Quantitative Analysis of Fast C Bend Startle Response in Larval Zebrafish for the Evaluation of Behavioral Responses and Neuropharmacological Effects

by

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"Man is a worm and food for worms. This is the paradox: he is out of nature and hopelessly in it; he is dual, up in the stars and yet housed in a heart-pumping, breath-gasping body that once belonged to a fish and still carries the gill marks to prove it"

- Ernest Becker, *The Denial of Death*

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ABSTRACT

Larval zebrafish behavior profiling provides a rapid and accessible method to gather data on diverse behaviors with relevance to human health, but analyzing and quantifying this information can be challenging. For use in tandem with the UCSF larval zebrafish behavioral profiling system, an open-source platform for flexible, user-friendly behavior analysis, named "Fish Spectacles", was developed. This platform readily quantifies and visualizes the differences in behavioral phenotypes such as those for the fast C bend startle response. To demonstrate this approach in characterizing behaviors and perturbations from neuroactive molecules, a small collection of molecules was used to probe the fast C bend startle response. This collection included both characterized neuroactives as well as novel molecules based on the neuroactive amino acid scaffold. These novel molecules seek to improve the bioavailability by replacing the problematic carboxylic acid and primary amine groups with tetrazole and dimethylamine isosteres. Evaluation of behavioral responses revealed these new molecules were neuroactive and resembled their amino acid counterpart when one was tested. These findings underscore the utility of zebrafish behavior phenotyping for advancing the discovery of neuroactive small molecules.

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UCSF : University of California, San Francisco

AMPA : α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

NMDA : N-methyl-D-aspartic acid

GABA : Gamma-aminobutyric acid

ACBC : aminocyclobutyl carboxylic acid

DCS : D-cycloserine

SUDAA : synthetic utility derivatives of amino acids

DCM : dichloromethane

TEA : triethylamine

$POCl_3$: Phosphorous oxychloride

TMS-azide : trimethylsilyl-azide

MeOH : methanol

PDF : portable document file

ms : milliseconds

EC_{50} : half-effective concentration

nM : nanomolar

uM : micromolar

pKa : acid dissociation constant

cLogP : calculated hydrophobicity

tPSA : total polar surface area

MAO : monoamine oxidase

CHAPTER 1 : QUANTIFICATION OF LARVAL ZEBRAFISH BEHAVIOR

Abstract

Behavioral profiling has the potential to capture large amounts of information about the behaviors of larval zebrafish in a rapid and accessible manner. Analyzing and quantifying this information is much more challenging. Fish Spectacles is an open-source quantitative behavior analysis platform designed to work in tandem with UCSF's behavioral profiling system. Fish Spectacles presents a user friendly and flexible way to quantify a diverse range of behaviors, and outputs information about the analysis through text and .txt files or graphs and .pdf files. In demonstrating the use of Fish Spectacles, several different experiments treating fish with etomidate or cotreatment of etomidate and glutamatergic molecules are analyzed and visualized.

Introduction

Studying Behavior

The brain is the least understood organ. It takes in information about the environment through highly specialized sensory cells and produces characteristic responses in the form of movement or lack thereof. These processes of using information to produce behaviors in the brain remain largely unknown. How can we study the brain? Experimental approaches in humans have been largely unsuccessful due to factors such as individual differences, uncontrolled exposure to environmental stimuli, the complexity and scale of the human brain, and the challenges in measuring and predicting behavioral outputs. Consequently, much of our understanding of behavior is derived from studies conducted in non-human animals.

Biosemiotics

To understand the behavior of an animal, one must first consider the perspective of the animal. Every animal perceives its environment through its specialized sensory cells. The types of sensory cells an animal has and how these sensory cells gather information about the environment determines what an animal perceives. These modes of perceptions can vary greatly among animals. Consider the world of the female tick[1]. She hatches from her egg ready to search for prey. Light triggers general photoreceptors on her skin to move in the direction of the light. This guides the tick to a high perch to wait for passing prey. As she waits, she is blind and deaf to approaching prey. Instead, she uses her sense of smell to detect butyric acid, a small molecule released by the skin of animals, to know when to launch from her perch. When she lands on an animal and the thermoreceptors on her body feel the warmth of the animal, she finds a hairless spot to attach and feed. Once she has fed and grown enough, she releases a scent and smells for the scent of a male. Upon finding a male, she mates and lays eggs, perpetuating this behavioral cycle. In this way, the perspective world of the female tick consists of the general detection of light, odor, and warmth. Her perceptions of these specific stimuli elicit corresponding behaviors: move toward light, jump towards butyric acid, feed towards warmth, and reproduce toward male tick smell. Therefore, studying animal behavior requires an understanding of both the stimuli animals detect and how the animals perceive the stimuli.

Quantifying Behavior in the Lab

In the United States, experimenters focused on this relationship between stimulus and behavior and the science of behaviorism emerged. This approach took the relationship to the extreme with ideas of the environment being the sole producer of behavior, behaviors being

universal among animals, and the sole shaping of behaviors based on reward and punishment consequences[2,3]. To test these ideas, scientists like B.F. Skinner focused on developing the first quantitative methods for studying behaviors in a controlled manner. Many of these methods pioneered experimental tools and apparatuses for delivering stimuli and measuring the response such as Skinner boxes (Figure 1.01). This quantitative approach to studying behavior produced much of our current knowledge of operant behaviors and conditional learning.

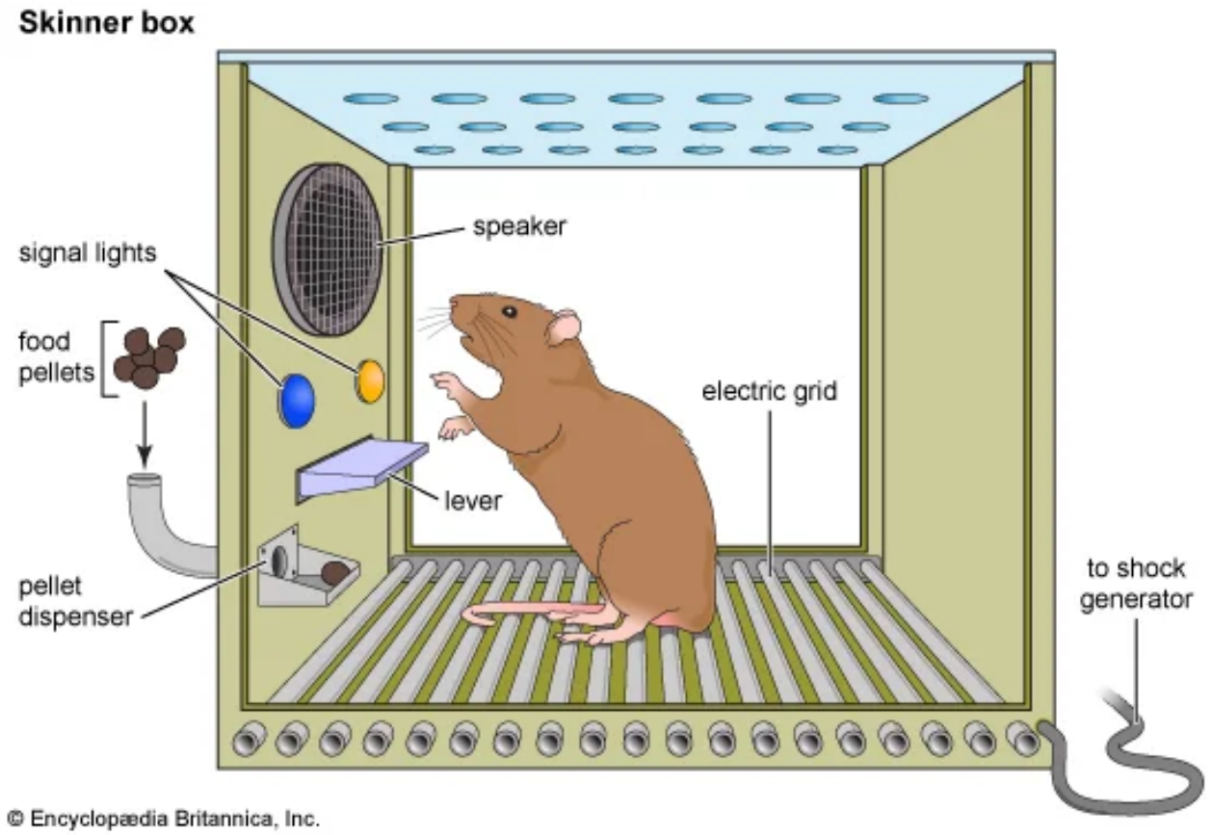

Figure 1.01 Skinner box. Used to expose rodents to stimuli and measure behavioral responses. Adapted from *Encyclopædia Britannica, Inc./Steven N. Kapusta*

Ethology

In Europe, a different approach was taken to studying behavior. Scientists instead focused on studying the different types of behaviors between species, and this science developed into ethology[4]. Ethologists characterized the behavior itself as well as the environmental stimuli that would cause the behavior. They would then probe how the brains of the animals detect and respond

to the environmental stimuli to produce the behavior. In this approach, they focused on studying animals in the natural environment and designing experiments in natural conditions to probe underlying behaviors. “Ethology is the science of interviewing an animal in its own language.”- Nikolaas Tinbergen[5]. For example, Karl von Frisch devised experiments in the language of the bee by moving the hive and sources of food[6]. These experiments revealed how the bees use the Sun and the position of the hive to communicate the location of food with each other (Figure 1.02). This approach produced radically different ideas about the relationship between animals and behavior. Each species of animal was identified as having its own unique perceptions and behaviors, and only the stimulus from the natural environment was able to trigger authentic behaviors.

By studying a wide range of behaviors, ethologists discovered behaviors fall into specific categories. These behaviors share characteristics among the detection of stimulus and response to the stimulus. One type of behavior is fixed action patterns which are a well-defined movement in response to an environmental stimulus which are conserved among animals of the same species or higher taxonomy[7]. These behaviors have a stimulus threshold that once surpassed, the behavior is triggered, and the behavior will continue even if the inducing stimulus is removed. All animals are born knowing fixed action patterns, but they must learn how to improve the behavior. Some examples of fixed actions patterns include squirrel nut cracking, gulls removing eggshells from the nest, and human smiling[8-10]. Non-fixed action pattern behaviors include imprinting of newborn goslings on a “mother” and fully learned behaviors such as making tools by apes[5, 9]. The characteristics of these fixed action patterns emerge as a result of the underlying biology.

