

High-throughput Screening in Larval Zebrafish Identifies Novel Potent Sedative-hypnotics

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ABSTRACT

Background: Many general anesthetics were discovered empirically, but primary screens to find new sedative-hypnotics in drug libraries have not used animals, limiting the types of drugs discovered. The authors hypothesized that a sedative-hypnotic screening approach using zebrafish larvae responses to sensory stimuli would perform comparably to standard assays, and efficiently identify new active compounds.

Methods: The authors developed a binary outcome photomotor response assay for zebrafish larvae using a computerized system that tracked individual motions of up to 96 animals simultaneously. The assay was validated against tadpole loss of righting reflexes, using sedative-hypnotics of widely varying potencies that affect various molecular targets. A total of 374 representative compounds from a larger library were screened in zebrafish larvae for hypnotic activity at 10 μ M. Molecular mechanisms of hits were explored in anesthetic-sensitive ion channels using electrophysiology, or in zebrafish using a specific reversal agent.

Results: Zebrafish larvae assays required far less drug, time, and effort than tadpoles. In validation experiments, zebrafish and tadpole screening for hypnotic activity agreed 100% ($n = 11$; $P = 0.002$), and potencies were very similar (Pearson correlation, $r > 0.999$). Two reversible and potent sedative-hypnotics were discovered in the library subset. CMLD003237 (EC_{50} , ~ 11 μ M) weakly modulated γ -aminobutyric acid type A receptors and inhibited neuronal nicotinic receptors. CMLD006025 (EC_{50} , ~ 13 μ M) inhibited both *N*-methyl-D-aspartate and neuronal nicotinic receptors.

Conclusions: Photomotor response assays in zebrafish larvae are a mechanism-independent platform for high-throughput screening to identify novel sedative-hypnotics. The variety of chemotypes producing hypnosis is likely much larger than currently known. (**ANESTHESIOLOGY 2018; 129:459-76**)

GENERAL anesthesia is an essential tool in modern medicine, and there is growing interest in developing new sedative-hypnotics with improved clinical utility.^{1,2} Current and past clinical sedative-hypnotics represent a variety of anesthetic chemotypes (volatile ethers, barbiturates, phenylacetates, alkyl phenols, arylcyclohexylamines, imidazoles, and steroids), many identified by empiric observation, before 1980. Most new hypnotics in clinical development are modifications or reformulations of these drugs.¹

Drug libraries may contain many more undiscovered sedative-hypnotics. Several screening strategies to identify hypnotics have been reported, most based on established molecular targets of anesthetics, particularly γ -aminobutyric acid type A ($GABA_A$) receptors. Direct target-based screening approaches detect modulation of specific $GABA_A$ receptor subtypes expressed in cells.³⁻⁶ *In silico* screening approaches calculate binding energies between candidate ligands and pharmacophores derived from high-resolution structures of $GABA_A$ receptor or homologs.⁷⁻⁹ Another drug screening strategy detected displacement of a fluorescent anesthetic from horse apoferritin binding sites formed among

What We Already Know about This Topic

- Recent efforts to identify new sedative-hypnotics are based on activity in established molecular targets of anesthetics, mostly γ -aminobutyric acid type A receptors. However, strategies focusing on specific targets may overlook potentially useful compounds that act through other or multiple mechanisms.
- A mechanism-independent screening approach that is also inexpensive and efficient is needed to accelerate discovery of new potent sedative-hypnotics.

What This Article Tells Us That Is New

- A screening approach that detects sedative-hypnotic drug activity in zebrafish larvae, based on inhibition of movements in response to brief bright light stimuli (photomotor responses), was established and used to screen several hundred organic compounds with drug-like biophysical properties. Two novel compounds were found to potentially produce reversible sedative-hypnotic effects, one of which demonstrated hypnotic activity in rodents.
- The results suggest that testing photomotor responses in zebrafish larvae is a mechanism-independent approach for efficient discovery of novel sedative-hypnotics. Further testing of the newly discovered drugs in mammals is needed.

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α -helical bundles, similar to those in GABA_A receptors.^{10,11} These indirect hypnotic discovery strategies have used secondary electrophysiologic tests for GABA_A receptor modulation.

However, not all clinical anesthetics modulate GABA_A receptors.^{12,13} Many sedative-hypnotics apparently act *via* other molecular targets,^{14,15} and these would likely be missed by target-based screening strategies.

Stimulus-response tests in animals potentially represent a mechanism-independent screening approach for sedative-hypnotic drug activity. The most common such test in vertebrates is loss of righting reflexes, in which drug-exposed animals are placed supine and observed for return to the normal prone or four-leg standing position. Accurate pharmacodynamic measurements using loss of righting reflexes tests require steady-state drug concentrations in an animal's nervous system. In rodents, establishing steady-state drug concentrations in tissues is easy with inhaled agents delivered at defined partial pressures, but very difficult with intravenous agents. Conversely, steady-state concentrations of nonvolatile drugs are easily established in water-breathing aquatic vertebrates immersed in drug solutions. Thus, *Xenopus* tadpoles are widely used for loss of righting reflexes testing of intravenous sedative-hypnotics, but this approach is impractical for primary sedative-hypnotic screening in large numbers of drugs. In contrast, young zebrafish have proven useful for high-throughput bioassays of psychoactive drugs in libraries,^{16,17} but have not been used to screen specifically for new sedative-hypnotics.

Here, we describe development of an approach to assess sedative-hypnotic drug effects in up to 96 zebrafish larvae simultaneously using computer-controlled stimuli and quantification of video-monitored motor responses. Automated zebrafish larva hypnosis assays based on photomotor responses perform nearly identically to manual *Xenopus* tadpole loss of righting reflexes tests for both hypnotic drug screening and potency determinations, while requiring far less material, time, and effort. Applying this novel approach to a library of 374 organic small compounds identified two with reversible hypnotic activity. These newly identified sedative-hypnotics were further characterized in *Xenopus* tadpoles and a panel of molecular targets thought to mediate general anesthetic actions. To explore translational potential, limited studies of intravenous administration in rats were also performed.

Materials and Methods

Animals

Xenopus tadpoles and frogs were purchased from Xenopus One (USA) and used with approval from the Massachusetts General

Hospital Institutional Animal Care and Use Committee (Boston, Massachusetts). Adult female frogs were used as a source of oocytes for two microelectrode voltage clamp electrophysiology, while tadpoles were used in manual loss of righting reflexes experiments, as previously described.¹⁸ Zebrafish (*Danio rerio*, Tubingen AB strain; gifted from Eric Liao, M.D., Ph.D., Center for Regenerative Medicine, Massachusetts Hospital, Boston, Massachusetts) were used with approval from the Massachusetts General Hospital Institutional Animal Care and Use Committee according to established protocols.¹⁹ Adult zebrafish were maintained in a specialized aquatic facility and mated to produce embryos and larvae as needed. Embryos and larvae were maintained in Petri dishes (140 mm diameter) filled with E3 medium (in mM: 5.0 sodium chloride, 0.17 potassium chloride, 0.33 calcium chloride, 0.33 magnesium sulfate, 2 HEPES, pH 7.4) in a 28.5°C incubator under a 14/10h light/dark cycle until used in experiments. The density of embryos and larvae was fewer than 100 per dish. Experiments were performed on zebrafish larvae at up to 7 days postfertilization. After either use in experiments or at 8 days postfertilization, larvae were euthanized in 0.5% tricaine followed by addition of bleach (1:20 v:v). Adult male Sprague-Dawley rats (250 to 400 g) were purchased from Charles River Laboratories (USA) and used with approval from the Massachusetts General Hospital Institutional Animal Care and Use Committee in loss of righting reflexes tests after intravenous drug administration. Female rats were excluded from these studies, because their sensitivity to anesthetics varies with estrus cycle.

Anesthetics and Test Compounds

Etomidate was a gift from Douglas Raines, M.D. (Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts), and was prepared as a 2 mg/ml solution in 30% propylene glycol:water (v:v). Alphaxalone was purchased from Tocris Bioscience (United Kingdom). Ketamine was purchased from Mylan Pharmaceuticals (USA) as a 10-mg/ml aqueous solution with 0.1 mg/ml benzethonium chloride as a preservative. Dexmedetomidine was purchased from U.S. Pharmacopeia (USA). Propofol, pentobarbital, atipamezole, and alcohols were purchased from Sigma-Aldrich (USA). A set of 11 potent GABA_A receptor modulators (table 1)^{8,9,20–22} was a gift from Erwin Sigel, Ph.D. (Institute of Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland). A chemical compound library “diversity” subset (374 compounds) was obtained from the Boston University Center for Molecular Discovery, Boston, Massachusetts (directed by J.A.P.). Physical properties^{23,24} of these compounds are as follows (mean \pm SD [range]): molecular weight [MW] = 390 \pm 101 (162 to 799); calculated LogP = 3.8 \pm 1.6 (–0.13 to 12.9); polar surface area (\AA^2) = 66 \pm 24 (16 to 160); hydrogen-bond (H-bond) donors = 0.87 \pm 0.85 (0 to 5); and H-bond acceptors = 3.9 \pm 1.5 (1 to 10). Library compounds were provided on 384-well plates as 0.2 micromoles dried film, and were reconstituted in 40 μ l dimethyl sulfoxide (DMSO) as 5-mM solutions. Examination under a dissecting microscope was used to confirm complete dissolution of each compound.

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Table 1. Potent γ -Aminobutyric Acid Type A Receptor Modulators Tested in Tadpoles and Zebrafish Larvae

Compound No.	Reference	Name of Compound in Reference
1	Ramerstorfer <i>et al.</i> ²⁰	CGS-9598
2	Middendorp <i>et al.</i> ⁸	Compound 11
3	Kopp <i>et al.</i> ²¹	Valerenic acid derivative-10
4	Middendorp <i>et al.</i> ⁸	Compound 20 (structure not shown)
5	Maldifassi <i>et al.</i> ⁹	Compound 31
6	Maldifassi <i>et al.</i> ⁹	Compound 132
7*	PubChem Compound Database	PubChem CID: 43947938
8	Middendorp <i>et al.</i> ⁸	Compound 67
9	Baur <i>et al.</i> ²²	4-O-methylhonokiol
10*	PubChem Compound Database	PubChem CID: 18593928
11*	PubChem Compound Database	PubChem CID: 3878620

*Compounds 7, 10, and 11 have not been described in previous publications. Modulation of γ -aminobutyric acid receptor type A by these compounds was confirmed by Constanza Maldifassi, Ph.D. (Center for Interdisciplinary Neuroscience, University of Valparaíso, Valparaíso, Chile; personal communication). Compound information and available commercial vendors can be found in PubChem Compound Database (<https://pubchem.ncbi.nlm.nih.gov/>).

Chemicals

Salts, buffers, γ -aminobutyric acid (GABA), acetylcholine, *N*-methyl-D-aspartate (NMDA), and glycine were purchased from Sigma-Aldrich.

