Therefore, carbaryl may dually regulate the synthesis of melatonin and also directly compete for binding at melatonin receptors to influence mediated processes. Context for melatonin receptor signaling and physiological relevance are also important considerations, as time of day (Benloucif and Dubocovich, 1996; Gillette and Mitchell, 2002) and protein composition of the system under study (Liu et al., 2019) can significantly affect interpretation of results. Lastly, we must also acknowledge the ability of the cholinergic system to influence phase of circadian rhythms on its own (Liu and Gillette, 1996). However, the peak of sensitivity for cholinergic regulation of circadian rhythms (CT 17–19) is outside the sensitive periods (CT 1–3 and 9–11) for modulation of circadian rhythms by melatonin receptor agonists (Benloucif and Dubocovich, 1996; Dubocovich et al., 2005).

Several other off-target receptors and proteins could contribute to the phase resetting effect of carbaryl. The PubChem BioAssay data base (<https://pubchem.ncbi.nlm.nih.gov>), with over 1700 entries from biologic screening platforms such as ChEMBL (<https://www.ebi.ac.uk/chembl/>), Tox21 (<https://ntp.niehs.nih.gov/whatwestudy/tox21/toolbox/index.html>), and the National Center for Advancing Translational Science (<https://ncats.nih.gov/etb>), lists potential carbaryl targets at concentrations below 10 μ M to include the pregnane X receptor (potency = 3.2 μ M; Tox21) and the aryl hydrocarbon receptor (potency = 5.3 μ M; Tox21). Environmental dioxins alter circadian rhythms via aryl hydrocarbon receptor-mediated interference

of clock gene transcription and reduce phase resetting induced by light pulses in mice (Xu et al., 2010, 2013; Tischkau et al., 2011). Dioxins affect clock genes and behavioral rhythms, contributing to altered glucose metabolism, insulin resistance, and sleep disorders. However, the time of circadian sensitivity for these effects has not been reported, making comparisons with the range of sensitivity for melatonin receptor-mediated phase shifts of circadian activity difficult. Action of carbaryl at other proteins was also investigated using the National Institute of Mental Health Psychoactive Drug Screening Program screening for inhibition of radioligand binding at 40 GPCR targets, which only revealed affinity for serotonin receptor 2B (5HT_{2B}) (Besnard et al., 2012). Carbaryl affinity for 5HT_{2B} ($K_i = 5.2 \mu\text{M}$) suggests that we cannot rule out actions at this receptor; however, serotonin maximally modulates circadian phase at ZT 6 (Prosser et al., 1993; Gillette and Mitchell, 2002) outside of the windows of sensitivity to melatonin receptor ligands and there is no evidence that 5HT_{2B} activity modulates circadian biology.

Routes of exposure for carbaryl in the general population range from inhalation and dermal exposure predominately at occupational sites to oral exposure from contaminated food and water supplies on the environmental side (Hazardous Substances Data Bank, National Library of Medicine, Gunasekara et al., 2008). The level of daily exposure to carbamate insecticides in humans not occupationally exposed is reportedly 0.02–0.12 $\mu\text{g/kg}$ body weight/day or 1.4–8.4 $\mu\text{g/day}$ from dietary sources (Duggan et al., 1983; Gartrell et al., 1985, 1986; Hazardous Substances Data Bank, National Library of Medicine). Carbaryl does not accumulate in the body, as it is rapidly metabolized to 1-naphthol, which is used as a biomarker of exposure (Shealy et al., 1997; Gunasekara et al., 2008). Doses of carbaryl required to produce cholinergic effects are generally higher than the dose used in the present study (10 mg/kg, i.p.) to compete for binding at brain melatonin receptors *ex vivo* as well as to phase-shift circadian activity rhythms in C3H/HeN mice. Doses of carbaryl that produce clinically significant cholinesterase inhibition in brain and blood are equal to or above 15 mg/kg i.p. or oral gavage (Ruppert et al., 1983), with one study finding no effects of carbaryl on brain cholinesterase activity with doses up to 42.5 mg/kg via oral gavage (Wang et al., 2014). Conversely, another study by Moser et al. (2015a) found that doses of 3 and 7.5 mg/kg carbaryl via oral gavage significantly reduced brain and blood cholinesterase activity by 10%–30% and 30%–50%, respectively, compared with controls. However, many of the behavioral and physiologic effects of carbaryl attributable to effects on the cholinergic system (altered gait, reduced motor responses, convulsions, ptosis, lacrimation, salivation, chewing, decreased body temperature, etc.) are not exhibited in rodent studies until doses of at least 20–30 mg/kg given intraperitoneally or via oral gavage, with effects only on decreased pupil response and increased ptosis seen below this range at 10 mg/kg i.p. (Moser et al., 1988). Thus, it seems possible that the toxicological implications of carbaryl could be more relevant to the melatonin versus the cholinergic system based on doses required for physiological and clinically relevant effects. Future studies will determine dose relevance below 10 mg/kg i.p. to address external validity issues with matching environmentally relevant exposure doses for effects of carbaryl on phase shift of circadian rhythms and other chronobiological behaviors.

Our data highlight novel pharmacological properties of carbamate insecticides at melatonin receptors. Carbamates like carbaryl display unique pharmacological properties at melatonin receptors, suggesting their potential to alter physiologically relevant responses independent of or in addition to canonical actions at AChE activity. Based on our data we suggest that pharmacological actions of carbamate insecticides at melatonin receptors should be investigated for potential to produce 1) mistimed melatonin receptor activation, 2) alterations of melatonin rhythms by activating and/or blocking timing cues at nonoptimal times of day or by mistimed phase shifts leading to circadian rhythm desynchronization, 3) disrupted timing of physiologic processes under control of melatonin-mediated time-of-day signaling (i.e., modulation of pancreas metabolic rhythmicity), and/or 4) modulation of core clock gene transcription or rhythmicity. These changes could predispose or contribute to relevant disease pathologies observed after exposure to carbamate insecticides linked indirectly to circadian disruption, particularly those not explained by actions at AChE, such as sleep apnea (Zirlik et al., 2013; Baumert et al., 2018).

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Jones, Glatfelter, Rajnarayanan, Dubocovich.

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