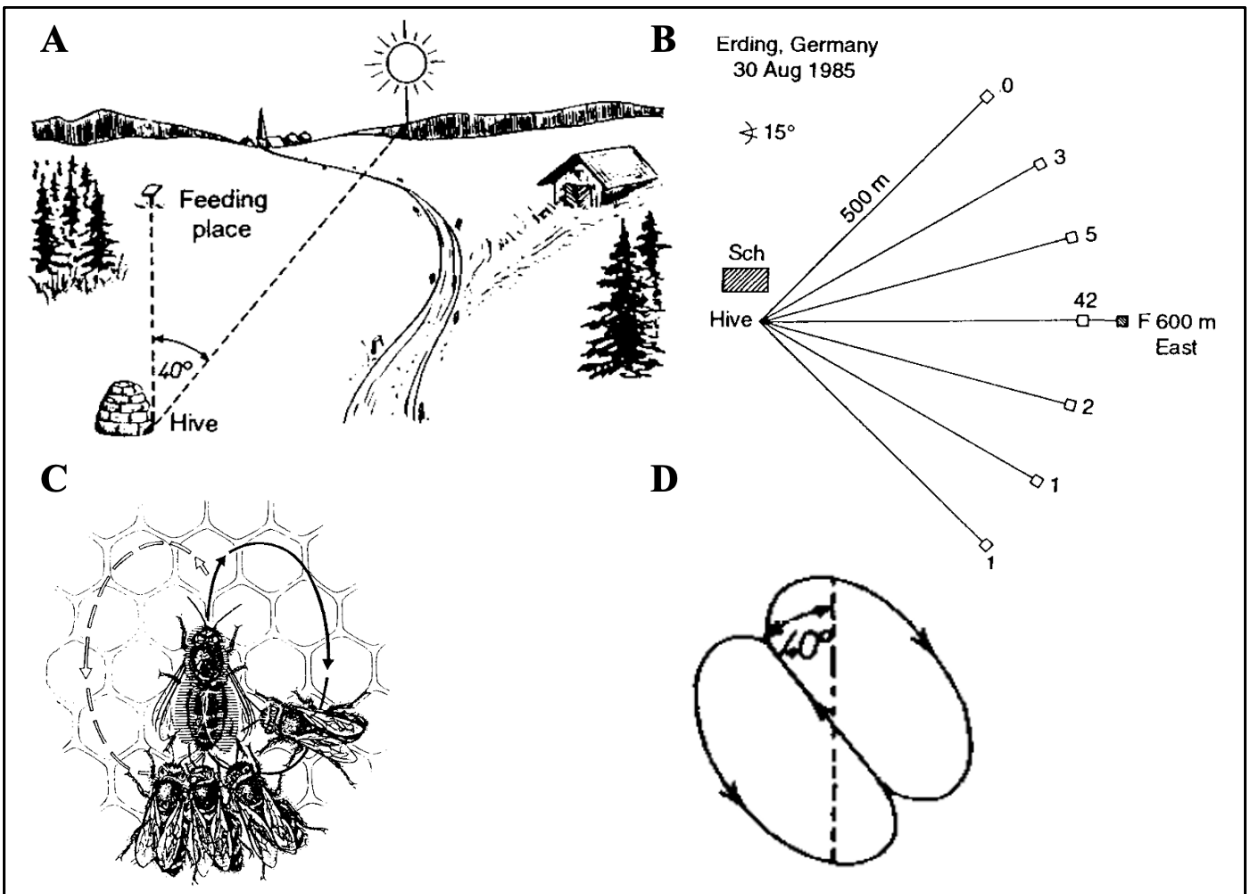

Figure 1.02 Ethology of Bee Communication. Karl Frisch devised experimentation by moving the hive (A) and the food source (B) to identify bees communicate to each by dances (C). These dances indicate the direction to the food source by rotating the dance (D) and the distance by the duration of the dance (not shown). Adapted from *Decoding the language of the bee*[6]

Neuroethology

When ethologists began studying these types of behaviors more closely, they found types of behaviors have the same neural circuit structures, and each behavioral characteristic is the result of a property in the underlying neural circuit[11, 12]. The types of stimuli that can trigger a behavior are determined by the particular sensory cells that are connected to the circuitry. The specificity in the stimulus, such as what noise is startling or normal, is set by the processing of information by neurons directly connected to the sensory cells, primary afferents, and interneurons connected to the primary afferents. The threshold of the stimulus represents the accumulation of multiple inputs

through different sensory cells, and the all or nothing response indicates the presence of command neurons or a command center in the brain which must be activated to trigger the behavior. Once command neurons or centers are activated, the motor neurons are irreversibly activated. Finally, the conservation of the behavior among members of a taxonomic order is representative of conserved genetic instructions for constructing the circuit.

An example of this type of neural circuit underlying fixed action patterns is the fast C bend startle response in larval zebrafish[13-17]. The fast C bend startle response is a "C" shaped movement by the fish in response to light, sound, and touch stimuli which match a startling characteristic[18] (Figure 1.03a,b). After the fish are startled by a sufficient stimulus, the C bend movement occurs even if the stimulus is removed. The neural circuit underlying the fast C bend startle response includes connections to the eyes, inner ears, lateral line, and sensory neurons in the skin[19-24]. The excitatory primary afferents and inhibitory interneurons determine specificity by only allowing "startling" stimuli to reach the command cells[25-27]. At the center of the neural circuit is a pair of command neurons, known as Mauthner cells (Figure 1.03a). They receive input from the primary afferents and if activated, stimulate a series of primary and secondary motor neurons to produce both the characteristic C motion and a subsequent swimming motion directly afterwards. The series of muscle movements that occur during this motion can be directly tied to patterns of activation of these primary and secondary motor neurons[28-32]. These connections between the neural circuits and behavior provided a powerful approach to answering questions about behavior and developed into its own field of neuroethology. Other active areas of neuroethology include bird song, and odor responses in worms[33, 34].

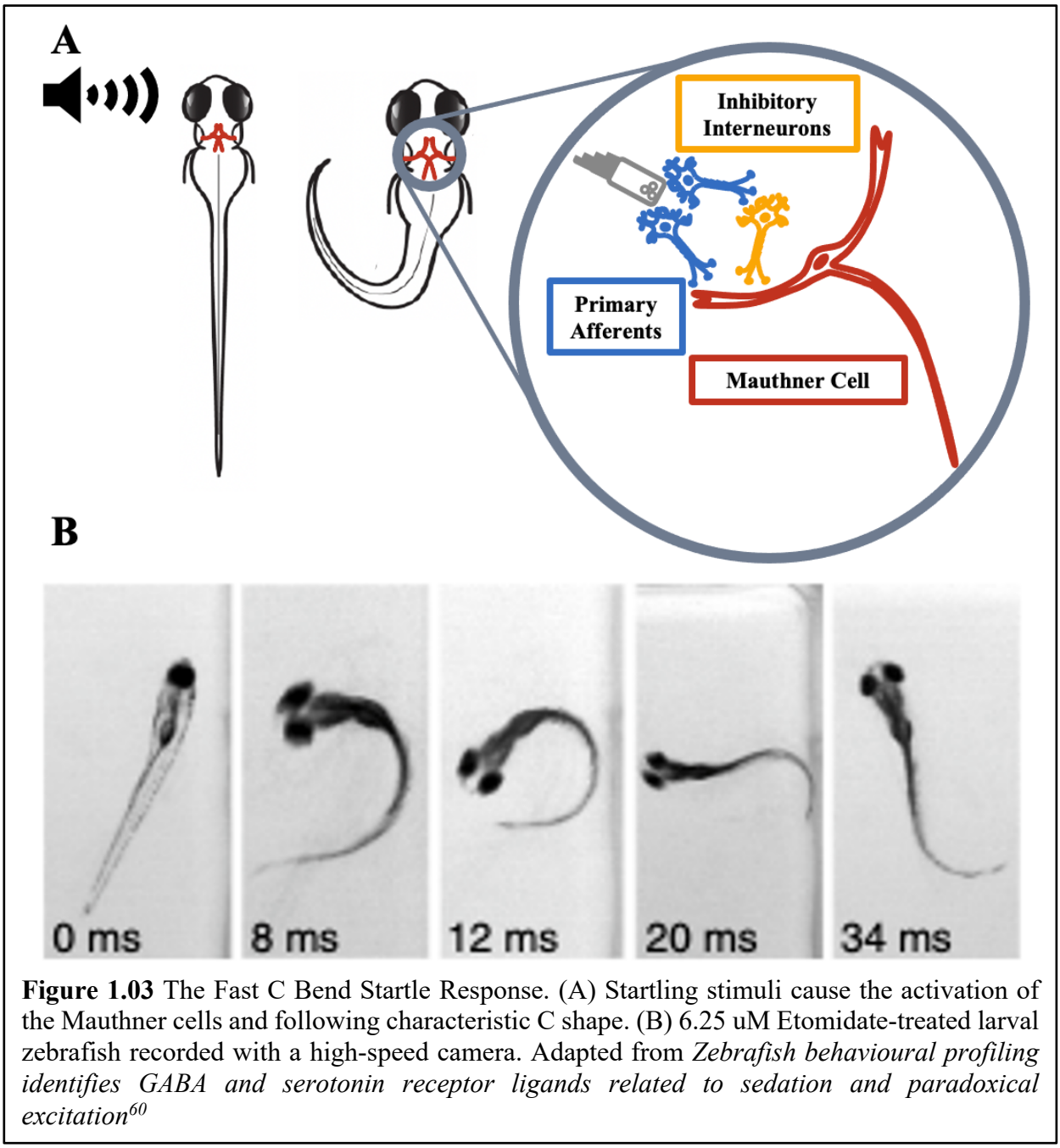

Figure 1.03 The Fast C Bend Startle Response. (A) Startling stimuli cause the activation of the Mauthner cells and following characteristic C shape. (B) 6.25 uM Etomidate-treated larval zebrafish recorded with a high-speed camera. Adapted from *Zebrafish behavioural profiling identifies GABA and serotonin receptor ligands related to sedation and paradoxical excitation*[60] *Neuropharmacology*

These biologies provide powerful approaches and tools for understanding behavior. Much has been learned about the organization of neural circuits and the connection between neural circuits and behavior. While this is a wealth of information, it does not provide insight to the

molecular mechanisms in neural circuits. Molecular mechanisms remained unidentified until small molecules with specific effects were discovered, often by accident through their behavioral impacts, and these neuroactive compounds were subsequently used to investigate neural circuits.

A prime example of this is the molecule iproniazid. Iproniazid was first used as an antibiotic to treat tuberculosis, but its behavioral effects were discovered when depressed tuberculosis patients recovered from both conditions simultaneously[35]. Investigating how this molecule could affect behavior in such a way, its molecular target of monoamine oxidases and the role of monoamine oxidases in the brain was uncovered. This role of monoamine oxidase led to the development of a whole class of monoamine oxidase inhibitors as antidepressants.

The role of glutamate in the brain was discovered in a similar manner. It was observed that injecting glutamate into the brains of animals caused seizures, indicating a neuroactive role[36]. This led to the evaluation of chemical derivates and natural products with bioorthogonal functionality to glutamate. These derivates, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid, N-methyl-D-aspartic acid (NMDA), and others, did not fully represent the glutamate phenotype but were able to accurately capture specific parts of the phenotype[37-40] (Figure 1.04). Studying these molecules revealed specific types of glutamate receptors, each with unique molecular mechanisms and functions in the brain. Without these chemical investigations, these receptors would not have been discovered.

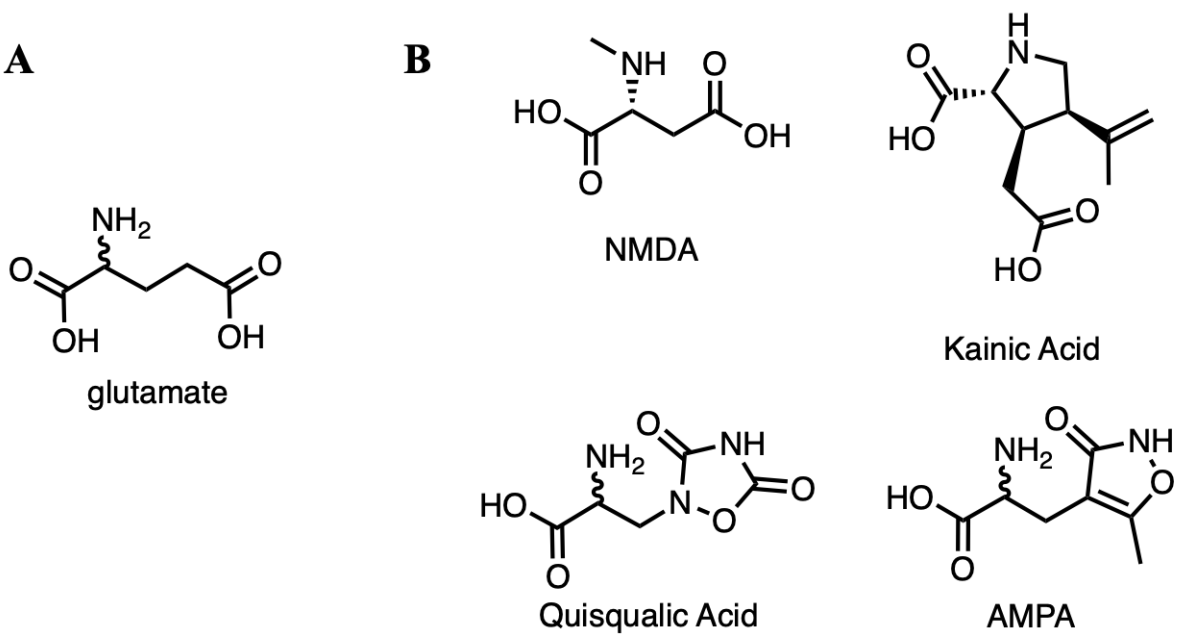

Figure 1.04 Glutamate and Neuroactive Derivates. (A) Glutamate and molecules (B) with relative specificity for certain glutamate receptors. NMDA : NMDA receptors, kainic acid : kainate receptors, quisqualic acid : metabotropic glutamate receptors, AMPA : AMPA receptors[37-40]

The large majority of neuroactive molecules were discovered in the mid-1900's. These produced a wealth of knowledge on both understanding behaviors and treating neurological diseases. Since then, the discovery of new classes of neuroactives has significantly slowed[41]. With the rapid advancement of target centered approaches possibly along with the simple and direct scientific narratives they bring, target centered approaches now dominate the field of drug discovery. They excel at developing molecules against known targets but cannot discover new targets and mechanisms which is needed for behavioral discovery, unless this happens by accident[42, 43]. To both further our understanding of molecular mechanisms in behavior and develop effective neuroactive therapeutics, new chemical matter needs to be evaluated for specific behavioral phenotypes.