Loss of Righting Reflexes Assays in Tadpoles and Rats

General anesthetic potency was assessed in *Xenopus* tadpoles at room temperature (22°C) as previously described.²⁵ For each anesthetic concentration studied, 8 or 10 animals were studied, based on previous experience. Groups of four to five tadpoles per container were placed in aqueous solutions (20 ml per animal; fig. 1A) containing known sedative-hypnotics or experimental compounds and tested every 5 min for 30 min. Loss of righting reflexes was assessed by gently turning each animal supine using a polished glass rod. Absence of swimming and/or turning prone within 5 s was counted as loss of righting reflexes. We recorded the loss of righting reflexes count/total animals as a function of time after immersion in drug. In screening tests for hypnotic activity, tadpoles were exposed to 10 μ M drug. Drugs were considered active if at least 50% of animals demonstrated loss of righting reflexes after 30 min of exposure. Concentration-dependent tadpole loss of righting reflexes results were analyzed based on results at 30 min. Individual binary results (1 for loss of righting reflexes; 0 otherwise) were tabulated and analyzed by fitting logistic functions [$Y = \text{Max} * 10^{\wedge} (\text{nH} * \log[\text{drug}]) / (10^{\wedge} (\text{nH} * \log[\text{drug}]) + 10^{\wedge} (\text{nH} * \log[\text{EC}_{50}]))$] using nonlinear least-squares (GraphPad Prism 6.0, GraphPad Software, USA). We report mean EC_{50} (95% CI). After 30 min of drug exposure and final loss of righting reflexes testing, animals were returned to clean water and

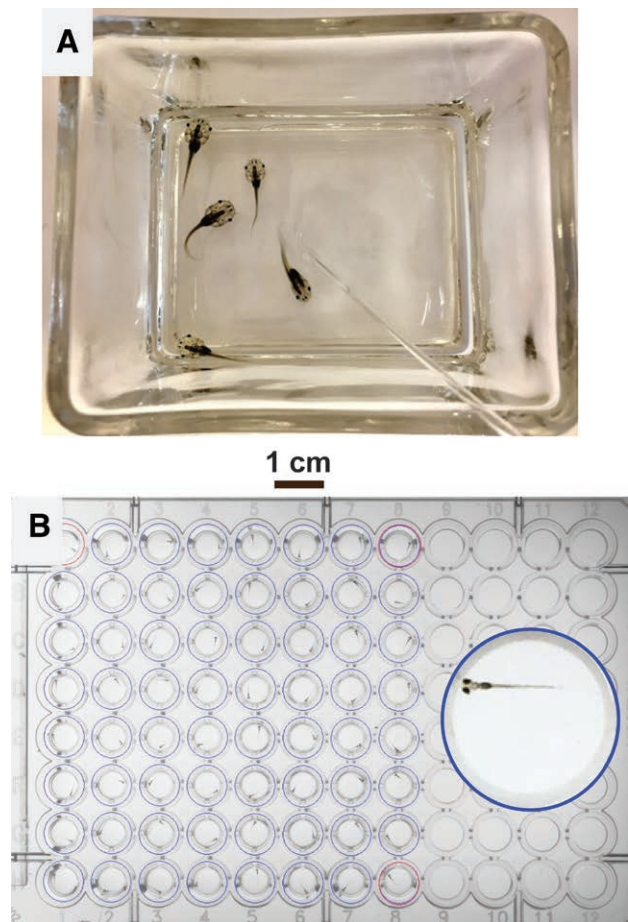


Fig. 1. Tadpoles versus zebrafish larvae. (A) A glass container holding five pre-limb bud stage *Xenopus* tadpoles in 100 ml water. The polished glass rod, visible in the lower right quadrant of the photograph, is used to manually turn the animals during loss of righting reflexes tests. (B) A 96-well plate loaded with 64 zebrafish larvae, 1 larva per well in 0.2 ml E3 buffer each. The inset shows a magnified view of one well containing a 7-day postfertilization larva.

observed for 24 h in order to establish whether drug effects were reversible. In cases where animals did not survive for 24 h after drug exposure, we repeated experiments in additional groups of animals to confirm whether toxic effects were consistently observed.

For two novel compounds that produced reversible sedation and hypnosis in both zebrafish larvae and tadpoles, limited initial tests of hypnotic efficacy were also performed in rats, using loss of righting reflexes assays.²⁶ Rats were briefly (less than 5 min) anesthetized by isoflurane inhalation in order to place a 24-gauge intravenous catheter in a tail vein. After recovery from isoflurane for at least 60 min in room air, rats were gently restrained. Before drug administration, intravenous cannulation was confirmed by gentle aspiration of blood and resistance-free injection of 0.25 ml normal saline. The desired dose of test drug in dimethyl sulfoxide vehicle (0.1 to 0.5 ml) was then injected in less than 5 s, followed by a 1-ml normal saline flush. Immediately after drug

delivery, rats were removed from restraint and turned supine. A rat was judged to have loss of righting reflexes if it failed to right (*i.e.*, turn itself back onto all four paws) within 5 s after being turned. Both the latency to loss of righting reflexes after saline flush and the duration of loss of righting reflexes, defined as the time from loss of righting reflexes onset until the animal spontaneously returned to a four-paw upright stance, were measured with a stopwatch. Because our goal was to establish whether these drugs could induce hypnosis using limited available amounts of the test compounds, only one or two rats were studied at each dose.

Zebrafish Larvae Photomotor Response Assays

Using a 1,000- μ l pipetter fitted with a cut and fire-polished tip, single zebrafish larvae (7 days postfertilization, sex indeterminate) were placed into wells of a standard 96-well plate containing 150 μ l E3 buffer. Known anesthetics and test compound stocks were prepared in DMSO, and then diluted in E3 buffer to four times the desired final concentration. A multipipette was used to load 50 μ l of four times solutions into wells, bringing the final volume to 200 μ l (fig. 1B). Final DMSO concentrations were no more than 0.2%.

Immediately after addition of drugs (less than 5 min), the 96-well plate loaded with larvae was placed in a Zebrabox (Viewpoint Behavioral Systems, Canada) and adapted at 28°C in the dark chamber for 15 min. During experiments, activity of individual larvae was recorded with an infrared video camera and analyzed using Zebralab v3.2 software (Viewpoint Behavioral Systems). Basal activity in the darkened chamber was recorded for 5 to 10 s, followed by a 0.2-s exposure to a 500-lux white light stimulus, and another 5 to 10 s in the dark. Each animal was tested in this manner up to 10 times at 3-min intervals. Zebralab software quantifies each animal's motor activity by assessing changes in infrared image pixel intensity (on a scale of 1 to 256) of all pixels corresponding to the image area of its circular well, between sequential video sweeps (every 40 ms). An activity score is calculated by summing the absolute values of pixel intensity changes over the whole well. Activity integration is a Zebralab output that sums activity scores over multiple video sweeps during an experimentally defined epoch. For larval photomotor response experiments, we used activity integration epochs of 0.2 to 1.0 s and normalized activity scores for 0.2-s epochs (*e.g.*, the activity score for a 1-s epoch was reduced fivefold).

To establish a binary photomotor response outcome, we calculated the mean and SD for prestimulus basal activity (5 to 10 s per trial, up to 10 trials, normalized to 0.2-s epochs) for individual larvae. Photomotor response for a single trial was scored as positive (1) if activity during any of the three 0.2-s epochs during and after the photic stimulus exceeded the upper 95% CI (mean + 2 \times SD) for basal activity. Otherwise, photomotor response was scored as negative (0). Cumulative photomotor response probabilities for each larva were calculated by pooling single trial photomotor response results from multiple sequential trials. For statistical analyses, results from

all larvae in an exposure group were pooled. D'Agostino and Pearson normality tests performed on cumulative photomotor response probabilities from studies using eight or more animals per group indicated normally distributed results when not naturally skewed toward either 1.0 (in control conditions) or 0 (with high concentrations of hypnotics).

Screening for Hypnotic Drug Activity Using Larval Zebrafish Photomotor Responses

The hypnotic effects of compounds at 10 μ M (in 0.2% DMSO) were tested in groups of 8 to 12 zebrafish larvae. Each plate included 6 to 10 test compounds, a negative control group in 0.2% DMSO, and a positive control group in 10 μ M etomidate. Individual larva single trial photomotor response responses were tabulated for four trials, and averaged to calculate cumulative photomotor response probabilities. The photomotor response probabilities for all larvae in a drug-exposed group were combined to calculate mean and variance (SD or 95% CI) statistics. Drug-exposed group results were compared to those from the negative control (no drug) group using one-way ANOVA (GraphPad Prism 6.0) with Dunnett's *post hoc* test. Pairwise *P* values were calculated using unpaired two-tailed Student's *t* tests. Compounds that inhibited photomotor response relative to control with *P* < 0.05 (adjusted using a Bonferroni correction for multiple comparisons) were studied further to establish hypnotic potency and reversibility.

To calculate the power of our drug screening approach, we performed an analysis as follows: Photomotor response experiments with no drug in 96 larvae tested four times each revealed mean cumulative photomotor response probability of 0.89 with SD of 0.17 (see Results). With eight larvae per group, and α = 0.005 (applying a Bonferroni correction for 10 comparisons to each control) in a two-tailed *t* test, a power calculation (using G*Power v 3.08, University of Dusseldorf, Dusseldorf, Germany) indicated 0.95 probability (1 – β) of detecting a 0.45 absolute (50% relative) reduction in photomotor response probability (effect size = 2.6).

Concentration-response Studies Using Larval Zebrafish Photomotor Responses

Larvae in groups of 8 to 12 were exposed to either control (no drug) or varying concentrations of drug. When drug stock solution (usually greater than 100 mM) was in DMSO, all control and final drug solutions included the same DMSO concentrations (less than or equal to 0.1%). Cumulative photomotor response probability for each animal was established as described above from four trials. Results (mean with 95% CI) for all animals in each exposure group were calculated and plotted against log[drug]. Concentration-dependent photomotor response inhibition was analyzed by fitting logistic functions to pooled photomotor response probability data using nonlinear least-squares (see above tadpole loss of righting reflexes analysis). We report mean hypnotic EC₅₀ (95% CI).

Drug Effects on Spontaneous Activity of Zebrafish Larvae

Spontaneous activity data from prestimulus baseline periods were used to assess the sedative potency of tested drugs. Activity integration values for all 25 0.2-s prestimulus epochs per trial were pooled across all four trials and all animals in a drug exposure group. These data were used to calculate mean and variance (SD or 95% CI) statistics. Combined spontaneous activity data were normalized to the mean value for no-drug controls on the same plate. Spontaneous activity in drug-exposed larvae were compared to no-drug controls, and drug-dependent inhibition of spontaneous activity was analyzed using logistic fits, as described above for photomotor response results.

Drug Effect Reversibility in Zebrafish Larvae

The reversibility of photomotor response inhibition was tested in zebrafish larvae exposed to the highest drug concentrations used in concentration-response studies. These larvae were carefully transferred to Petri dishes containing fresh E3 medium and placed in an incubator used to maintain embryos and larvae. Larvae were repeatedly tested for motor reactivity to a gentle tap on the Petri dish at 15 and 30 min after drug exposure, and again 24 h later. In cases where drug-exposed zebrafish larvae did not survive for 24 h, additional groups of animals were tested to establish whether toxic effects were reproducible.

Library Hit Validation

Active library compounds from zebrafish screening were validated using new freshly supplied aliquots of the original stock from the Boston University Center for Medical Discovery, Boston, Massachusetts. Compound identity and purity were confirmed using ultra-performance liquid chromatography–mass spectrometry with a “passing” threshold of 90% purity as measured using evaporative light scattering detection. Activity and potency of the fresh aliquots were confirmed in zebrafish photomotor response assays. Tadpole and rat loss of righting reflexes assays were performed using freshly prepared compounds, generated using published methods, at greater than 98% purity as determined by nuclear magnetic resonance spectroscopy and ultra-performance liquid chromatography–mass spectrometry analysis. CMLD003237 was prepared according to the published protocol, which produces the (+) enantiomer in 90% enantiomeric excess and greater than 11:1 diastereomeric ratio.²⁷ CMLD003237 prepared for tadpole and rat studies was further purified by flash column chromatography to greater than 20:1 diastereomeric ratio. CMLD006025 and its enantiomer CMLD011815 were prepared according to published protocols.^{28–30}

Ion Channel Expression

DNA plasmids encoding human NMDA receptor subunits NR1B and NR2A were obtained from Steven Treistman, Ph.D. (University of Massachusetts Medical School, Worcester, Massachusetts). Plasmids encoding the human neuronal nicotinic acetylcholine receptor subunits $\alpha 4$ and $\beta 2$ were obtained from

James Patrick, Ph.D. (Salk Institute, La Jolla, California). Plasmids encoding human hyperpolarization cyclic nucleotide-gated (HCN1) channels were a gift from Peter Goldstein, M.D. (Weill Cornell Medical College, New York, New York). Human $\alpha 1$, $\beta 3$, and $\gamma 2$ GABA_A receptor subunits were inserted into pCDNA3.1 expression vectors (Thermo Fisher Scientific, USA). Human glycine receptor $\alpha 1$ subunit coding DNA was cloned from whole brain messenger RNA (mRNA; Thermo Fisher Scientific) using polymerase chain reaction and inserted into pCDNA3.1. Capped mRNAs were transcribed *in vitro* using mMessage Machine kits (Thermo Fisher Scientific). For NMDA receptor studies, oocytes were injected with 15 ng in 1:1 mRNA mixtures of NR1B:NR2A; for neuronal nicotinic acetylcholine receptor studies, 15 ng of $1\alpha 4:1\beta 2$; for GABA_A receptors, 5 ng total of $1\alpha 1:\beta 3:\gamma 2$; for HCN1 channels, 15 ng HCN1; and for glycine receptors, 0.015 ng $\alpha 1$ subunit mRNA. Oocytes were incubated in ND96 solution (in mM: 96 sodium chloride, 4 potassium chloride, 1.8 calcium chloride, 1.0 magnesium chloride, and 5 HEPES, pH 7.5) supplemented with 100 μ g/ml gentamicin at 18°C for 48 to 96 h before electrophysiology.

Voltage-clamp Electrophysiology

Two microelectrode electrophysiology techniques for GABA_A receptors, NMDA receptors, and neuronal nicotinic acetylcholine receptors have been described previously.^{31,32} Experiments were performed at 20 to 22°C in ND96 buffer (Mg^{2+} -free ND96 was used in NMDA receptor experiments). Positive modulation of $\alpha 1\beta 3\gamma 2$ GABA_A receptors was assessed using activation with EC5 γ -aminobutyric acid (GABA; 3 μ M, activating 5% of maximal current). Positive modulation of glycine $\alpha 1$ receptors was assessed with EC5 glycine (1 μ M). Inhibition of $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors was tested using maximal activation conditions (1 mM acetylcholine). Inhibition of NMDA receptors was tested using maximal activation (100 μ M NMDA plus 10 μ M glycine) in Mg^{2+} -free ND96. Voltage-dependent HCN1 currents were stimulated using a previously described voltage-jump protocol, starting and ending at a holding level of -40 mV.³³ In all cases, oocytes were pre-exposed to drug for 30 s before receptor activation. Voltage-clamped currents were low-pass filtered at 1 kHz, digitized at 100 Hz, recorded, and analyzed offline to evaluate baseline-corrected peaks. Peak currents were normalized to responses measured in the same cell without drug. All hypnotic compounds were tested at $2 \times EC_{50}$ for loss of righting reflexes in zebrafish or tadpoles. Drug effects on GABA_A receptors were compared to those produced by 3.2 μ M etomidate ($2 \times EC_{50}$) in the same cells. Drug effects on glycine receptors and HCN1 channels were compared to 4.5 μ M propofol ($2 \times EC_{50}$). Drug effects in NMDA and neuronal nicotinic acetylcholine receptors were compared to 120 μ M ketamine ($2 \times EC_{50}$) in the same cells. The number of electrophysiologic experiments needed to detect either a doubling of GABA or glycine receptor EC5 responses (an effect size of about three) or 30% inhibition of maximal responses in

other channels (also an effect size of about three) was determined to be four, based on power analysis (using G*Power v 3.08) for a two-tailed t test with $1 - \beta = 0.8$ and $\alpha = 0.025$ (using a Bonferroni correction for two comparisons). Thus, at least four cells were used for each experimental condition in each receptor type. The number of cells in specific experiments is reported in the figure legends. One-way ANOVA was applied for statistical comparisons and Student's t tests were used to calculate pairwise P values.

Atipamezole Reversal Tests

To test compounds for α_2 -adrenergic receptor agonist activity, we used the selective α_2 -adrenergic receptor antagonist atipamezole at 10 nM and tested for reversal of hypnosis in groups of zebrafish larvae ($n = 8$ or 12 per group). The 10-nM atipamezole concentration was chosen based on concentration-response studies in combination with $2.5 \times EC_{50}$ dexmedetomidine (1.0 μ M) and control experiments with other sedative-hypnotics that confirmed specificity (see Results).

Statistical Analyses

Statistical methods used in drug screening and concentration-response analysis are described above. In comparing zebrafish photomotor response inhibition and tadpole loss of righting reflexes results, the concordance of binary (significant inhibition or not) drug screening outcomes was assessed using Cohen's Kappa with Fisher exact test for statistical significance. Multiple drug potencies (mean EC_{50} s or $\log[EC_{50}$ s]) in zebrafish *versus* tadpoles were compared with Pearson correlations. Drug effects on ion channels were compared to positive and negative controls using ANOVA with Dunnett's test for multiple comparisons, and pairwise P values were calculated using two-tailed paired Student's t tests. These analyses and nonlinear least-squares logistic fits were performed using GraphPad Prism 6.0. Results are reported as mean \pm SD or 95% CI. Some graphs display unidirectional 95% CI, for clarity. In these cases, the CIs are symmetrical around the mean. There were no missing data associated with statistical analyses. No outlier data were detected in our analyses.

Results

Development of a Photomotor Response Assay Using Zebrafish Larvae

Our initial goal was to develop a high-throughput assay for hypnotic drug activity based on stimulus-response in zebrafish larvae, and to validate it against standard *Xenopus* tadpole loss of righting reflexes tests. Based on previous published work with zebrafish larvae,^{16,34,35} we tested both acoustic/vibration and photic stimuli in larvae ranging in age from 4 to 7 days postfertilization. Motor responses to acoustic/vibration stimuli (taps delivered with a solenoid) were consistent under control conditions, but were not fully extinguished by 10 μ M etomidate or 10 μ M propofol (data not shown), both

of which fully inhibit righting reflexes in pre-limb bud stage *Xenopus* tadpoles. Brief flashes of bright (500-lux) white light also elicited motor responses in dark-adapted zebrafish larvae (fig. 2A). The magnitude of activity after photic stimuli was smaller and less consistent than that after tap stimuli, but was fully extinguished by either etomidate or propofol at 10 μ M. Experiments in larvae from 3 to 7 days postfertilization indicated that photomotor responses were more consistent in older animals with more mature visual systems (data not shown). All subsequent experiments used 7 days postfertilization larvae. We used a single animal per well in 96-well plates, to avoid activity triggered by contact with other moving animals.

To quantify hypnotic drug effects on the photomotor response, we first tried averaging the peak activity level during and after light stimulus for drug-exposed groups and normalizing to the nondrug control group. However, stimulated activity levels varied widely among animals and among repeated trials in single animals (e.g., fig. 2B). Baseline motor activity also varied among larvae and was inhibited by increasing sedative-hypnotic drug concentrations. To minimize these sources of variability and mimic tadpole loss of righting reflexes tests, we established a rigorous binary outcome for each photomotor response trial. Each larva's motor activity in three 0.2-s epochs both during and immediately after photic stimulus was compared to the upper 95% CI for spontaneous activity in all 0.2-s epochs during prestimulus baseline periods (fig. 2B). By testing each animal in multiple trials, we calculated cumulative photomotor response probabilities. Drug effects on spontaneous motor activity, as a measure of sedation, were independently analyzed (see "Materials and Methods").

We tested the effect of repeating photomotor response trials up to 10 times using 96 zebrafish larvae, aiming to minimize outcome variance. Under control conditions, desensitization to the light stimulus was observed with repeated trials. This effect weakened as the intertrial interval increased from 30 s to 3 min. However, intervals of 3 to 12 min all produced similar drops in photomotor response probability from more than 90% in the first trial to less than 60% at the tenth trial (fig. 3A; *open symbols*). Cumulative photomotor response probability with increasing numbers of trials at 3-min intervals is also shown in figure 3A (*solid symbols*). Linear regression analysis indicated a nonzero slope for cumulative photomotor response probability from four trials (slope = -0.0247 ± 0.0022 ; $P = 0.0082$) to 10 trials (slope = -0.0301 ± 0.0009 ; $P < 0.0001$). The cumulative photomotor response probability variance (SD) remained stable for up to four trials, and then monotonically increased with each added trial as the effects of desensitization grew (fig. 3B). Cumulative photomotor response probability associated with four trials was only 7% lower than that from the initial control trial. Thus, we used four trials with a 3-min interval in subsequent photomotor response experiments. With this approach, desensitization to repeated photic stimuli was absent in larvae exposed to hypnotic concentrations

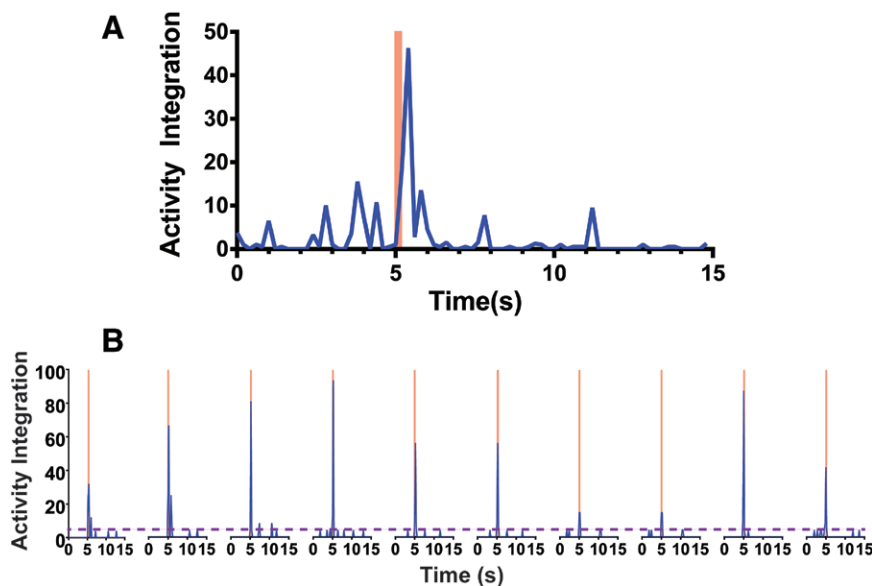


Fig. 2. Photomotor responses in 7 days postfertilization zebrafish larvae. Activity integration (blue lines) is a Zebralab experimental output totaling the number and intensity of pixel changes in a designated image area between sequential infrared video sweeps during a 0.2-s experimental epoch (each epoch includes five video sweeps lasting 0.04 s). (A) Average activity integration for eight larvae in E3 buffer with 0.2% DMSO (control conditions) is plotted at 0.2-s intervals during a single photomotor response trial. A 500-lux white light stimulus was activated at 5 s and discontinued at 5.2 s (pink bar). Note that activity dramatically increased during the photic stimulus and diminished within 1 s. (B) Activity of a single larva during a series of 10 photomotor response trials, with 3-min intervals between trials, is shown. Pink bars indicate photic stimuli. The purple dashed line indicates the upper 95% CI for mean baseline activity during all 10 prestimulus epochs (5 s each). Activity during and after photic stimuli was consistently above the 95% baseline threshold in all trials, while varying in magnitude from trial to trial.