Animal Models

Generally, behaviors are investigated for their relevance to human behaviors since humans are difficult to study. Thus, the animal being used must have analogous behaviors and neural circuitry. The most common animals used are rodents. These mammals are close evolutionary relatives of humans and share many commonalities in neural circuits and behaviors. Behaviors relating to analgesia, depression, anxiety, learning and memory, substance abuse, and others are used to evaluate the effects of small molecules. While useful, these experiments take long amounts of time to perform and only a limited amount of chemical matter can be evaluated. These facts greatly limit behavioral discovery using rodents.

An alternative behavioral animal model is larval zebrafish. While not mammals, these vertebrates have all the same neurotransmitters as humans and have analogous brain regions to humans[44]. As a result, not only do they have many analogous behaviors, but they also share many neuropharmacological phenotypes with humans. The effects of antipsychotics, antidepressants, and hypnotics on zebrafish and humans are similar[45-49]. The real advantage of larval zebrafish is their rate of development, size, and high numbers produced from breeding. These animals hatch and are able to swim at four days post-fertilization, and by seven days post-fertilization they have a wide behavioral repertoire including hunting food and conditional learning[49, 50]. At seven days post-fertilization, these animals are approximately four millimeters in length which allows for easy experimental manipulation. For example, up to ten of these animals can fit in the well of a 96-well plate. These properties allow rapid experimentation for insights relevant to humans, and with this animal model, behaviors can be evaluated in a systematic and quantitative manner.

Larval Zebrafish Behavioral Profiling

Larval zebrafish behaviors are commonly studied through video analysis before follow-up experimentation on the neural circuit underlying the behavior. One of the first behaviors to be studied, the fast C bend startle response, the behavioral characteristics was used to identify the role of Mauthner cells and the circuit itself. Since then, this approach has been expanded to a wide range of behaviors including light responses, seizures, and circadian behaviors. Given the ease of experimentation of larval zebrafish, these behaviors can be evaluated in a high throughput manner to test a range of conditions. In this aim, behavioral profiling systems were developed for the high throughput evaluation of larval zebrafish behaviors[51-53].

These behavioral profiling systems have allowed side by side comparisons of different conditions on specific behaviors. Evaluating different genetic, environmental, and small molecule conditions on these behaviors has revealed insights into the workings of the behavior and the neural circuit underlying it. Given this potential, a larval zebrafish behavioral profiling system was developed at UCSF[54, 55]. This system holds a 96-well plate on a stage underneath a high-speed camera (Figure 1.05). Various types of stimuli are played to the animals though lights, speakers, and solenoids on the stage while recording, and the video is analyzed by cropping the wells and calculating a motion index from the frame-by-frame pixel intensity differences for each well. This information is then stored in a database for subsequent access and analysis.

To evaluate behaviors using this system, stimuli played to the fish are grouped into assays designed to evaluate a specific response, and assays are grouped together into batteries capable of evaluating multiple types of responses in a single experiment. In previous approaches, these responses have been successfully used to predict and discover molecular interactions in behavior

using custom analysis scripts. To advance experimentation and quantitative methods for diverse behavioral profiling, the Fish Spectacles platform was developed.

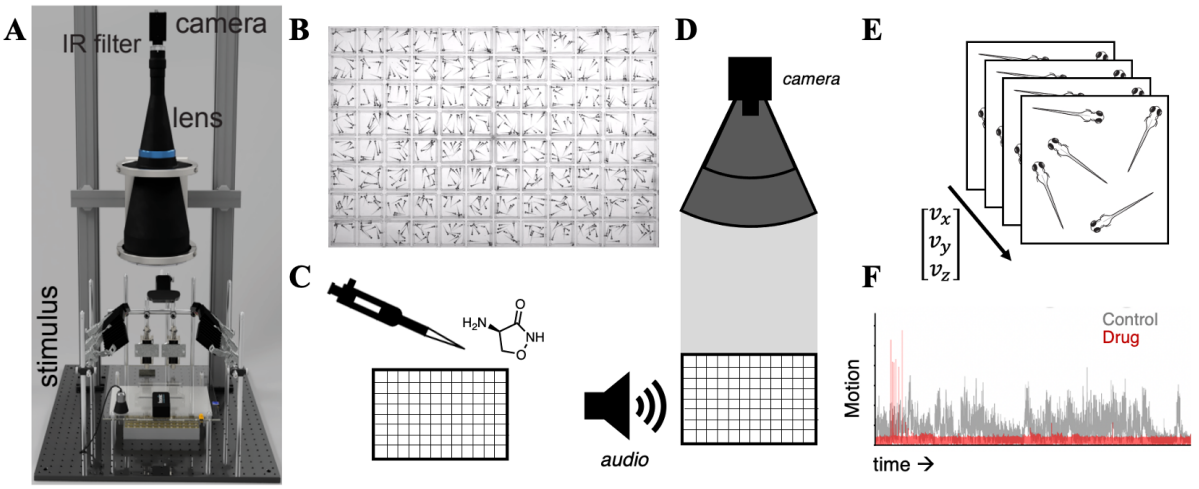

Figure 1.05 Behavioral Profiling System. (A) photograph of system. Experimental workflow of plating larval zebrafish six fish per well in a 96-well plate (B), treating the animals with drugs (C), using the profiling system (D), calculating the motion index (E), and visualizing the results (F).

Results

Fish Spectacles Design

Fish Spectacles is an open-source, user-friendly, lightweight, and flexible analysis application written in Python for analyzing data from the behavioral profiling system. It is fully open source and designed to prioritize user accessibility and data analysis flexibility while maintaining lightweight Python files with minimum requirements. If a user is able to collect raw data, download the application, place the data in the correct folders, and open Fish Spectacles from the terminal, no further knowledge of the code or Python is needed to complete an analysis (Figure 1.06). The platform uses a terminal prompt with functionality to check user input against correct

options and reprompt the user when needed to collect information from the user on how to visualize and/or analyze the data.

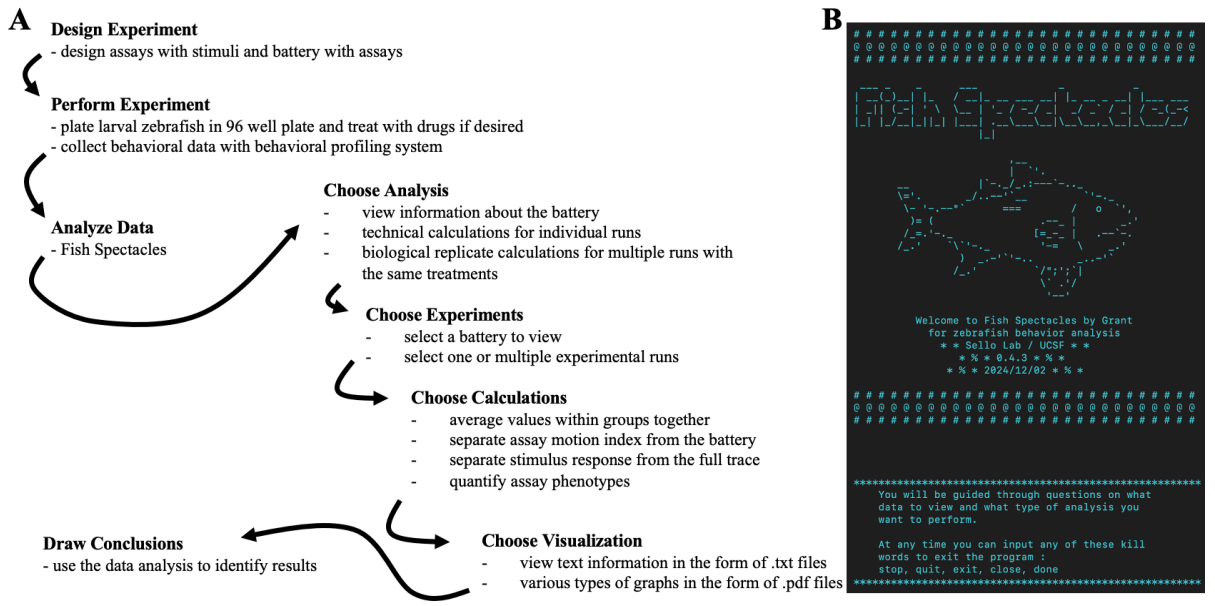

Figure 1.06 Use of Fish Spectacles. Scientific process (A) and initial display of Fish Spectacles (B)

Raw Behavioral Data

When analyzing raw data, it is important to remember how the number relates to the measured quantity. Our behavioral profiling system captures the motion of the animals in 96-well plates by evaluating differences in pixel intensities frame by frame in the video recorded during the experiment. While information about the shape of the fish, shape of fish movements, and contributions of individual larvae when more than one is present in a well is lost in the course of analysis, the bulk behavior is still readily discernible.

It is also critical to realize the relationship between the quantified motion index value and the number of fish is limited by the area of the well. This relationship is maximized when the area of the well is half covered by fish and each fish moves to a new location in the other half. When fewer fish are in a well, not all the pixels will change, and the motion index will be lower. When

more fish are in a well, some of the area previously covered by a fish will again be covered by a fish and the motion index value will be lower. Inherent to the maximum motion index condition, it occurs when the fish respond simultaneously. If responses are staggered, the corresponding motion in the well will be distributed over more frames. The motion index changes from a single peak for the simultaneous response to a broadened peak for staggered responses.

Finally, the frame rate of the camera is important in the quantification of the motion index. The frame rate must be sufficiently fast to capture the responses of the animals. Specifically, it must be fast enough to capture a frame during the response motion of the animals to the stimulus. This requirement can be variable depending on the behavior. The fastest of these behaviors in larval zebrafish is the fast C bend startle response. This behavior occurs within 10 ms after the stimulus is played to the animals. To be able to capture this behavior on a motion index, the camera must capture at least one frame every 10ms or 100 frames per second. Information about motion that occurs within the frame rate, 10ms for 100 fps, is not captured.

The information retained by the motion index is solely the movement occurring per frame in the well, and despite the information lost, it is still highly informative on the behaviors and responses of animals as shown in previous publications. With the quantified motion index, the information is stored by assigning each plate an experiment or "run" number and creating a table with rows for each well in the plate and columns for any information with that well, along with the list of motion index values. These lists are as long as there are frames in the recorded video, meaning a 10-minute experiment recorded at 100 fps produces approximately 60,000 motion index values. Information about the assays and stimuli played to the animals is saved as the assay or stimulus name and the time it started and stopped under a battery number in two respective files. To analyze and visualize the full results of the experiment, this information must be combined.

Behavioral Profiling Experiments

To demonstrate Fish Spectacles, three simple experiments were performed evaluating the enhanced acoustic responsiveness of larval zebrafish when treated with the sedative etomidate. Etomidate promotes the fast C bend startle response as identified by the characteristic response within 10 milliseconds (ms) and C shape motion. The first experiment used six fish per well and compared untreated fish to those treated with various concentrations of etomidate. The second evaluated the effect of a NMDA receptor ligand, D-cycloserine[57-59], on the habituation phenotype, and the final experiment compared different numbers of fish per well on the response of the animals when they were treated with etomidate.