of etomidate (fig. 3C; slope = -0.008 ± 0.0195 ; $P = 0.67$; $n = 12$) or equihypnotic solutions of dexmedetomidine (slope = -0.002 ± 0.012 ; $P = 0.85$; $n = 12$), ketamine (slope = -0.002 ± 0.019 ; $P = 0.88$; $n = 12$), alphaxalone (slope = 0.013 ± 0.019 ; $P = 0.48$; $n = 12$), tricaine (slope = -0.002 ± 0.015 ; $P = 0.86$; $n = 12$), and butanol (slope = -0.002 ± 0.019 ; $P = 0.88$; $n = 12$). These results indicate both that all these sedative-hypnotics inhibit mechanisms underlying photomotor response desensitization to repeated stimuli and that a 15-min drug exposure before photomotor response testing establishes steady-state drug concentrations in larval nervous tissues.

The optimized photomotor response assay provided two measures of concentration-dependent drug action in a single experiment: sedation measured from inhibition of spontaneous motor activity, and hypnosis from inhibition of the photomotor response. Figure 3D shows results and logistic analyses from combined data in groups of zebrafish larvae exposed to varying concentrations of etomidate. Sedation by etomidate requires sixfold lower concentrations than hypnosis, while Hill slopes are comparable for both effects.

Validation of Photomotor Response Inhibition Against Tadpole Loss of Righting Reflexes

Our first validation of the zebrafish larva photomotor response assay used a set of nine sedative-hypnotic compounds with previously published potencies in tadpole loss of righting reflexes tests: ethanol,³⁶ butanol,³⁶ hexanol,³⁶

ketamine,³⁷ propofol,³⁸ etomidate,³⁹ pentobarbital,⁴⁰ dexmedetomidine,⁴¹ and alphaxalone.⁴² These hypnotics are characterized by loss of righting reflexes EC_{50} s ranging from low micromolar to high millimolar and effects at a variety of molecular targets.^{14,43} Photomotor response concentration-response experiments ($n = 10$ larvae per condition) showed that for all drugs except dexmedetomidine, EC_{50} for photomotor response inhibition in zebrafish larvae was within a factor of three of the published EC_{50} for tadpole loss of righting reflexes (fig. 4A). The large discrepancy between the published loss of righting reflexes EC_{50} for dexmedetomidine (mean \pm SD, $7 \pm 1.1 \mu\text{M}$)⁴¹ and the photomotor response EC_{50} (mean, $0.4 \mu\text{M}$) led us to retest dexmedetomidine in *Xenopus* tadpoles, resulting in an EC_{50} of $0.66 \mu\text{M}$ (95% CI, 0.28 to $1.56 \mu\text{M}$; $n = 10$ per concentration). The Pearson correlation coefficient for drug potencies in zebrafish versus tadpoles (using our value for dexmedetomidine in tadpoles) was 0.999 ($P < 0.0001$), reflecting remarkably close agreement.

To test the utility of zebrafish photomotor responses in screening new potent hypnotic compounds, we used a second group of 11 compounds that were all recently identified as potent modulators of GABA_A receptors (table 1), and that had been tested for hypnotic activity and potency using tadpole loss of righting reflexes tests.⁹ Results of previous tadpole loss of righting reflexes tests at $10 \mu\text{M}$ identified five compounds with hypnotic activity and six without. Screening these 11 compounds for hypnotic activity using zebrafish

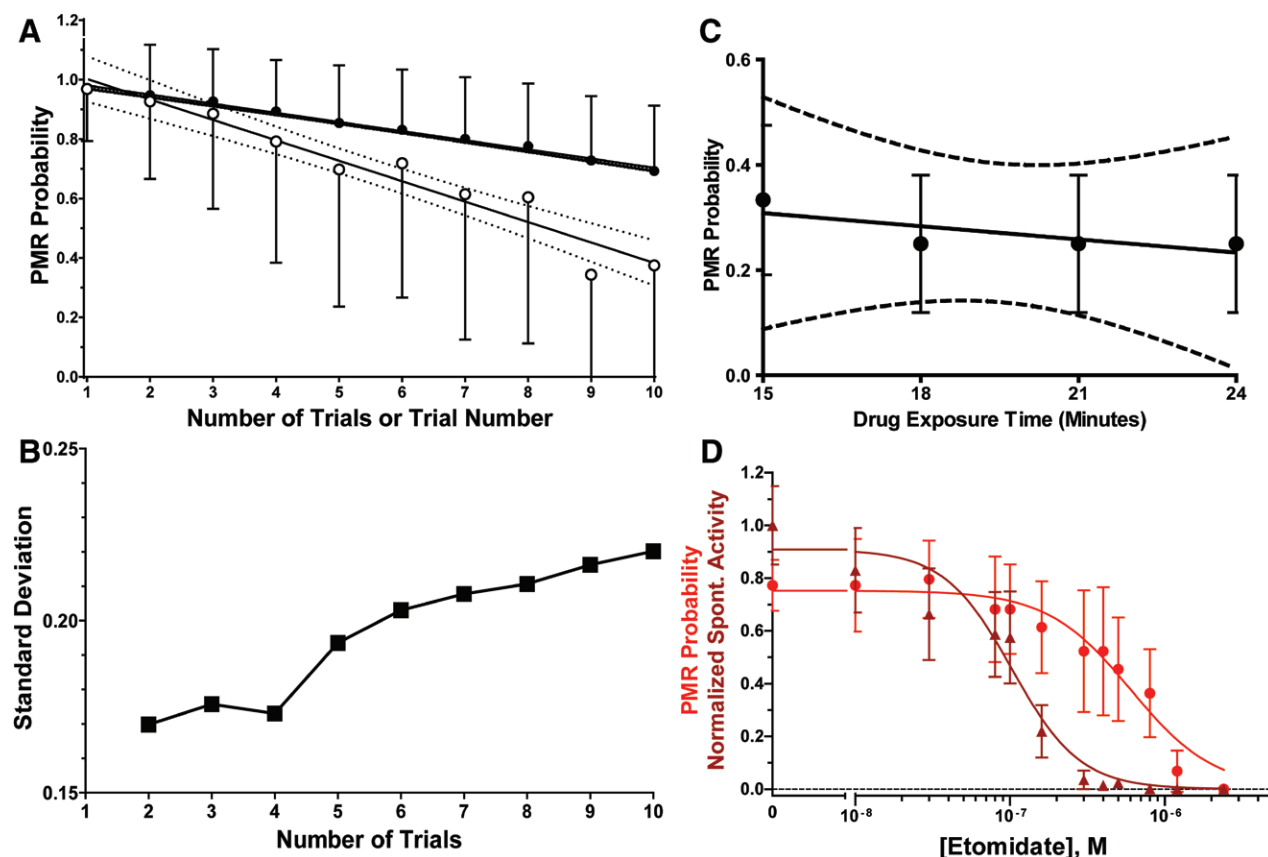


Fig. 3. Photomotor response probability and variance with repeated trials are affected by sedative-hypnotic drugs. (A) Photomotor responses (PMRs) were tested 10 times with 3-min intervals between trials in larvae ($n = 96$) in E3 buffer with 0.2% DMSO (no-drug control conditions). The single trial PMR probability (open circles; mean \pm SD) monotonically decreases with each repetition (trial number) and cumulative PMR probability (solid circles; mean \pm SD) decreases with the number of included trials, as larvae desensitize to the photic stimulus. Solid lines through the plotted points are linear regression fits, and dashed curves are 95% CI for the fitted lines. The single PMR trial slope (mean \pm SD) = -0.069 ± 0.0062 and cumulative PMR trial slope = -0.0301 ± 0.0009 . Both slopes are nonzero ($P < 0.0001$ by linear regression). A linear fit to cumulative PMR probabilities for only the first four trials gives slope (mean \pm SD) = -0.025 ± 0.0022 , which is also nonzero ($P = 0.0082$). (B) SDs from cumulative PMR probability data in A are plotted against trial number, showing that variance increases after more than 4 trials. (C) Single PMR probability trial results (mean \pm SD; $n = 16$) in a group of zebrafish larvae exposed to $1.5 \mu\text{M}$ etomidate and tested four times at 3-min intervals are plotted against drug exposure time, which includes a 15-min pretest exposure period. The fitted (solid) line to data has a slope (mean \pm SD) = -0.008 ± 0.020 , which is not significantly different from zero ($P = 0.67$ by linear regression). The 95% CIs for the fitted line are drawn as dashed lines. (D) An example of experimental results showing etomidate-dependent inhibition of both spontaneous (Spont.) activity (dark red triangles) and cumulative PMR probability (red circles). Points represent mean with 95% CI for 10 animals per concentration, each tested in four PMR trials at 3-min intervals. Lines through data represent logistic fits. Inhibition of spontaneous activity is characterized by $\text{EC}_{50} = 0.10 \mu\text{M}$ (95% CI, 0.080 to $0.12 \mu\text{M}$) and $nH = 2.0 \pm 0.37$. Inhibition of PMR probability is characterized by $\text{EC}_{50} = 0.6 \mu\text{M}$ (95% CI, 0.45 to $0.81 \mu\text{M}$) and $nH = 1.6 \pm 0.40$.

photomotor response assays produced identical positive and negative screening results (table 2). The concordance of the two approaches was 100% with Cohen's Kappa = 1.000 ($P = 0.0022$ by Fisher exact test).

Potencies (EC_{50} s) for the five hypnotic GABA_A receptor modulators in both tadpoles and zebrafish agreed remarkably closely (fig. 4B; Pearson correlation $r = 0.999$; $P < 0.0001$).

Discovery of New Sedative-hypnotic Compounds in a Drug Library Screen

Our second major aim was to use zebrafish larvae photomotor response assays to screen for new sedative-hypnotic

compounds in a drug library. We obtained a library of 2,651 compounds from the Boston University Center for Medical Discovery (Boston, Massachusetts), including a "diversity set" of 374 compounds selected randomly to represent the variety of chemotypes in the larger collection. We screened the diversity set using eight larvae per compound, comparing photomotor response results for up to 10 test compounds to a negative (no-drug) control group on the same plate. We found two compounds that, at $10 \mu\text{M}$, inhibited photomotor response probability in zebrafish larvae by more than 50%. Larvae exposed to the first of these compounds (DS68; CMLD003288) at $10 \mu\text{M}$ died within 24 h of exposure.