To evaluate the acoustic responsiveness, a custom battery was designed to capture four behavioral characteristics: general movement, acoustic responsiveness, pre-pulse inhibition, and habituation. This was done with four types of assays. The first type, background assay, is the simplest, and it plays no stimuli to the animals for two minutes. This records the baseline activity of the animals before and after assays. Acoustic responsiveness is measured with four soft solenoid taps played every 30 seconds to evaluate how the animals are responding to the acoustic stimulus. This soft solenoid tap produces an acoustic stimulus capable of inducing the fast C bend startle response[60]. The pre-pulse inhibition is measured by the ability of a non-behavior inducing stimulus to inhibit a triggering stimulus. In this case, a soft speaker noise to which the fish do not respond was played at a range of 100 ms intervals before a soft tap stimulus: simultaneous, 100 ms, 200 ms, 300 ms, 400 ms, 500 ms, 600 ms, 700 ms, 800 ms, and 900 ms. It is important to note, while the software triggers the speaker and soft tap simultaneously, they do not play to the animals simultaneously. The speaker plays noise more readily from electronic signals than the soft tap and

it is expected to play slightly before the soft tap when triggered at the same time (~5 ms). For the habituation, several assays were designed playing a soft solenoid tap at various intervals to induce habituation of the fast C bend startle response. The fish begin habituating when taps are played every 10s and habituate quicker as the rate of tapping increases. Thus, three habituation assays of different intervals, 10s, 5s, and 2.5s were used to visualized changes in habituation.

Viewing Raw Behavioral Data

The most basic functionality of Fish Spectacle is the output of raw data. To capture data in a readable, permanent, and transportable format, text information is saved in text files and graphs are saved in portable document files (PDFs). Text information includes treatments and concentrations used in the experiment and assays, and stimuli played during the battery with their start and stop times. Graphs for raw information are motion index vs time plots. Fish Spectacles can generate this graph in various contexts. First, the plots of a single well of six fish untreated can be visualized (Figure 1.07a).

In addition to this raw motion index data, Fish Spectacles also reads the battery and assay data to gather when assays and stimuli were played to the animals. In the most basic visualization, the stimuli are overlaid on the motion index plots as vertical lines (Figure 1.07b). This overlay allows for a visual connection between the motion index values and the stimulus plays, and is useful for qualitative analysis of the behavioral responses. The battery for the current experiments is relatively simple, but other batteries have been developed with more stimuli to evaluate a wide range of responses.

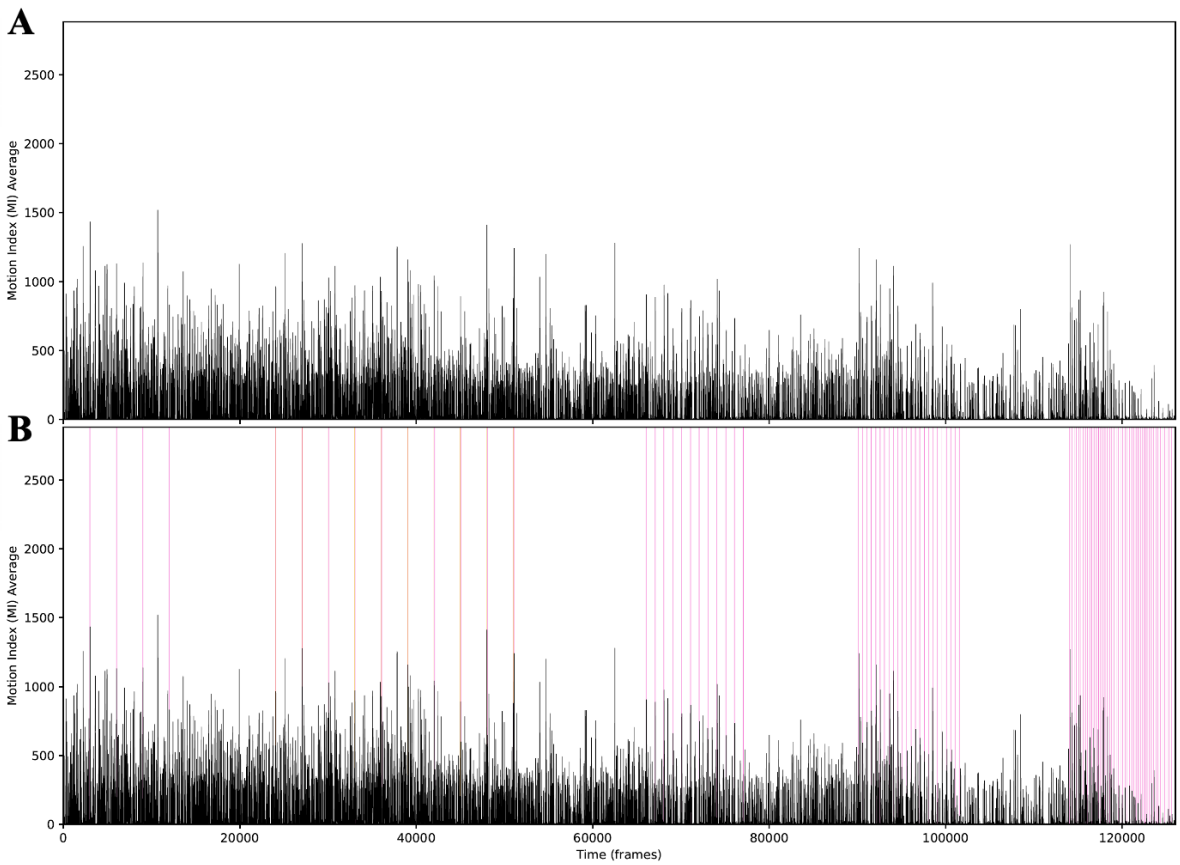

Figure 1.07 Full Battery Motion Index for a Single Well of Six Fish. Full trace shown (A) and the full trace shown with stimulus plays overlaid as vertical lines (B). Soft taps shown in magenta and MP3 speaker noise shown in orange.

Primary Analysis of Behavioral Data

These functionalities are useful, but conditions are typically performed in multiple technical replicates. Conditions are usually a drug treatment, and the treatment and concentration information is saved in the raw behavioral data. Fish Spectacles reads this information and sort the wells into treatment and concentration groups. These collections of motion index values can be overlaid on the same plot to visualize differences within the group (Figure 1.08a). Fish Spectacles also averages these motion index values together to generate an average trace for the condition (Figure 1.08b).

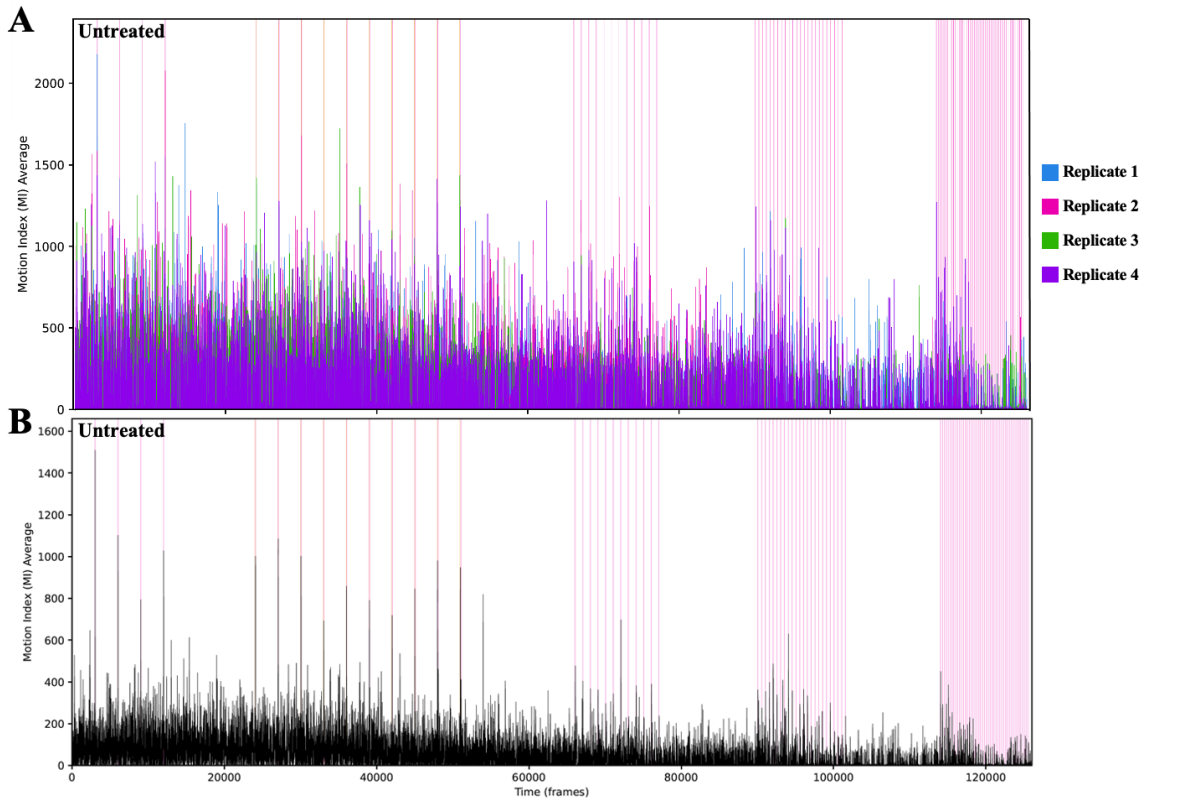

Figure 1.08 Four Technical Replicates of Six Fish per Well. Each replicate shown on the same graph (A) and the average of the replicates (B)

For some experiments, conditions are not drug treatments. An example is evaluating different numbers of fish per well for the effect on the measured response. Fish Spectacles accounts for this by being able to take in custom user-defined groupings. A user only needs to put in the name of the group and the well or list of wells in that group, and the platform can use this as input for sorting wells. Again, these groups of wells can be plotted with each individual well or averaged together. These motion index plots are useful for viewing how fish behave across the whole battery.

Fish Spectacles further analyzes this information. The battery information is used to identify the motion index values associated with each individual assay. These individual assays can be visualized (Figure 1.09). The responses to stimuli are identified as well, and they are quantified to a single value. This calculation is highly customizable and can calculate the mean, maximum, or sum of the motion index values. Each calculation is useful for different types of response analysis. The mean represents the average motion occurring during the stimulus window (Figure 1.10a). The maximum represents the greatest amount of motion that occurred in one frame (Figure 1.10b), and the sum represents the total motion that occurred during the stimulus time frame (Figure 1.10c). For each calculation, the relationship between the value and the standard deviation is unique. These differences in calculations are shown for averaged untreated fish during the baseline soft tap response assay. The unaveraged responses of groups or conditions can be

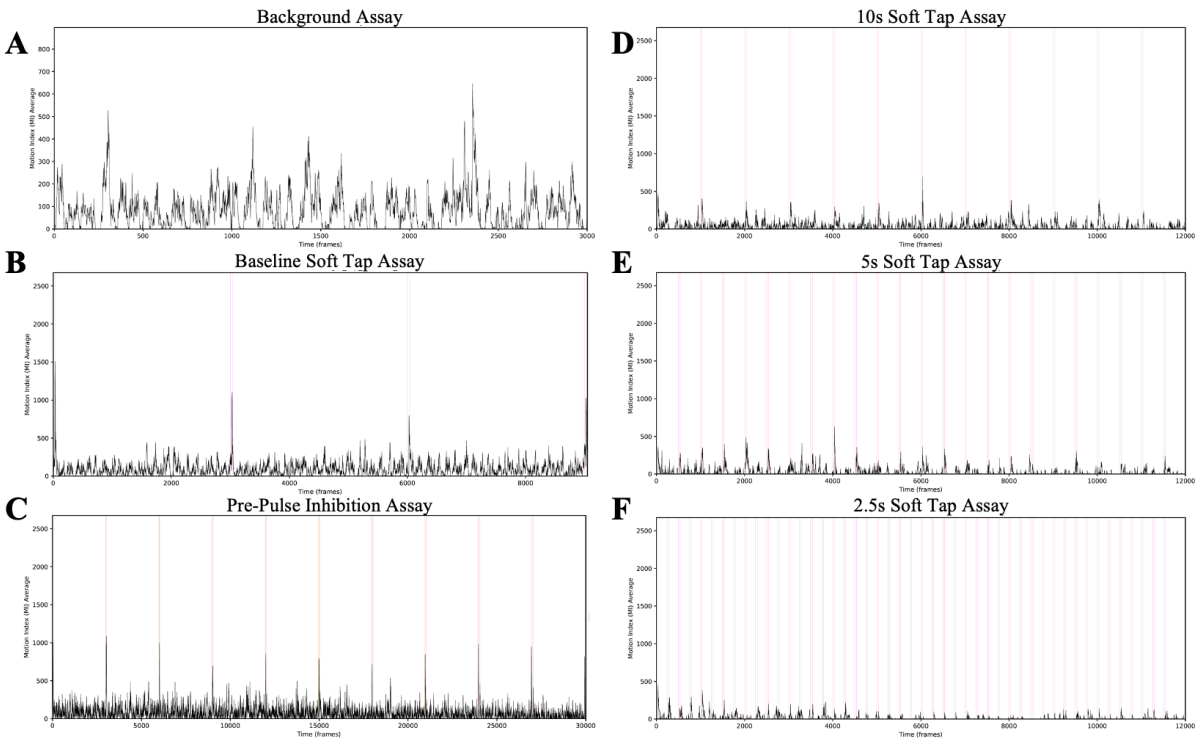

Figure 1.09 Individual Motion Indices by Assay. Shown are the background assay (A), baseline soft tap assay (B), pre-pulse inhibition assay (C), 10s soft tap assay (D), 5s soft tap assay (E), 2.5s soft tap assay (F).

visualized as well. Using the maximum calculation, each response in the same group is plotted side by side and is useful for viewing the variability within a condition (Figure 1.11). While these calculations can be customized, the default calculations are to calculate the maximum response when the stimulus is a sound and the average response when the stimulus is a light. Thus, the maximum value is used to quantify the soft tap response. This point will be further showcased in the secondary analysis.