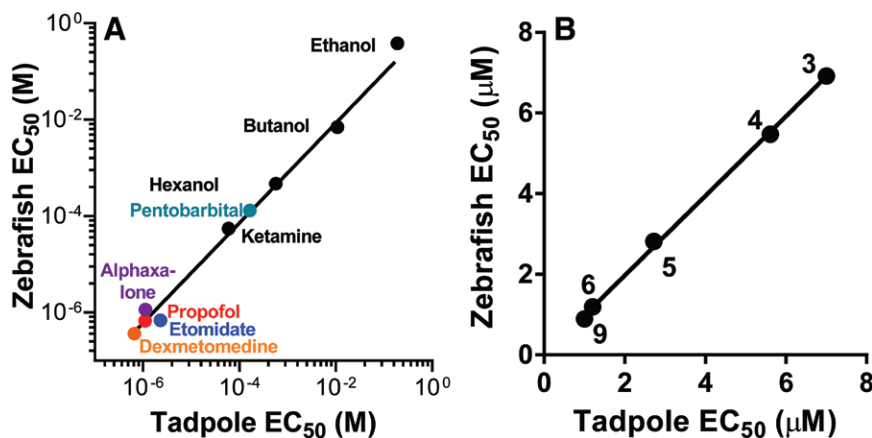


Fig. 4. Correlation of hypnotic potencies in tadpoles and zebrafish. (A) Zebrafish photomotor responses were reversibly inhibited by known anesthetics with potencies closely correlated to published tadpole loss of righting reflexes EC₅₀s (log[EC₅₀] Pearson correlation $r = 0.999$, $R^2 = 0.998$, $P < 0.0001$). Citations for tadpole EC₅₀s are as follows: ethanol,³⁶ butanol,³⁶ hexanol,³⁶ ketamine,³⁷ propofol,³⁸ etomidate,³⁹ pentobarbital,⁴⁰ and alphaxalone.⁴² Dexmedetomidine EC₅₀ in tadpoles was determined by the authors. (B) In a set of five γ -aminobutyric acid type A receptor modulators displaying potent hypnotic activity in tadpole screening tests (table 2), a very strong correlation is observed in comparison to potencies in zebrafish (EC₅₀ Pearson correlation $r = 0.9995$, $R^2 = 0.999$, $P < 0.0001$). Table 1 provides citations for the specific compounds, indicated by label number.

Table 2: Zebrafish versus Tadpole Screening for Hypnotic Activity in γ -Aminobutyric Acid Type A Receptor Modulators

		Tadpole LoRR		
		Yes	No	Total
Zebrafish PMR	Yes	5	0	5
	No	0	6	6
	Total	5	6	11

A set of 11 potent γ -aminobutyric acid type A receptor modulators (table 1 for details and references) were scored as demonstrating or lacking hypnotic activity at 10 μ M. Manual tadpole loss of righting reflexes (LoRR) tests were scored as positive if 5 or more of 10 animals lost righting reflexes after 30 min of immersion in test solution. Zebrafish larvae photomotor response (PMR) inhibition was performed on eight animals per compound with five or six experimental drugs simultaneously tested against a negative control (E3 buffer with 0.2% dimethyl sulfoxide) and a positive control (10 μ M etomidate). The PMR outcomes were scored based on ANOVA comparisons to the negative control group ($P < 0.05$). Concordance between zebrafish larva PMRs and tadpole LoRRs for identifying hypnotics among this group of compounds was 100% (Cohen's Kappa = 1.000; $P = 0.0022$ by Fisher exact test).

After confirmation of this toxicity in a second group of zebrafish larvae, we discontinued study of this compound. A second active compound (DS85; CMLD003237; methyl ((3*S*,4*R*,*E*)-4-nitro-1-phenylpent-1-en-3-yl)carbamate) induced fully reversible photomotor response inhibition in zebrafish larvae at 10 μ M (fig. 5A). A third compound (DS151; CMLD006025; (1*R*,4*S*,4*aS*,9*aS*,11*R*)-11-hydroxy-3-isopropyl-11-methyl-4,4*a*,9,9*a*-tetrahydro-1*H*-1,4-ethanofluoren-10-one) produced only about 30% inhibition of photomotor response when screened at 10 μ M (fig. 5B). However, the screening data revealed that this compound inhibited spontaneous motor activity by more than 90% at 10 μ M (fig. 5C). Retesting CMLD006025 at 20 μ M revealed an 85% reduction of control photomotor response probability (mean \pm SD, 0.13 ± 0.21 vs. 0.83 ± 0.13 ; $P < 0.0001$; $n = 8$ per group).

Characterization of New Sedative-hypnotics

Physical properties of CMLD003237 are as follows: MW = 264.1 Da; calculated LogP = 2.5; polar surface area = 81.5 Å²; 1 H-bond donor; and 3 H-bond acceptors. Concentration-dependent studies of CMLD003237 in zebrafish larvae revealed EC₅₀ = 3 μ M for inhibition of spontaneous activity and EC₅₀ = 11 μ M for inhibition of photomotor response (fig. 6A). A second fresh sample of CMLD003237 was retested to confirm activity in zebrafish, and a newly synthesized batch was used to test hypnotic effects in *Xenopus* tadpoles. CMLD003237 at up to 30 μ M reversibly inhibited tadpole righting reflexes, with an EC₅₀ of 12 μ M (fig. 6B), close to the value for photomotor response inhibition. Tadpole loss of righting reflexes results also confirm that CMLD003237 inhibits responses to multiple sensory stimuli.

To investigate possible molecular mechanisms underlying the sedative-hypnotic actions of CMLD003237, we tested effects of hypnotic concentrations ($2 \times$ photomotor response EC₅₀ = 22 μ M) on the activity of various neuronal receptors that are sensitive to potent sedative-hypnotic drugs and also likely mediators of their effects. CMLD003237 modulated $\alpha 1\beta 3\gamma 2L$ GABA_A receptors, enhancing EC5 GABA-elicited currents by a factor of 3.0 ± 0.50 (fig. 6C; mean \pm SD; $n = 5$; $P < 0.0001$ by one-way ANOVA). For comparison, an equipotent concentration of etomidate (3.2 μ M) produced much more gating enhancement in GABA_A receptors (14 ± 1.7 -fold; mean \pm SD; 95% CI, 12.2 to 16.5; $n = 5$; $P < 0.0001$ vs. both control and CMLD003237). CMLD003237 inhibited glycine $\alpha 1$ receptor currents by around 50%, in contrast to positive modulation by propofol (fig. 6D). CMLD003237 did not affect the activity of NR1B/NR2A NMDA receptors (fig. 6E) and inhibited human $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors by around 20% (fig. 6F). CMLD003237

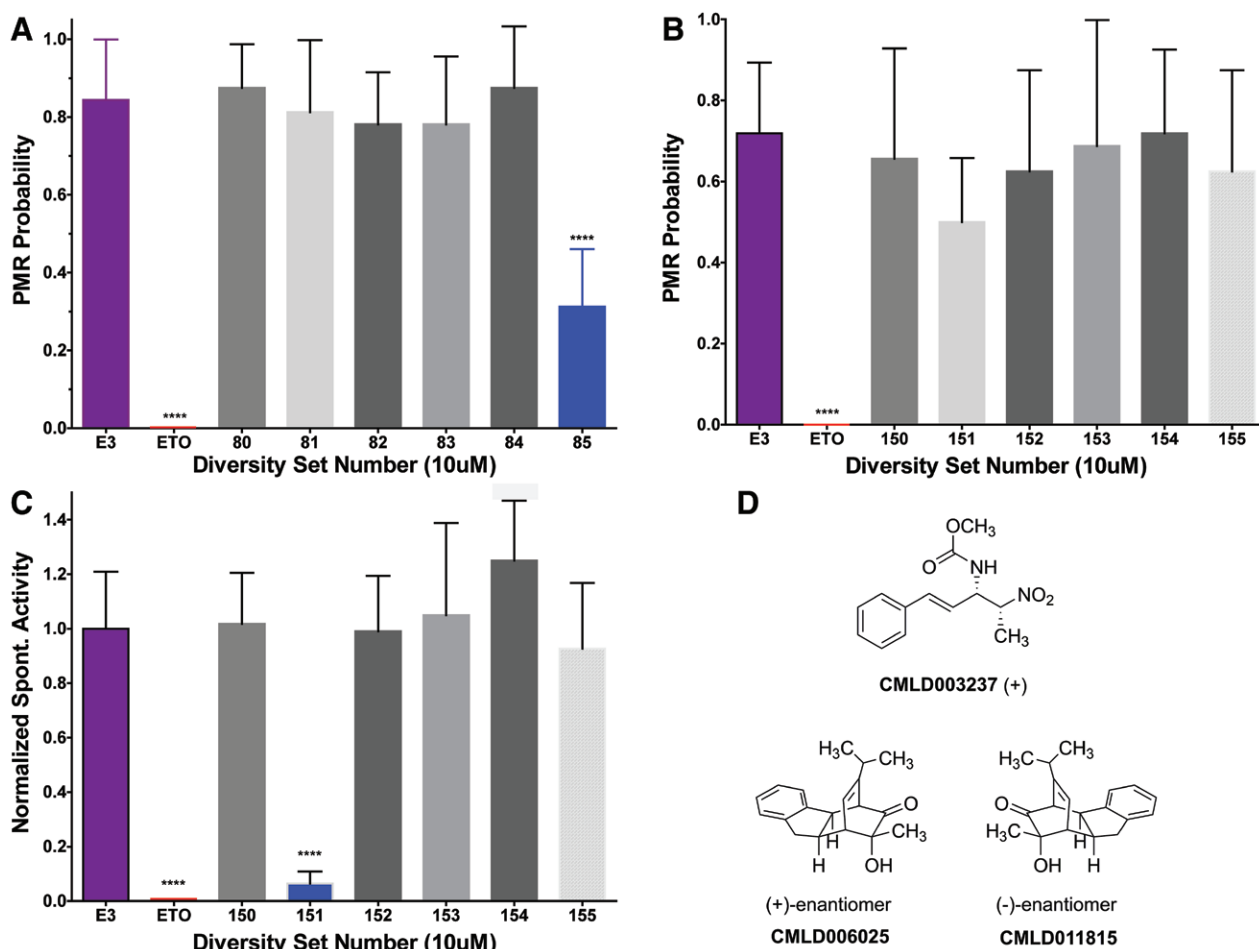


Fig. 5. Discovery of novel sedative-hypnotics using zebrafish larvae photomotor responses. (A–C) Bars represent cumulative photomotor response (PMR) probability or normalized spontaneous activity from four trials at 3-min intervals (mean with symmetrical 95% CI; $n = 8$). (A) Screening PMR results from an experiment including negative (E3 with 0.2% DMSO) and positive (10 μ M etomidate [ETO]) control groups, and six groups of larvae exposed to test compounds at 10 μ M. Diversity set (DS) no. 85 (DS85; CMLD003237) inhibits larval photomotor responses by more than 60% ($P < 0.0001$ by unpaired Student's t test). (B) Screening PMR results for DS150 through DS155 are shown. Note that DS151 (CMLD006025) does not significantly inhibit PMR probability ($P = 0.045$ by unpaired Student's t test, above the $P = 0.0024$ significance threshold after Bonferroni correction for seven comparisons). (C) Spontaneous activity, normalized to that of the negative control group from the same experiment shown in B. DS151 (CMLD006025) inhibited spontaneous (Spont.) activity by more than 90% ($P < 0.0001$ by Student's t test). (D) Chemical structures of CMLD003237, CMLD006025, and CMLD011815, the (-)-enantiomer of CMLD006025, are shown. **** $P < 0.0001$.

inhibited HCN1 currents less than 10% (fig. 6G), but produced no shift in voltage sensitivity (fig. 6H).

To determine whether CMLD003237 acted *via* α -adrenergic receptors, we tested whether the selective inhibitor atipamezole reversed its hypnotic effects in zebrafish larvae. To establish valid conditions for these experiments, zebrafish were first exposed to dexmedetomidine at $2.5 \times EC_{50}$ (1.0 μ M; fig. 7A) combined with varying concentrations of atipamezole (0.3 nM to 300 nM). This experiment identified 10 nM as the lowest atipamezole concentration that fully reverses dexmedetomidine hypnosis. Furthermore, 10 nM atipamezole alone produces no change in zebrafish larvae photomotor response probability and no reversal of hypnosis induced with various anesthetics that act through

other mechanisms (fig. 7B). Atipamezole produced no reversal of CMLD003237-induced hypnosis (fig. 7B).

Properties of CMLD006025 are as follows: MW = 254.1 Da; calculated LogP = 3.0; polar surface area = 37.3 \AA^2 ; 1 H-bond donor; and 2 H-bond acceptors. CMLD006025 was tested for its concentration-dependent inhibition of photomotor responses in zebrafish larvae (fig. 8A) and tadpole loss of righting reflexes (fig. 8B), resulting in similar EC_{50} s of 13 μ M and 10 μ M, respectively. Review of the Boston University Center for Medical Discovery library data revealed that CMLD006025 is a highly pure enantiomer and that its mirror image enantiomer was another library compound, CMLD011815. The photomotor response inhibitory potency of CMLD011815 (EC_{50}

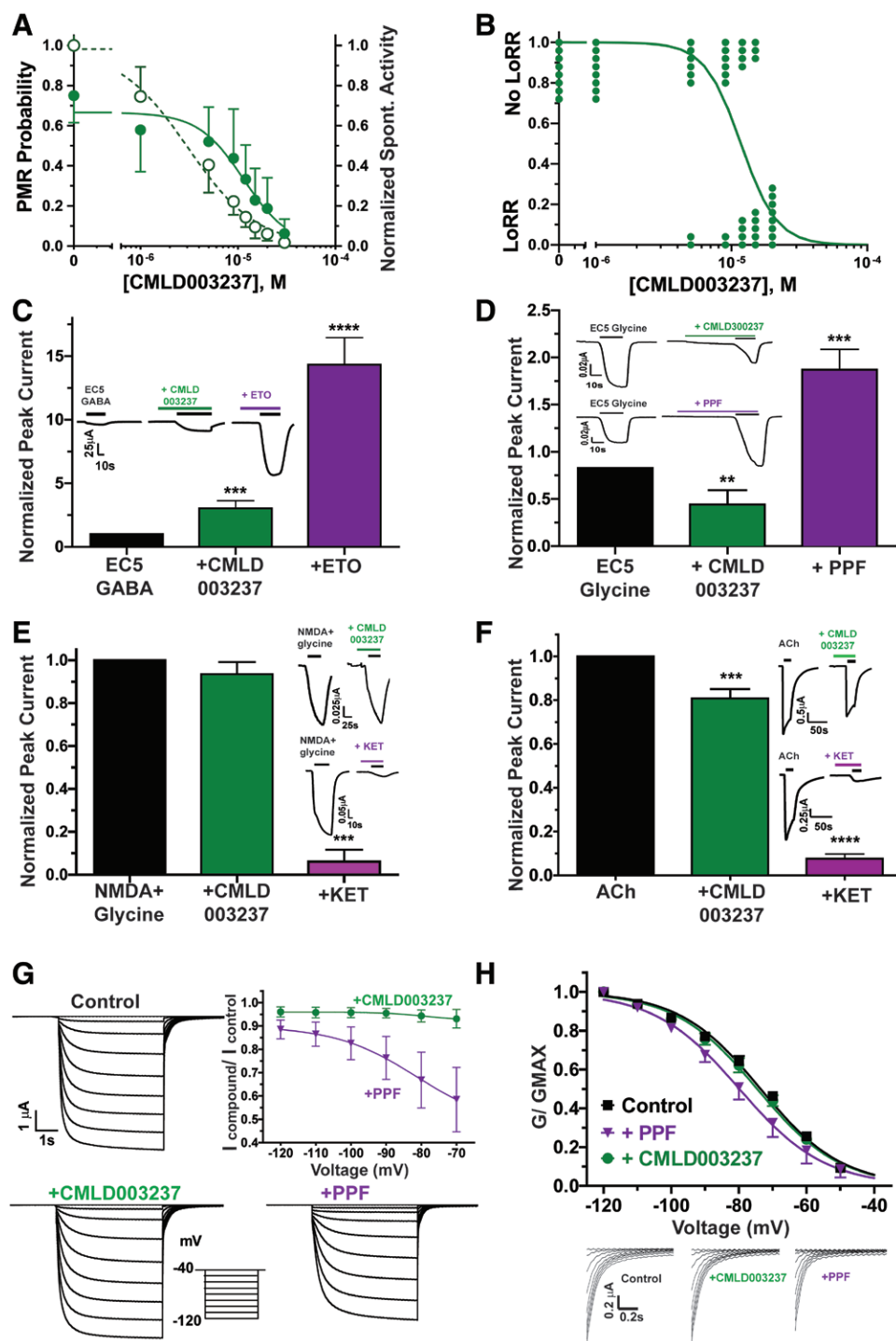


Fig. 6. Characterization of CMLD003237 in zebrafish larvae, *Xenopus* tadpoles, and ion channels. (A) Points represent mean with 95% CI for zebrafish larvae ($n \geq 8$ per group) photomotor response (PMR) probability (solid circles) and normalized spontaneous (Spont.) activity (open circles). Lines are logistic fits. PMR inhibition $EC_{50} = 11 \mu M$ (95% CI, 8.2 to 16 μM). Spontaneous activity inhibition $EC_{50} = 3 \mu M$ (95% CI, 2.1 to 4.5 μM). (B) Tadpole loss of righting reflexes (LoRR) results ($n = 8$ per group) are shown as binary outcomes. The line is a logistic fit with $EC_{50} = 12 \mu M$ (95% CI, 9.6 to 14.3 μM). (C–F) Bars represent control-normalized ion channel currents (mean with symmetrical 95% CI) measured in *Xenopus* oocytes. Currents in the presence of CMLD003237 or comparison drugs, both at $\approx 2 \times$ hypnotic EC_{50} , were normalized to paired control currents in the same oocyte, and outcomes with drugs were compared to controls using one-way ANOVA. Insets show examples of paired control versus drug oocyte currents. (C) Bars represent control-normalized EC5 γ -aminobutyric acid (GABA)-induced currents through human $\alpha 1\beta 3\gamma 2L$ γ -aminobutyric acid type A receptors. CMLD003237 (22 μM) enhanced currents elicited with EC5 GABA (3 μM) about threefold ($P = 0.0007$; $n = 5$). An equihypnotic etomidate solution (ETO; 3.2 μM) enhanced EC5 currents about 14-fold (Continued)

Fig. 6. (Continued). ($P < 0.0001$; $n = 5$). The inset shows currents recorded under all three conditions in one oocyte. (D) Bars represent control-normalized EC5 currents through human glycine receptor $\alpha 1$ receptors. CMLD003237 (22 μM) inhibited currents elicited with EC5 glycine (1 μM) about 50% ($P = 0.0013$; $n = 4$). An equihypnotic propofol solution (PPF; 4.5 μM) enhanced EC5 glycine currents 1.9-fold ($P = 0.0010$; $n = 4$). (E) Bars represent control-normalized peak currents through human NR1A/2B *N*-methyl-D-aspartate (NMDA) receptors. CMLD003237 (22 μM) does not affect control currents elicited with 100 μM NMDA + 10 μM glycine ($P = 0.32$; $n = 6$), while an equihypnotic ketamine (KET) solution (120 μM) inhibits control currents by about 95% ($P = 0.0002$; $n = 4$). (F) Bars represent control normalized peak currents through human $\alpha 2\beta 4$ neuronal nicotinic acetylcholine (ACh) receptors. CMLD003237 (22 μM) inhibited control currents elicited with 1 mM ACh by about 20% ($P < 0.0001$; $n = 9$). Equihypnotic ketamine (120 μM) inhibited control currents by more than 90% ($P < 0.0001$; $n = 8$). (G) Plotted symbols represent control-normalized peak currents (mean with 95% CI) through human HCN1 receptors. Raw currents from a single oocyte studied under control conditions, with CMLD003237, and with PPF are displayed along with an inset showing the voltage-jump activation protocol. CMLD003237 (22 μM ; $n = 8$) inhibited control currents by less than 10% at all test voltages. Propofol (4.5 μM ; $n = 5$) inhibited HCN1 currents by up to 40% in a voltage-dependent manner ($P < 0.0001$ vs. CMLD003237 at -70 mV). (H) Current traces are tail currents recorded at -40 mV from G, normalized to the tail current amplitude after activation at -120 mV. Normalized tail current amplitudes (G/GMAX) are also plotted against activation voltage ($n = 8$ oocytes for CMLD003237 and 5 oocytes for propofol). Lines through these data represent nonlinear regression fits to Boltzmann equations. Fitted propofol control V50 (mean [95% CI], -79.9 [-81.0 to -78.8]) differs from control (-73.6 [-72.9 to -74.3]; $P < 0.001$ by F test). Fitted CMLD003237 V50 (-74.6 [-73.9 to -75.4]) did not differ from control ($P = 0.142$ by F test). ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

> 30 μM ; fig. 8C), was lower than that of CMLD006025. Loss of righting reflexes tests in *Xenopus* tadpoles confirmed that CMLD006025 was more potent than CMLD011815 (fig. 8D).

We tested equal concentrations (26 μM) of CMLD006025 and CMLD011815 on various ion channels, seeking evidence of differential effects that might account for the stereoselective hypnotic actions in zebrafish and tadpoles. GABA_A receptor EC5 currents were enhanced similarly by both compounds (fig. 8E; 1.5- to 1.7-fold; $n = 8$ each; both $P < 0.001$ vs. control). Glycine $\alpha 1$ receptor EC5 currents were enhanced by propofol, unaffected by CMLD006025, and inhibited about 65% by CMLD011815, demonstrating stereoselective effects (fig. 8F). NMDA receptor currents were inhibited by $\sim 25\%$ in the presence of either CMLD006025 or CMLD011815 (fig. 8G). Neuronal nicotinic acetylcholine receptors were also inhibited by $\sim 65\%$ in the presence of either compound, without stereoselectivity (fig. 8H). Human HCN1 receptors were inhibited more by CMLD011815 than CMLD006025, but these effects were both much weaker than those of propofol (fig. 8I). Atipamezole did not reverse the hypnotic effects of either enantiomer at equipotent ($2 \times \text{EC}_{50}$) concentrations (fig. 8J).