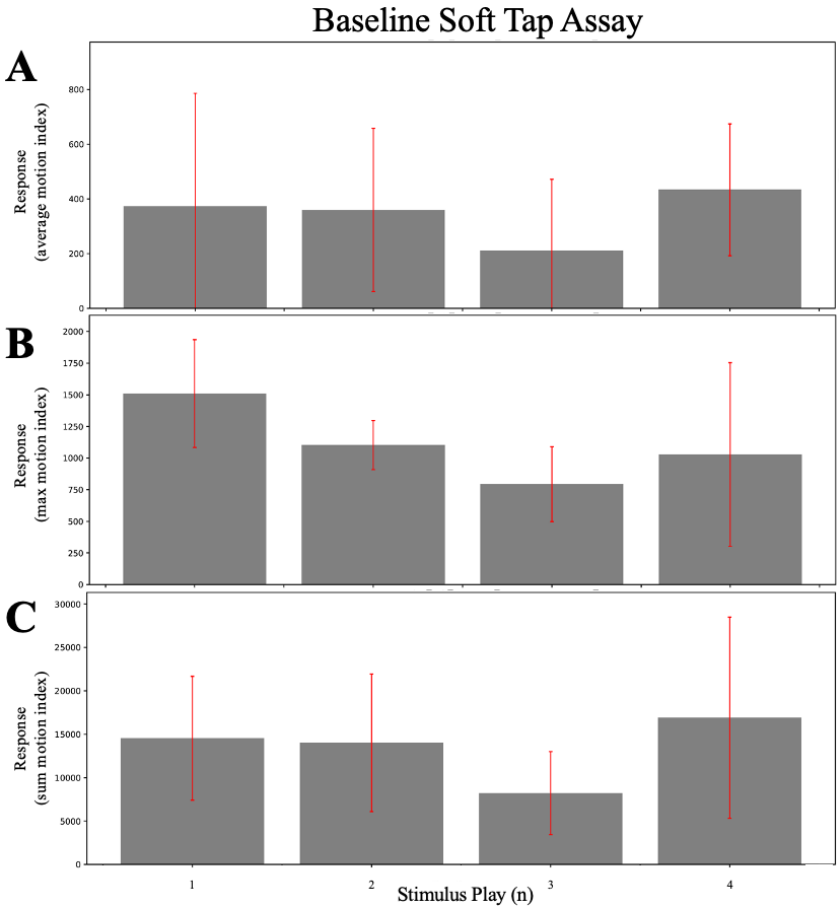

Figure 1.10 Variable Calculations on Response Motion Indices. Calculated are the average (A), maximum (B), and sum (C) for each response in the baseline soft tap assay.

With these types of analysis, graphs for different conditions are compared side by side. To compare the effect of etomidate treatments on the fish, a seven-point threefold dilution series of etomidate was given to the fish. With the averaged motion index for each condition, the background assay shows sedation as the progressive loss of general motion as the concentration of

etomidate increases (Figure 1.12a). The soft tap baseline assay shows that even though the etomidate-treated fish are sedated, they still respond to soft tap stimuli (Figure 1.12b). Comparing the graphs of the stimulus responses between etomidate-treated and non-treated fish shows the responses are approximately the same until increasing from 3 to 11 micromolar (uM) and then decreasing (Figure 1.12c). Differences in the soft tap responses between untreated fish and etomidate-treated fish become more apparent when the soft taps are closer together (Figure 1.13). Etomidate-treated fish continue responding to soft tap stimuli to a greater degree than their non-treated counterparts. This is possibly because the sedated fish only do the fast C bend startle response while non-treated fish are fully awake. The non-treated fish can still perform other behaviors such as different types of escape responses, and these behaviors might not be able to be interrupted by a soft tap stimulus.

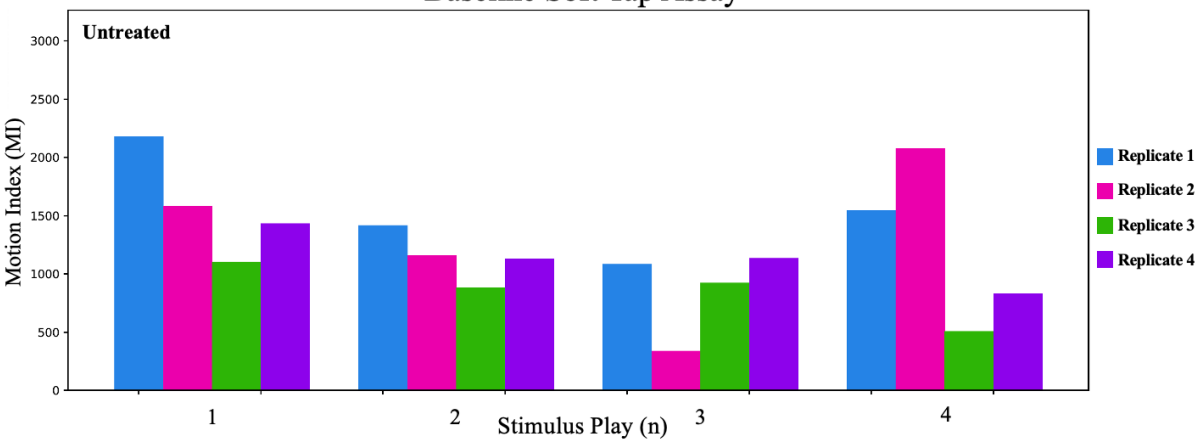

Figure 1.11 Technical Replicate Response Plot Side by Side. Side by side replicates of 6 fish per well during the baseline soft tap assay.

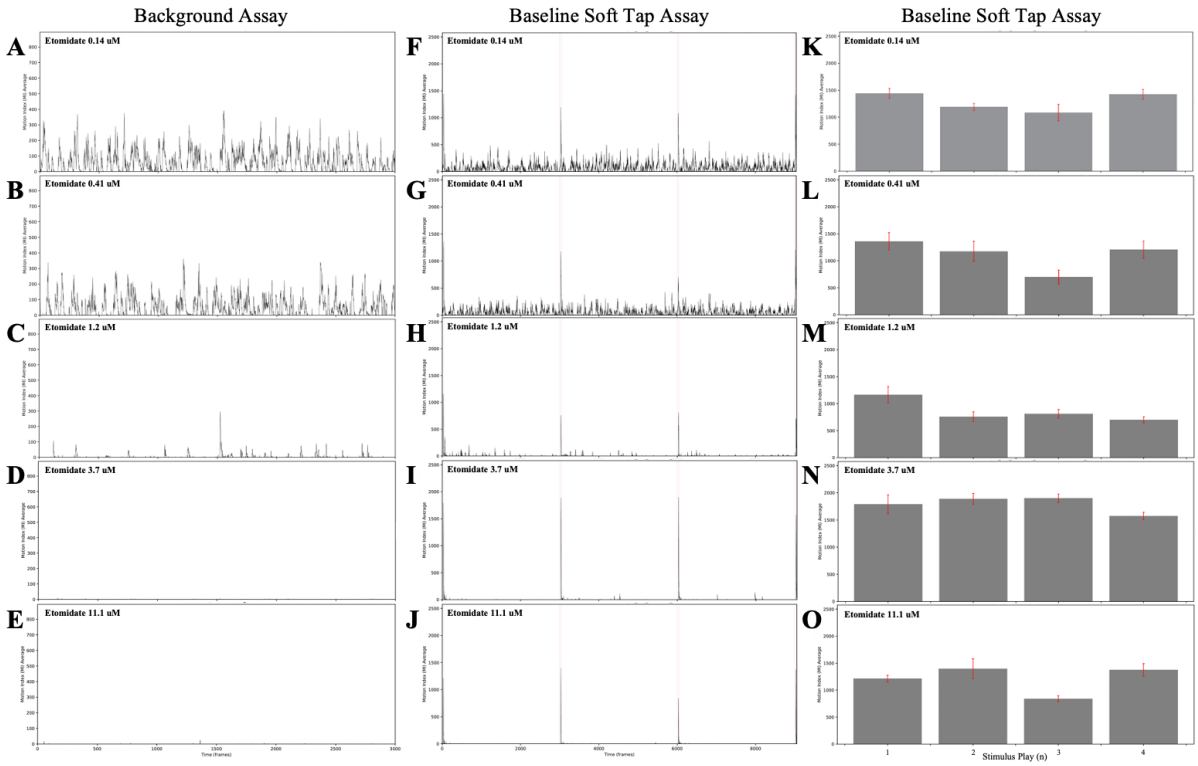

Figure 1.12 Behavior Effects of Etomidate Titration. Shown are increasing concentrations of Etomidate during the background assay (A-E), baseline soft tap assay (F-J), and responses of the baseline soft tap assay (K-O)

Secondary Analysis of Behavioral Data

The above qualitative analysis is useful for visualizing the effects of different conditions on behaviors. Fish Spectacles also quantifies this data by calculating a responsiveness value for each assay. These calculations are highly customizable. The whole assay motion index values or the stimulus responses in the assay can be used. With the whole assay motion index values, the mean, maximum, and sum can be calculated. Similar to the previous variable calculations, the mean represents the average motion during the assay. The maximum represents the greatest amount of motion occurring at the same time, and the sum is the total amount of movement during the assay. For example, to determine the baseline movement of the animals, the sum of the motion index values of the background assay is used (Figure 1.14). This responsiveness can be plotted in

a concentration-dependent manner for the etomidate titration. The half-effective concentration (EC50) is approximately 500 nanomolar (nM) for sedation.