The translational potential of CMLD003237 and CMLD006025 as intravenous sedative-hypnotics was explored in Sprague-Dawley rats. The dosages and number of animals tested was limited by the amount of compounds available. Three rats were each given a single intravenous bolus of CMLD003237 in DMSO, at increasing doses. The first rat received 9 mg/kg and displayed no loss of righting reflexes. A second rat that received 26 mg/kg lost righting reflexes 47 s after the injection and returned to an upright prone position after another 64 s. A third rat that received a 39 mg/kg intravenous bolus lost righting reflexes 25 s after injection and returned to an upright stance after another 5 min, 30 s. However, while the hypnotic potencies of CMLD003237 and CMLD006025 were similar in aquatic

animals (figs. 6 and 8), CMLD006025 injected intravenously at 40 mg/kg did not impair righting reflexes in rats ($n = 2$).

Discussion

Major Results

Goals for developing new sedative-hypnotics include facilitating efficiency in outpatient procedural settings and reducing anesthetic toxicities, particularly in vulnerable populations.⁴⁴ Improving current sedative-hypnotics through rational drug design or mechanism-based drug screening strategies may exclude potentially useful drugs that act through novel mechanisms. We have developed and validated a high-throughput stimulus-response screening approach for sedative-hypnotics in zebrafish larvae and used it in a small library of compounds to discover two drugs with reversible sedative-hypnotic activity in aquatic vertebrates, with one effective in rodents. Our novel approach represents a mechanism-independent primary anesthetic drug discovery strategy based on vertebrate animal stimulus-response assays.

Zebrafish Larvae Photomotor Responses versus Tadpole Loss of Righting Reflexes

Photomotor responses in zebrafish embryos or larvae have been used previously for neuromodulatory drug screening experiments.^{16,45–47} Embryonic zebrafish responses to intense light stimuli are mediated by photosensors in the developing hindbrain, not the eyes.⁴⁸ Our approach differs from previous studies in using zebrafish larvae, which have more developed vision and neural circuits than embryos,⁴⁹ and in specifically measuring both sedation (unstimulated motor activity) and hypnosis (inhibition of stimulated motor responses). Importantly, development of vision in zebrafish requires exposure to both light and dark, and visual transduction in larvae shows diurnal variation.^{49,50} A weakness of our photomotor response test is the possibility

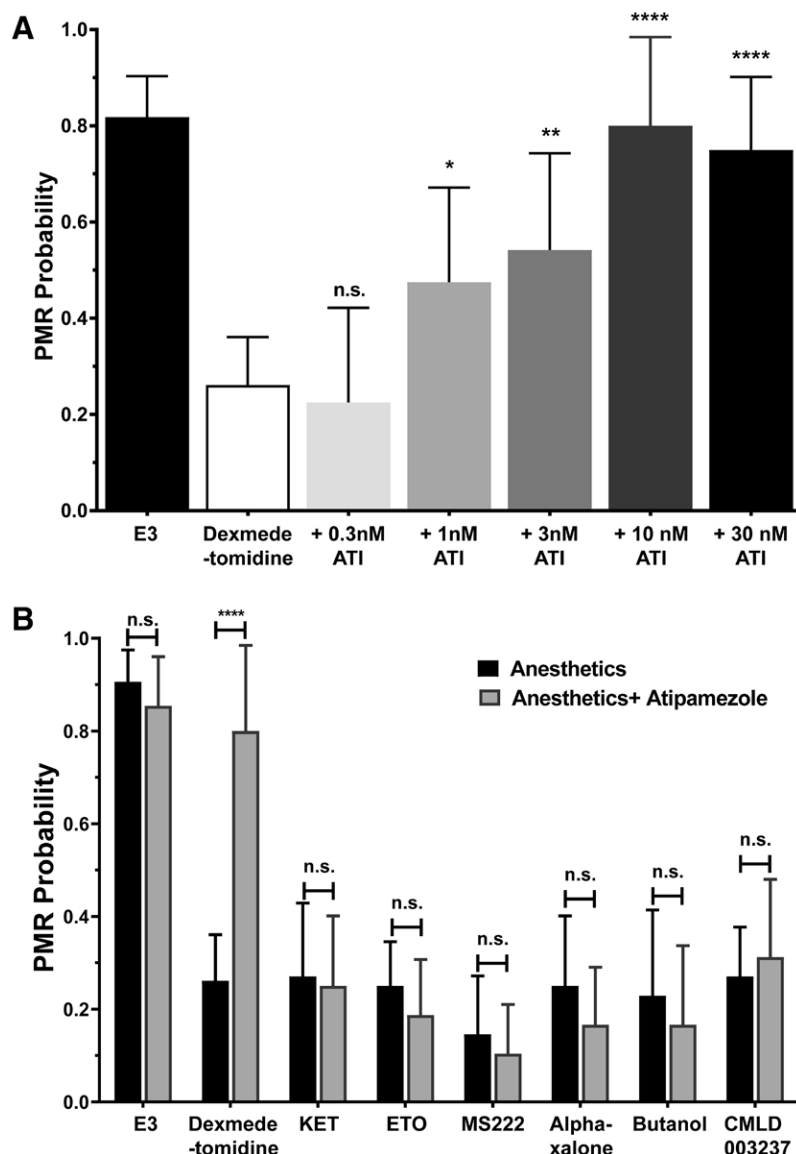


Fig. 7. Atipamezole reversal of zebrafish photomotor responses is a specific test for α_2 -adrenergic receptor agonism. (A) Atipamezole reverses photomotor response (PMR) inhibition by 1.0 μM ($2.5 \times \text{EC}_{50}$) dexmedetomidine. Each bar represents mean and symmetrical 95% CI of cumulative PMR probability in groups of 12 zebrafish larvae, each tested in four trials. One-way ANOVA was used to compare the dexmedetomidine group to those exposed to atipamezole. Atipamezole at 10 nM or higher concentrations fully reverses dexmedetomidine hypnosis ($P < 0.0001$ vs. dexmedetomidine alone). (B) Atipamezole at 10 nM does not reverse photomotor response inhibition by other sedative-hypnotics. Each bar represents mean and 95% CI of PMR probability in groups of eight zebrafish larvae, each tested in four trials. The black bars show the effect of each hypnotic drug at $2.5 \times \text{EC}_{50}$, and the paired gray bar shows the effect of the same drug combined with 10 nM atipamezole. Atipamezole alone does not affect the control PMR probability (E3 buffer; $P = 0.37$), nor does it reverse the hypnotic effects of ketamine (KET; $P = 0.84$), etomidate (ETO; $P = 0.38$), tricaine (MS222; $P = 0.58$), alphaxalone ($P = 0.36$), butanol ($P = 0.84$), or CMLD003237 ($P = 0.65$). ATI = atipamezole. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. n.s. = not significant.

of identifying drugs that selectively inhibit visual transduction. To address this issue, we validated sedative-hypnotic effects of both known and novel drugs in *Xenopus* tadpole loss of righting reflexes assays. We can also use zebrafish larvae responses to acoustic or tactile stimuli to validate hypnotic drug effects.

Our study demonstrated that zebrafish larvae are far more suitable for high-throughput drug screening than tadpoles.

Tadpole loss of righting reflexes assays use about 20 ml of water per animal, so a 10- μM drug solution for 10 tadpoles requires 2 micromoles. In comparison, 10 zebrafish larvae, each in 0.2 ml, require only 20 nanomoles of drug, 100-fold less than tadpoles. As a practical constraint, we were provided 0.2 micromoles of each drug we screened, tenfold less than needed for screening at 10 μM in 10 tadpoles, but tenfold more than needed for 10 zebrafish larvae. The zebrafish

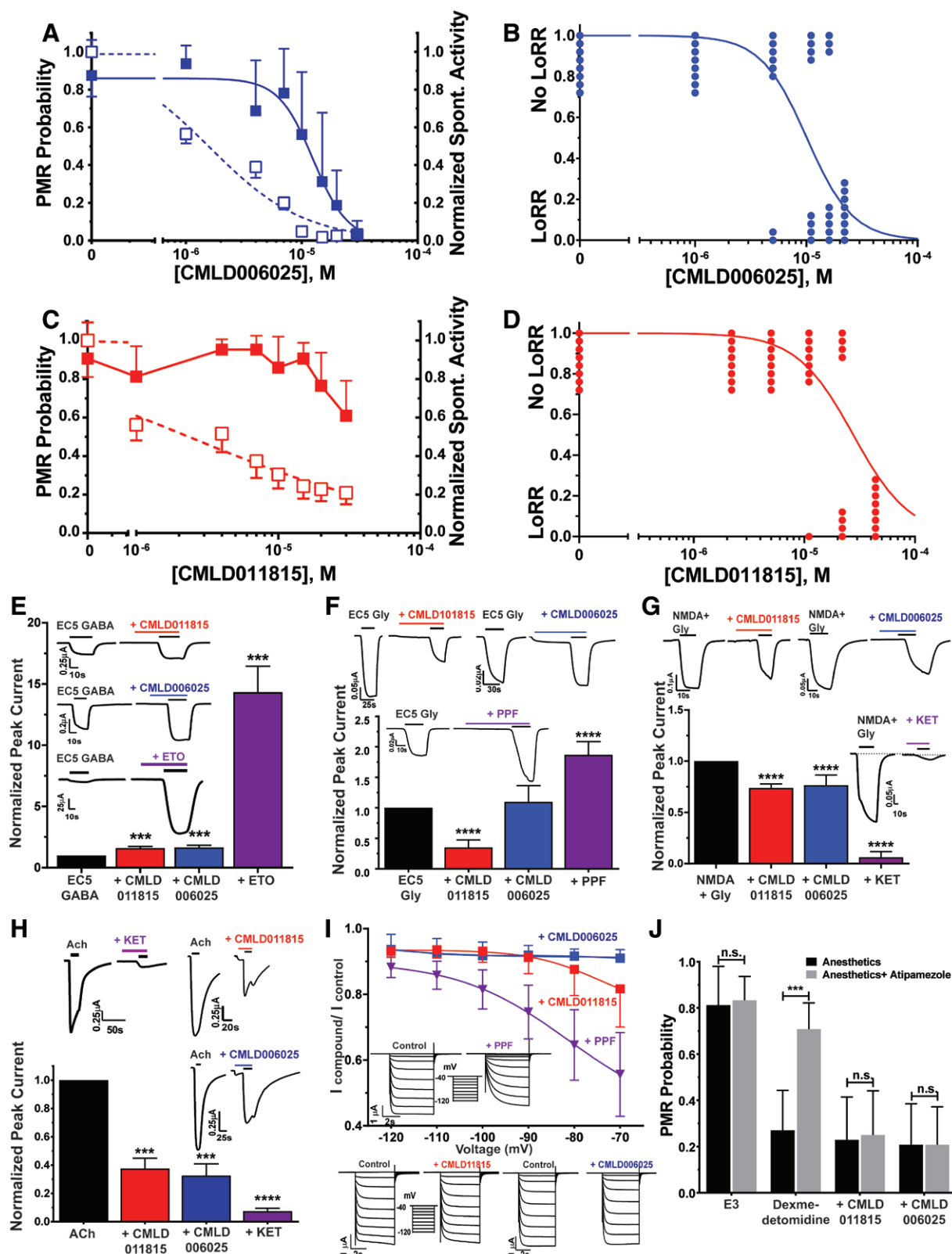


Fig. 8. Characterization of CMLD006025 and CMLD011815 in zebrafish larvae, *Xenopus* tadpoles, and molecular targets. (A) CMLD006025 inhibition of zebrafish larvae photomotor response (PMR) and spontaneous (Spont.) activity. Points represent mean with symmetric 95% CI ($n = 12$ per group), and lines are logistic fits. PMR inhibition (solid squares): $EC_{50} = 13 \mu M$ (95% CI, 9.9 to 16 μM). Spontaneous activity inhibition (open squares): $EC_{50} = 1.6 \mu M$ (95% CI, 1.2 to 2.1 μM). (B) Tadpole loss of righting