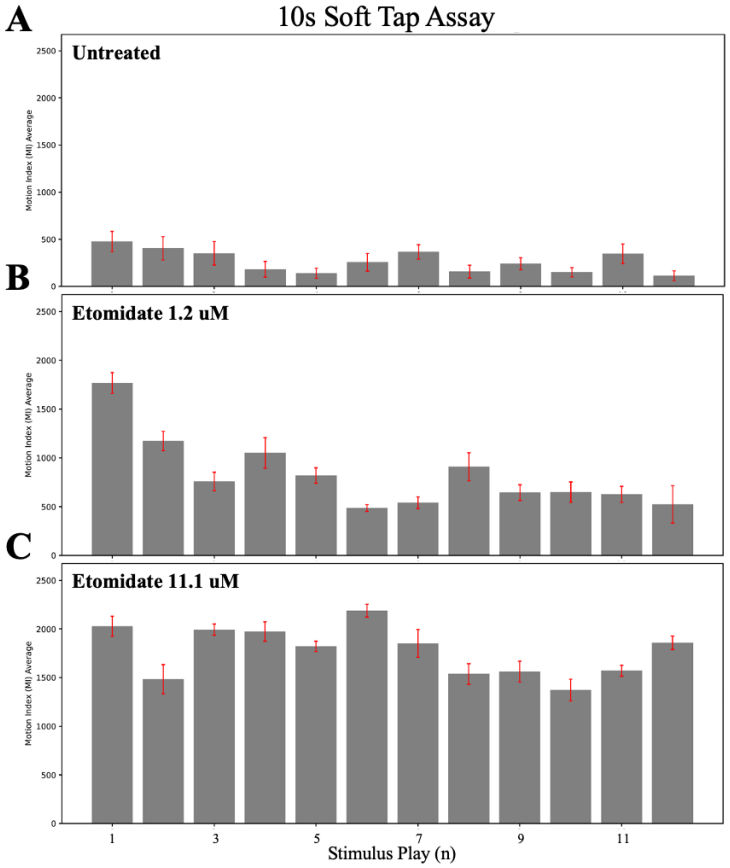

Figure 1.13 Select Etomidate Concentrations on Responses During 10s Soft Tap Assay. Shown are untreated fish (A), 1.2 uM etomidate-treated fish (B), and 11.1 uM etomidate-treated fish (C)

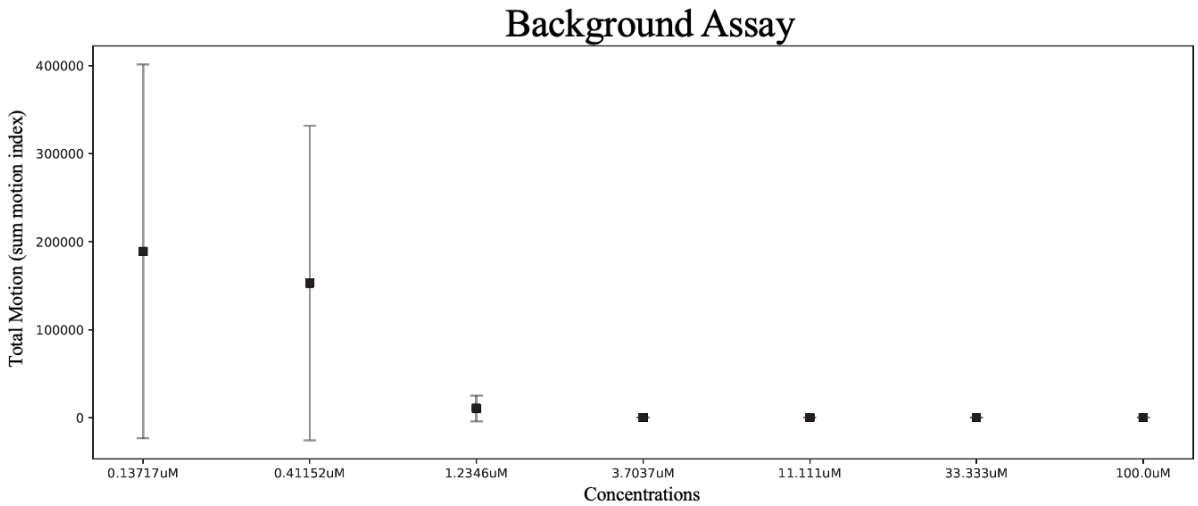

Figure 1.14 Etomidate Concentration-Dependent Effects on Total Motion During the Background Assay. Shown is the sum of the motion index values for each concentration.

To determine a responsiveness value from the individual stimulus responses, the mean, maximum, and sum can be calculated. It is important to remember that this analysis builds upon the calculation for the stimulus response. These values are already the result of a custom calculation of the motion index values, and as a result, the average of the individual stimulus responses is used to calculate responsiveness from the stimuli as the default. For the baseline soft tap assay, the responsiveness as the average of soft tap responses is shown for non-treated animals and the titration of etomidate (Figure 1.15). The qualitative results seen in the primary analysis are quantified, and the aforementioned trend in responsiveness can be seen.

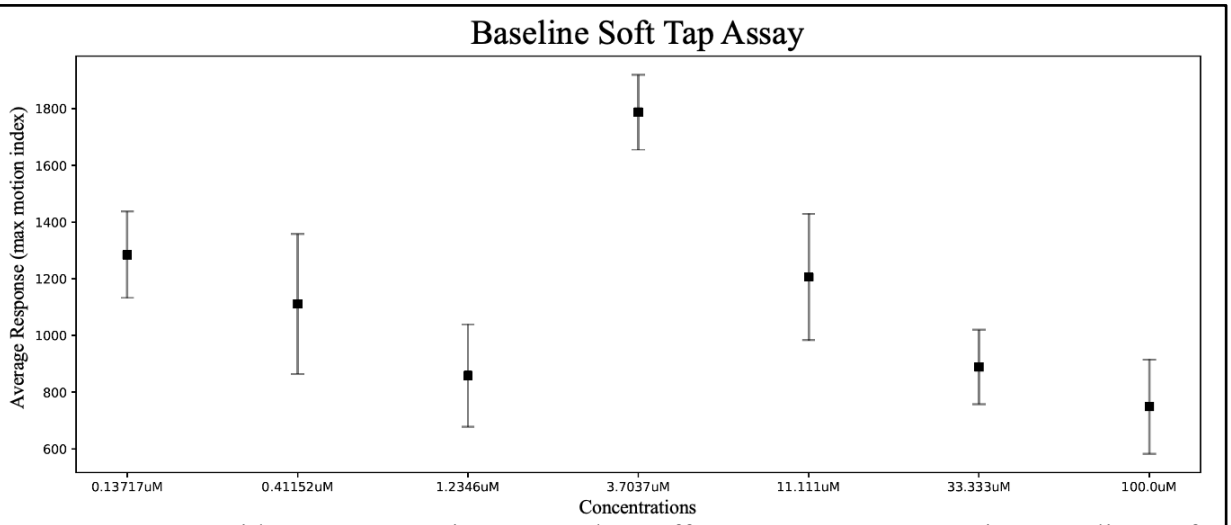

Figure 1.15 Etomidate Concentration-Dependent Effects on Responses During Baseline Soft Tap Assay. The average of the responses during the baseline soft tap assay is shown. The individual responses were calculated from the maximum motion index value.

Additionally, Fish Spectacles calculates the habituation, or rate of change in responses, for a given assay. This quantification can be used to evaluate the acoustic startle response caused by etomidate. In the 10s, 5s, and 2.5s soft tap assays, etomidate-treated fish habituate slow, medium, and rapid (Figure 1.16). The phenotype of habituation in the fast C bend startle response results from plasticity in the underlying circuit, and one receptor that controls plasticity in this circuit is the NMDA receptor. Its activation drives plasticity and habituation, while blocking it inhibits these processes, allowing NMDA receptor ligands to modulate the rate of habituation [61-67]. To probe this

effect, D-cycloserine, a partial agonist, was titrated against fish. The side-by-side comparisons on the stimulus responses of untreated fish to those treated with D-cycloserine (DCS) is shown with lines representing the calculated rate of change in the responses (Figure 1.17a). Additionally, the concentration-dependent changes are graphed by Fish Spectacles (Figure 1.17b). These show the increasing effect of DCS on the rate of habituation and the usefulness of quantification.

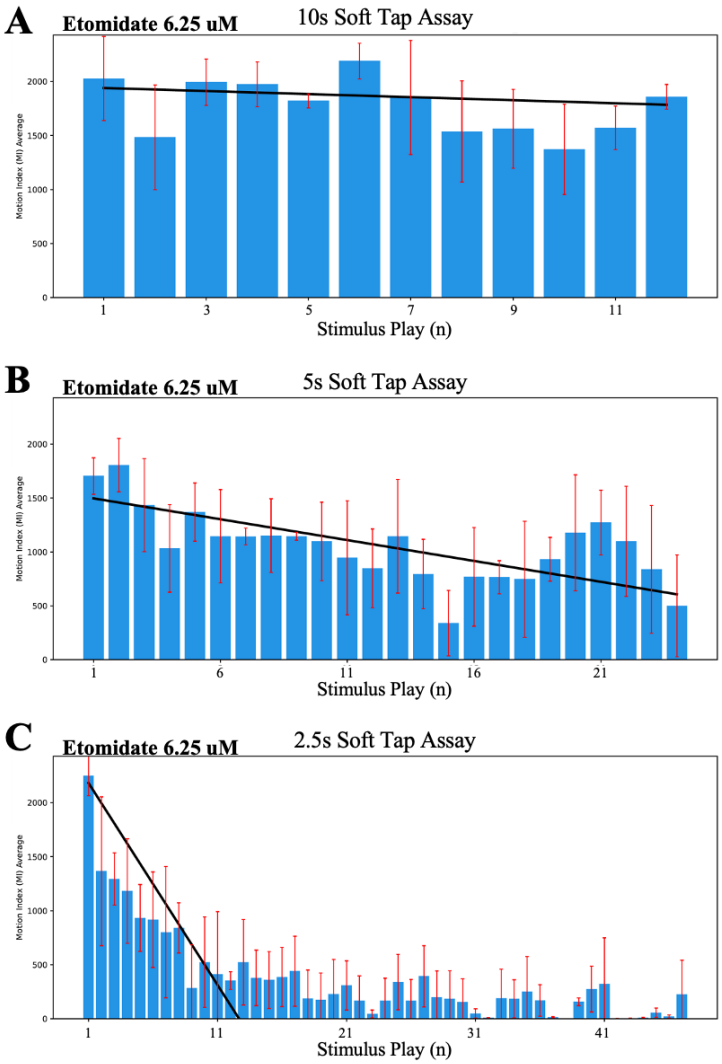

Figure 1.16 Responses and Rate of Change for 6.25 uM Etomidate Treatment. Show are the responses for the 10s (A), 5s (B), and 2.5s (C) soft tap assay along with the calculated rate of change in the responses (black line)

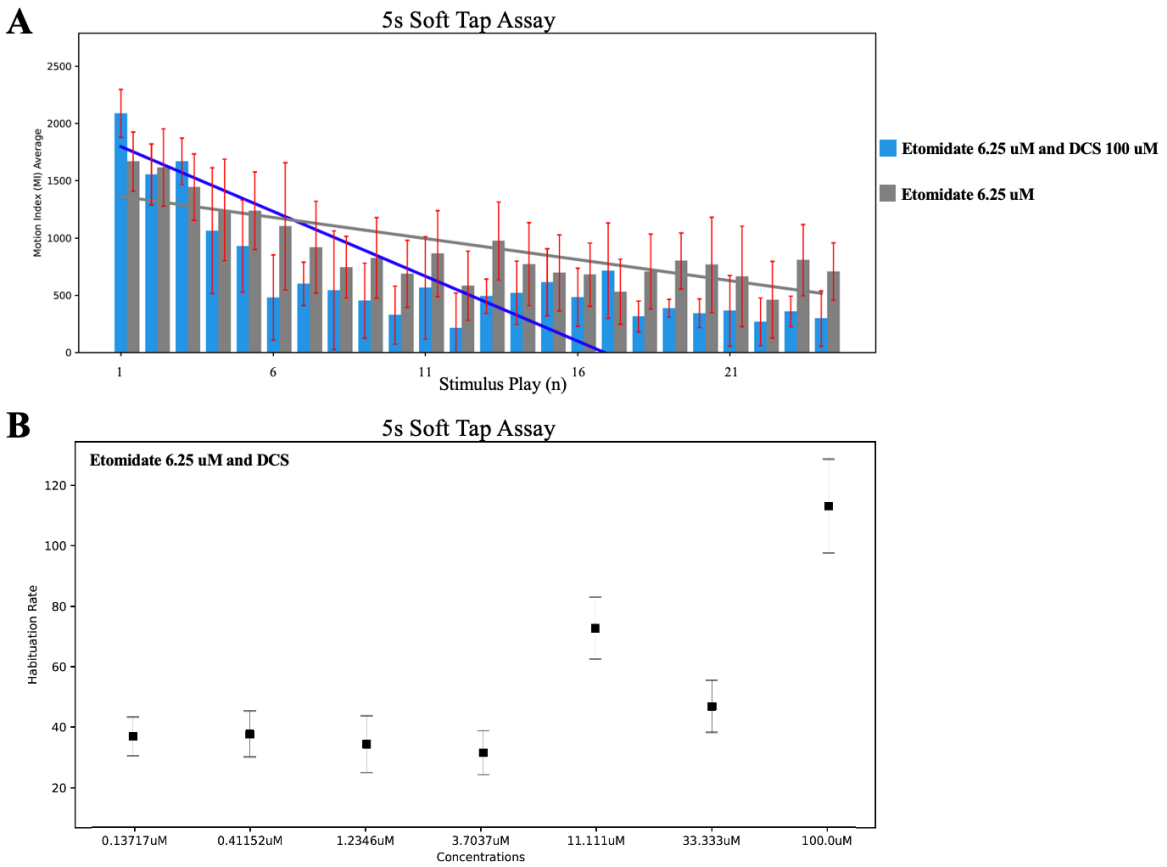

Figure 1.17 Effect of DCS on Responses During 5s Soft Tap Assay. Side by side comparison of responses of 6.25 uM etomidate (gray) and co-treatment of 6.25 uM etomidate and 100 uM DCS (blue) (A), and the concentration-dependent effects of 6.25 uM etomidate co-treatment with DCS (B).