(Continued)

Fig. 8. (Continued). reflexes (LoRR) results in the presence of CMLD006025 ($n = 8$ per group), shown as binary outcomes. The line is a logistic fit with $EC_{50} = 10.1 \mu\text{M}$ (95% CI, 7.2 to 14.1 μM). (C) CMLD011815 weakly inhibits larval zebrafish PMRs at concentrations above 10 μM (solid squares; mean with 95% CI; $n = 16$ per group). A logistic fit to PMR data did not converge. CMLD011815 inhibition of normalized spontaneous activity is plotted as open squares (mean with 95% CI) with logistic fit $EC_{50} = 2.3 \mu\text{M}$ (95% CI, 1.4 to 3.9 μM). (D) Tadpole LoRR results in the presence of CMLD011815 ($n = 8$ per group), shown as binary outcomes. The logistic fit EC_{50} is 28 μM (95% CI, 18 to 49 μM). (E–H) Bars represent control-normalized ion channel currents (mean with symmetric 95% CI) measured in *Xenopus* oocytes. Currents in the presence of drugs at $\approx 2 \times$ hypnotic EC_{50} were normalized to paired control currents in the same oocyte, and outcomes with drugs were compared to controls using one-way ANOVA. Insets show examples of paired control versus drug oocyte currents. (E) Bars represent control-normalized EC5 γ -aminobutyric acid (GABA)-induced currents through human $\alpha 1\beta 3\gamma 2\text{L}$ γ -aminobutyric acid type A receptors. CMLD011815 and CMLD006025 (both at 26 μM) similarly enhanced currents elicited with EC5 GABA (3 μM) by about 60% ($P < 0.001$ vs. control; $n = 5$, for both drugs). An equihypnotic etomidate solution (ETO; 3.2 μM) enhanced EC5 currents about 14-fold ($P < 0.0001$; $n = 5$). (F) Bars represent control-normalized currents through human glycine $\alpha 1$ receptors. CMLD011815 (26 μM) inhibited currents elicited with EC5 glycine (1 μM) by about 65% ($P < 0.0001$; $n = 4$). In contrast, CMLD006025 (26 μM) did not significantly alter current amplitude ($P = 0.29$; $n = 4$). An equihypnotic propofol solution (PPF; 4.5 μM) enhanced EC5 currents about 1.8-fold ($P < 0.0001$; $n = 4$). (G) Bars represent control-normalized currents through human NR1A/2B *N*-methyl-D-aspartate (NMDA) receptors. CMLD011815 and CMLD006025 (both at 26 μM) similarly inhibited currents elicited with 100 μM NMDA + 10 μM glycine by about 25% ($P < 0.0001$; $n = 4$ for both drugs). An equihypnotic ketamine solution (KET; 120 μM) inhibited currents about 95% ($P < 0.0001$; $n = 4$). (H) Bars represent control-normalized currents through human $\alpha 4\beta 2$ neuronal nicotinic acetylcholine (ACh) receptors. CMLD011815 and CMLD006025 (both at 26 μM) similarly inhibited currents elicited with 1 mM ACh by about 65% ($P = 0.0001$; $n = 4$ for both drugs). An equihypnotic ketamine solution (120 μM) inhibited currents more than 90% ($P < 0.0001$; $n = 4$). (I) Symbols represent control-normalized peak currents (mean with 95% CI) through human HCN1 receptors. Currents were inhibited less than 10% in the presence of 26 μM CMLD006025 ($n = 9$), while an equal concentration of CMLD011815 ($n = 9$) inhibited currents by about 18% with activation at -70 mV. An equihypnotic solution of propofol (4.5 μM ; $n = 5$) inhibited HCN1 currents by more than 40%. Boltzmann nonlinear regression of conductance-voltage (G/V) relationships (not shown) indicate V50 shifts with both propofol ($P < 0.0001$ by F test; $n = 5$) and with CMLD011815 ($P = 0.0015$ by F test), but not with CMLD006025 (both $P = 0.11$ by F test). (J) Bars represent zebrafish larvae PMR responses (mean with 95% CI; $n = 12$ per group) measured in the absence and presence of atipamezole (10 nM). Atipamezole reverses PMR inhibition by dexmedetomidine (1.0 μM ; $P = 0.0001$ by unpaired Student's *t* test), but not by equihypnotic concentrations of CMLD011815 (70 μM ; $P = 0.86$) or CMLD006025 (33 μM ; $P > 0.999$). *** $P < 0.001$; **** $P < 0.0001$. n.s. = not significant.

larvae photomotor response assay also required less glassware and benchtop space in comparison with tadpole loss of righting reflexes tests. Time and effort required for drug potency assays were also lower for zebrafish than tadpoles. Manually pipetting buffer, animals, and drugs into a 96-well plate took about 30 min, similar to the setup time for multiple groups of tadpoles. Loading zebrafish larvae into 96-well plates can be further accelerated through automation.⁵¹ Our computer-controlled photomotor response tests proceeded with multiple trials for up to 96 animals in parallel. With a 15-min adaptation and equilibration period before four trials at 3-min intervals, computerized data acquisition lasted under 30 min, and analysis of results took under 10 min after we developed approaches for processing Zebrolab outputs for standardized screening and concentration-response experiments. In comparable tadpole experiments, each animal was manually tested and observed for loss of righting reflexes for 5 s every 5 min for 30 min. This limited a single worker to testing no more than 20 animals at a time. Tadpole loss of righting reflexes tests also involve a degree of judgment, which can introduce bias or error, and with multiple lightly anesthetized animals together in a single container, errors related to tracking movements of individual tadpoles inevitably occur. Tadpole results were manually recorded and manually entered for computational analysis, introducing additional potential for human error.

While all healthy tadpoles exhibit brisk righting reflexes in the absence of hypnotic drugs, flashing bright white light

onto dark-adapted 7 days postfertilization zebrafish larvae did not elicit motor responses 100% of the time. We also found that undrugged larvae exhibited a diminishing photomotor response probability with repeated trials (fig. 3A). This desensitization to photic stimuli diminished in the presence of hypnotics (fig. 3C), introducing a potential source of bias into concentration-response analyses. We minimized this bias by limiting the number of repeated trials to four, resulting in a less than 10% drop in control photomotor response probability, with stable variance (fig. 3, A and B). Importantly, the absence of desensitization in larvae exposed to hypnotic drugs (fig. 3C) indicates that a 15-min pretest drug exposure is sufficient to establish steady-state pharmacodynamic effects and thus, effect-site concentrations. Zebrafish larvae desensitization to repeated stimuli has also been used as a method for studying learning and memory,³⁵ another neural process inhibited by general anesthetics. In this study, the commercial system used to track activity imposed limitations on the time-resolution of video recordings and the types of data analyses we could perform. In future experiments, more refined behavioral analyses may be achievable using high-speed video recording and customizable video analysis tools for zebrafish behaviors, which are available in public databases.⁵²

Our experiments comparing zebrafish larvae photomotor response tests and tadpole loss of righting reflexes indicate that both assays provide essentially the same information for

drug screening (table 2) and potency determination (fig. 4). Combined with its advantages in drug sample size and work time, these results support adoption of zebrafish larvae as a rapid and reliable platform for screening and initial characterization of sedative-hypnotic drugs.

Discovery of New Potent Sedative-hypnotics in a Drug Library

Our screen of 374 compounds from a larger library identified two compounds, CMLD003237 and CMLD006025, that reversibly and dose-dependently inhibit both zebrafish larvae photomotor responses and tadpole righting reflexes (figs. 6, A and B; and 8, A and B). If the frequency of sedative-hypnotics found in the diversity set (0.53%) is representative of all 2,651 compounds in the library, then screening the remaining compounds should identify another 12 new sedative-hypnotics. A survey of CMLD003237 effects on six neuronal receptors (figs. 6 and 7) suggests that both GABA_A receptors (fig. 6C) and neuronal nicotinic acetylcholine receptors (fig. 6F) could contribute to its hypnotic actions. However, inhibition of glycine receptors by CMLD003027 (fig. 6D) might antagonize its anesthetic actions in the spinal cord.⁵³ Comparing the hypnotic potencies in aquatic animals of CMLD006025 and its mirror-image enantiomer, CMLD011815, reveals stereoselectivity (fig. 8, A–D). Weak modulation of GABA_A receptors (fig. 8E), modest inhibition of NMDA receptors (fig. 8G), and inhibition of neuronal neuronal nicotinic acetylcholine receptors (fig. 8H) could all contribute to hypnosis by both enantiomers. The relatively low hypnotic potency of CMLD011815 in animals may be due to its inhibition of glycine receptors, which CMLD006025 lacks (fig. 8F). An intriguing and important feature of both CMLD003237 and CMLD006025 is that both apparently act through mechanisms different from established potent sedative-hypnotics, such as etomidate, propofol, alphaxalone, and dexmedetomidine (fig. 2A), all of which selectively target GABA_A or α 2-adrenergic receptors.⁴³ Additional molecular mechanisms other than those we tested in this initial study may also contribute to the hypnotic effects of these new sedative-hypnotics.

CMLD003237 and CMLD006025 display similar hypnotic potency in aquatic animals and comparable physical properties. However, exploratory translational experiments in rats receiving intravenous injections show that CMLD003237 produces reversible loss of righting reflexes, while CMLD006025 at similar doses does not. It is not surprising that sedative-hypnotic efficacy in small aquatic animals equilibrated for 15 to 30 min in a drug solution does not reliably predict the effects of bolus intravenous dosing in mammals. Pharmacokinetic limitations such as blood solubility, protein binding, and transport across the blood-brain barrier influence the latter far more than the former. It is conceivable that one or more important targets for CMLD006025 differ in rats and the two aquatic species we tested, but this type of pharmacodynamic difference is

far less likely than a pharmacokinetic difference. Thus, based on these results, we plan to further explore both the unusual hypnotic pharmacology and the translational potential of CMLD003237 and its structural variants. CMLD006025 presents more barriers than CMLD003237 to translational development, while its mechanism of hypnosis is of scientific interest.

Conclusions and Future Directions

Zebrafish represent an animal model with great potential for anesthetic drug discovery as well as basic and translational research on general anesthetics. Further screening of drug libraries using the approach we developed is likely to reveal many more potent sedative-hypnotics. Those that act through novel mechanisms will be of great scientific interest. Neuroscience techniques combining recordings from or stimulation of neuronal circuit activity^{54,55} with behavioral tracking, including photomotor responses,⁴⁸ have been developed for zebrafish, and these approaches could reveal important details about anesthetic mechanisms in neural networks. Methods for site-directed genetic manipulation of zebrafish have also been developed.^{56–58} Zebrafish with knockout or site-directed mutations in putative anesthetic target genes have the potential to provide new insights into anesthetic mechanisms and efficient screening strategies to find target-selective anesthetics. These approaches are being actively explored in our laboratory.

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Competing Interests

Massachusetts General Hospital, Boston, Massachusetts, and Boston University, Boston, Massachusetts, have filed a pat-

ent application for compounds related to the new hypnotics described here. Drs. Brown, Forman, Jounaidi, Porco, Schaus, and Yang are named as coinventors. The other authors declare no competing interests.

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