Pre-pulse inhibition is also analyzed in the secondary analysis. In the assay, pre-pulses of the speaker noise at intervals 100ms to 400ms before a soft tap fully inhibited the response, while the response gradually recovered at later intervals (Figure 1.18). This interval of inhibition is in agreement with the established literature (52). To evaluate the effect of different numbers of fish on these intervals, the responses of specific intervals are shown by condition (Figure 1.19). As noted, irrespective of numbers of fish, the responses are inhibited at the 200ms interval. By the 800ms interval, the responses are normal and resemble the baseline responsiveness of the soft tap baseline assay.

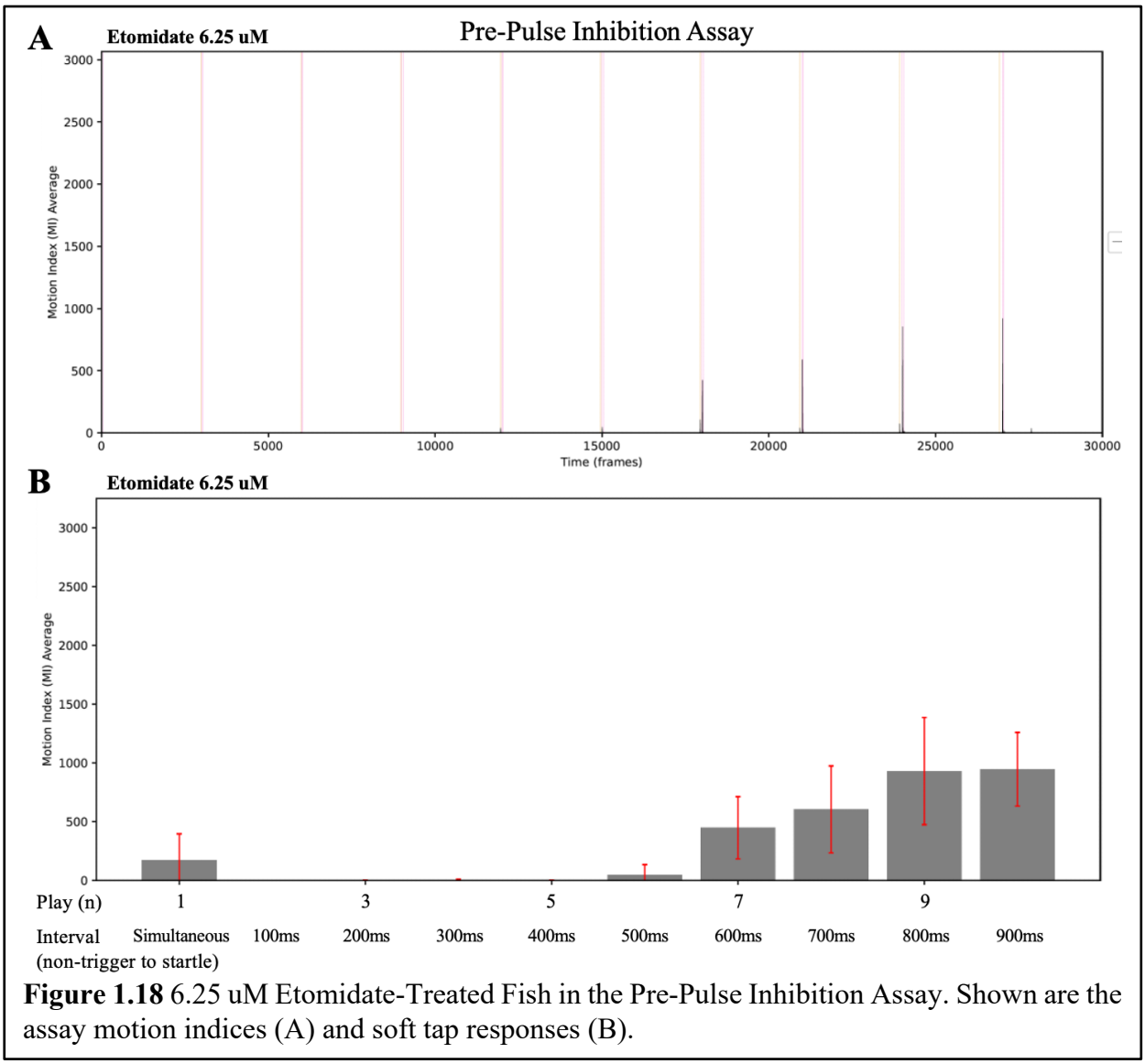

Figure 1.18 6.25 uM Etomidate-Treated Fish in the Pre-Pulse Inhibition Assay. Shown are the assay motion indices (A) and soft tap responses (B).

The final part of the secondary analysis is an automatic activity-based grouping function. This grouping finds conditions with values that are separated by at least one standard deviation and uses these conditions to define a group. It then finds all the conditions with values that fit within each group. This is highly useful when visualizing distinct effects on responses. As an example, an experiment was performed on etomidate-treated fish where each row of a 96-well plate had different numbers of fish. As expected, the responses increase with increasing numbers of fish (Figure 1.20a). These results were easily grouped by the grouping function (Figure 1.20b).

This experiment also makes an excellent case to look at the differences in the stimulus response calculations, mean, max, and sum, and how they affect the responsiveness calculation since increasing numbers of fish should lead to higher response and responsiveness values. The results highlight how these values differ between conditions (Figure 1.21). It is clear that the maximum captures the response of the fast C bend startle response. It shows the most resolution in the conditions, and in most assays, each condition is well separated from the others. The sum of the stimulus motion index values to calculate the response worked the least for this behavior. It is not clear why the sum does not capture the fast C bend response well, but one explanation is while fish respond simultaneously and perform the C bend

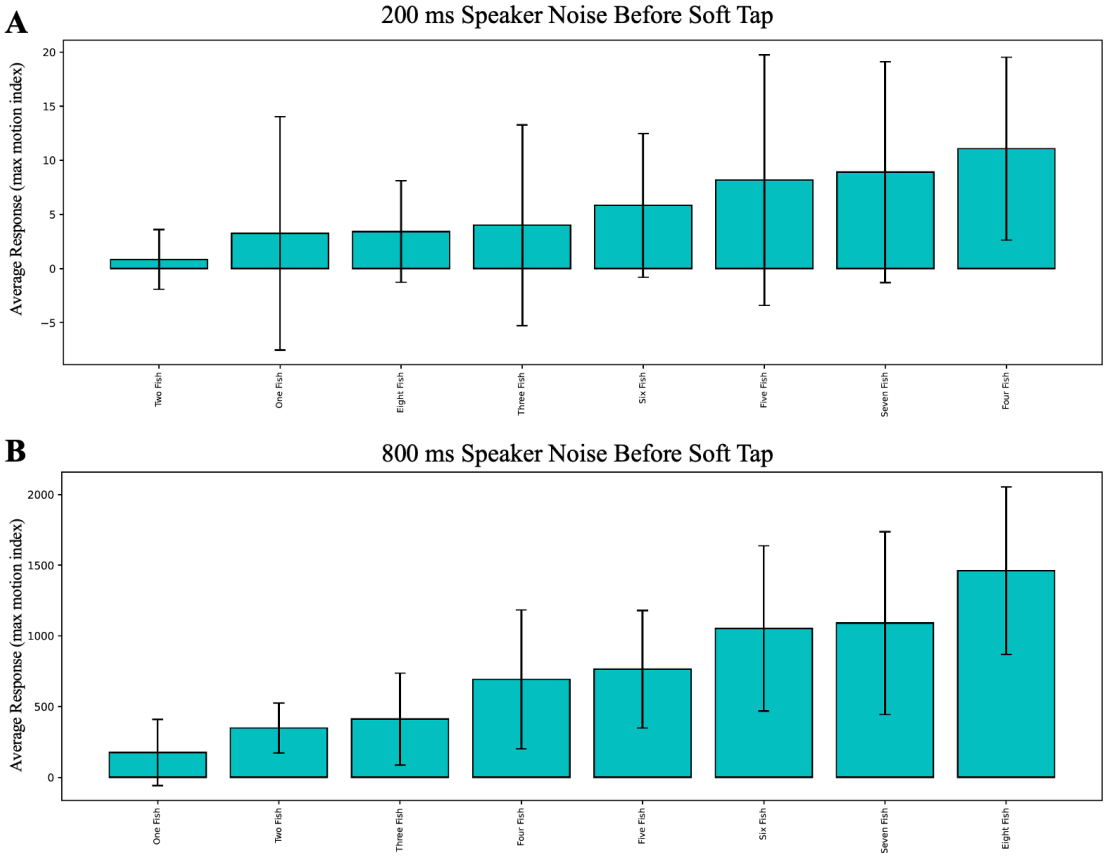

Figure 1.19 Pre-Pulse Responses at Different Intervals for Increasing Numbers of Fish. No responses independent of fish number seen when the speaker noise was play 200ms before the soft tap (A), but the fish number dependent responses can be seen during the later 800ms interval

may be more variable. This would produce the same effect of a high motion index value during the initial response to the stimulus and then a variable amount of motion index values after the C bend.

Finally, Fish Spectacles works with both individual experiments for technical replicates and multiple experiments for biological replicates. This enables experiments with the same battery to be analyzed for statistically significant effects. To do this, the list of experiments is input, and Fish Spectacles averages the results of the primary analysis together and performs the secondary analysis. All of the same functionality is available for the biological analysis as the technical analysis.

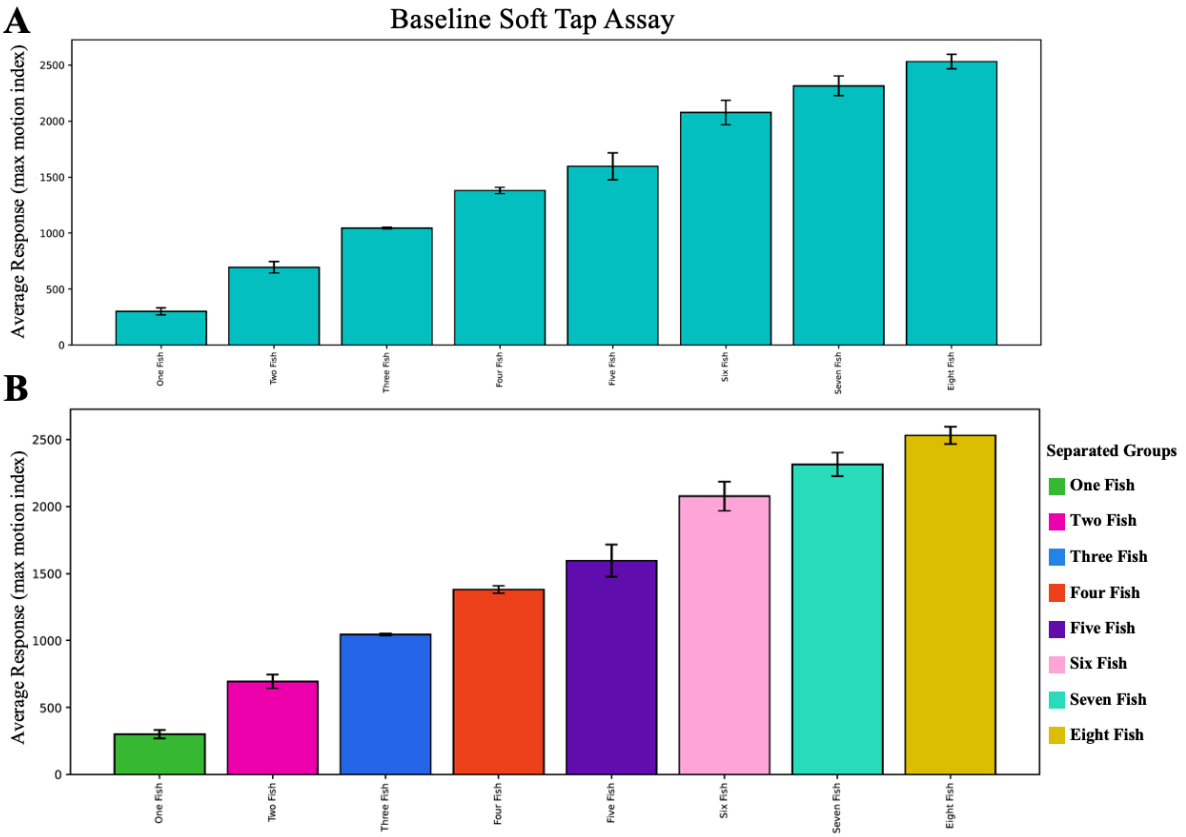

Figure 1.20 Grouping Analysis Applied to Responsiveness Calculations for the Baseline Soft Tap Assay. The responsiveness calculated from the average responses to soft taps during the baseline soft tap assay for different numbers of fish (A), and the Fish Spectacles grouping algorithm applied to the results of A (B).

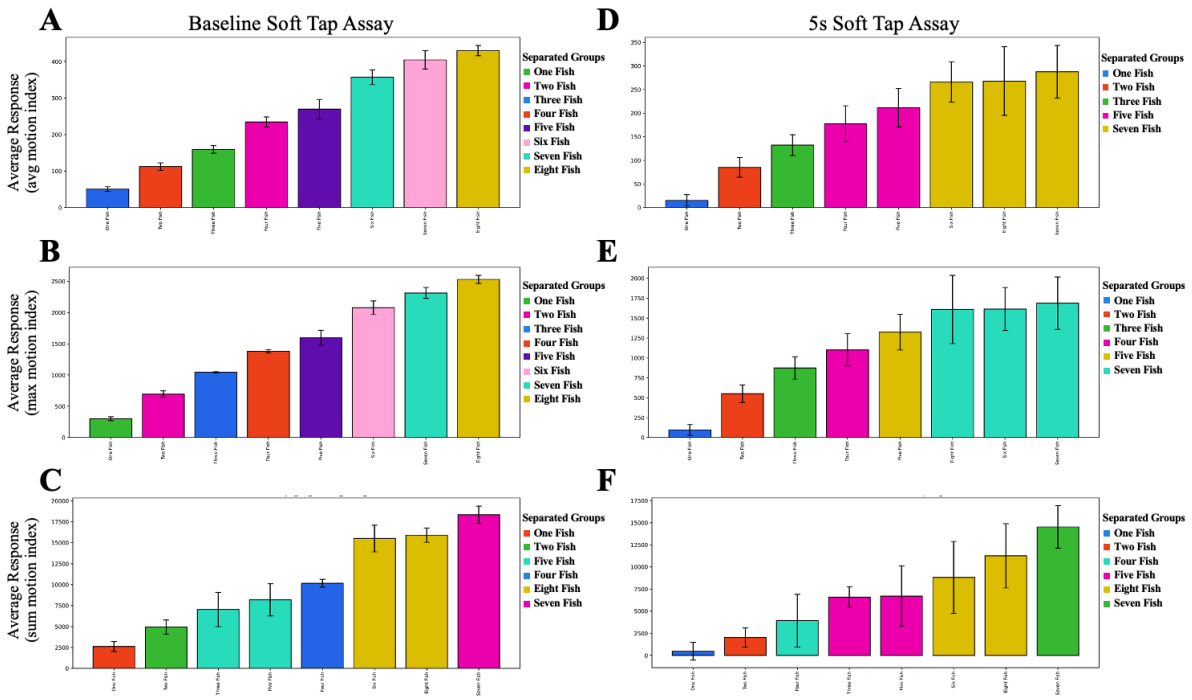

Figure 1.21 Grouping Applied to Different Responsiveness Calculations. The average of the average (A), maximum (B), or sum (C)of the individual responses during the baseline soft tap assay or the 5s soft tap assay (D, E, F respectively) are shown. The grouping algorithm successfully found separate groups with similar responsiveness.

Discussion

Accessible and Systematic Approach for Quantifying Larval Zebrafish Behavioral Data

Fish Spectacles is designed to enable quantitative analysis and visualization of raw behavioral data from UCSF's behavioral profiling system in a manner accessible to those with little knowledge of coding. The platform is highly flexible and accounts for each stage of analyzing behavioral data from viewing the whole experimental battery motion index to calculating rates of change in isolated stimulus responses all while maintaining efficient computer memory use. Many types of calculations are available and adding additional custom calculations is a simple process detailed in the README file. Viewing the results is also simple. Text information is saved in text files and graphs are saved in PDF files. These outputs enable long-term storage of data in a standard format, use in additional scripts for further analysis, and generation of figures for publication.

Potential for Machine Learning

Fish Spectacles excels at the processing, primary analysis, and secondary analysis of raw behavioral data which can generate large amounts of data on compounds. Viewing one or a few different conditions is manageable by a single user, but the data for commercial libraries in batteries with many different assays is a great case for machine learning. Machine learning models need clean data with well-defined features to be correctly trained. Fish Spectacles does this through its collection of groups in and between experiments and isolation of stimulus responses. These results can be used either through the text files, or Fish Spectacles itself can be modified. The primary and secondary data analysis classes have well defined methods and instances that can be called in a new analysis. Fish Spectacles in combination with machine learning has the potential to prevent many common problems such as training on non-representative features in the behavioral motion indices.

Experimental

Zebrafish Husbandry

Up to 10,000 fertilized zebrafish embryos were collected per day from group matings of wild-type zebrafish. Larvae were raised on a 14/10 hour light/dark cycle at 28 °C until 7 day post-fertilization. Larvae were anesthetized with cold egg water and distributed into square 96-well plates. Plates were incubated at room temperature for 30 minutes for animals to become active. Molecule stock solutions were applied directly to the egg water, and larvae were incubated with the drug at room temperature for 30 minutes before behavioral analysis. The zebrafish-related procedures were conducted according to established protocols approved by UCSF's Institutional

Animal Care and Use Committee (IACUC) and in accordance with the Guide to Care and Use of Laboratory Animals (National Institutes of Health 1996).

Molecules

Etomidate was dissolved in DMSO and diluted to the fish from 300X stock solutions for a final concentration of 0.3% final concentration of DMSO. DMSO was diluted accordingly for comparisons between drug treatments. DMSO did not produce any noticeable behavioral effects upon comparison to untreated fish (data not shown). DCS was dissolved in water.

Fish Spectacles Source Code

The full open-source code and README documentation are available on GitHub. https://github.com/grantburley/SelloLab_Fish_Spectacles

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CHAPTER 2 : DESIGN AND SYNTHESIS OF NEUROACTIVE AMINO ACID LIKE MOLECULES

Abstract

Much of our molecular knowledge of the nervous system comes from using small molecules as probes for neuroactivity. The most effective of these probes mimic endogenous neurotransmitters, amino acids or close derivates, to bind to specific receptors. This methodology of probing the nervous system has slowed with the lack of new neuroactive small molecules. Developing new probes requires strong considerations of pharmacodynamics and pharmacokinetics as many molecules are blocked from the brain with the blood brain barrier or interfere with endogenous neurotransmitter metabolism to produce off-target effects. A biological isostere of carboxylic acids with improved pharmacodynamic properties, tetrazoles, and secondary amines to prevent interference with metabolism is synthesized on the amino acid scaffold in the aim of developing new neuroactive probes.

Introduction

Nervous System Organization

The nervous system is complex. While specific areas of the nervous system are responsible for specific functions like other organ systems, the individual cells in the nervous system have unique connections and properties. This means two cells of the same type in the same area are likely to function differently and covey unique information to its neighbors. Compared to the digestive system where goblet cells all secrete mucus for lubrication or the urinary system where all glomerular endothelial cells filter the blood for waste products, this property of individual uniqueness makes understanding the nervous system much more difficult. The nervous system's properties arise from the network of cell connections and the connections between cells being malleable themselves, changing based on the activity of the individual cells[1]. As a result, much of

the scientific community's understanding of the nervous system comes from probing the connections between cells in living organisms, as this property has yet to be accurately recreated *in vivo*.

Molecular Communication

Communication in the nervous system is governed by small molecules called neurotransmitters. The majority of neurotransmitters are amino acids or derived from them (Figure 2.01). These neurotransmitters each bind to specific types of receptors in the nervous system such as glutamate with AMPA, Kainate, NMDA, and metabotropic receptors; gamma-aminobutyric acid (GABA) with ionotropic GABA-A and metabotropic GABA-B receptors; and glycine with ionotropic and potentially metabotropic receptors. Each type of receptor has a unique function from how it changes the cell to be more or less active and how it induces cell signaling through ionotropic depolarization or metabotropic secondary g proteins. Probing the activity and function of these proteins has been quite difficult. To probe activity of a specific receptor, a small molecule that binds specifically to that receptor is needed, and this quality is very hard to come by considering many of these proteins bind the same ligand. Despite this, ligands with relative specificity have been discovered and used to understand the function of some of these proteins[2-5].

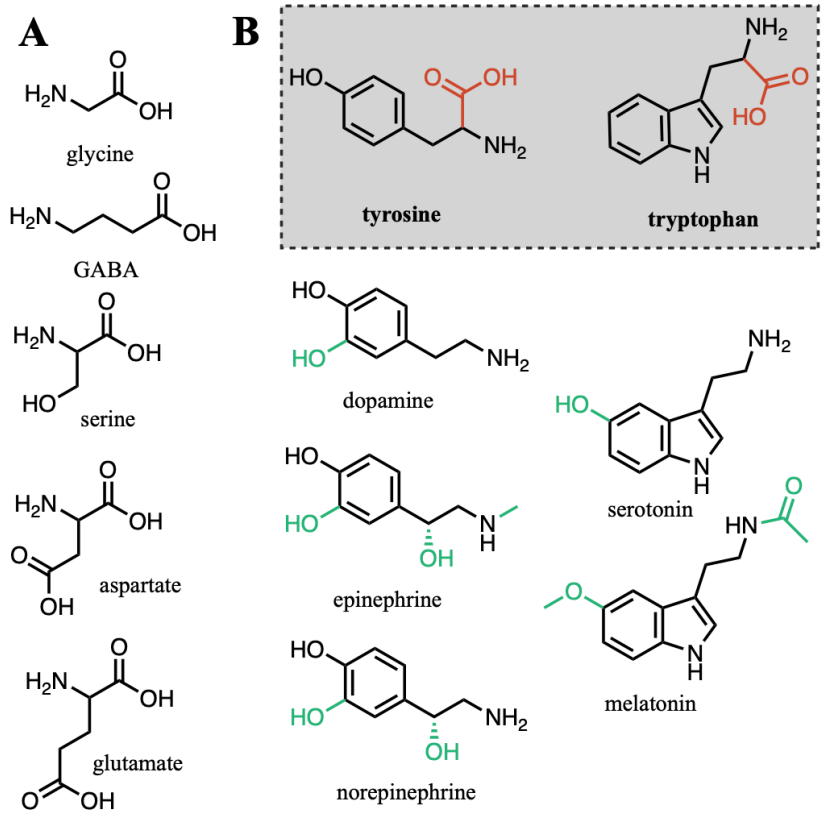

Figure 2.01 Neuroactive Amino Acids and Derivates. Some neuroactive amino acids (A) and neuroactive derivates of amino acids commonly tyrosine and tryptophan (B)

As we understand how these receptors function, the way these receptors become dysregulated during neuropathologies becomes clearer. The receptors of the glycinergic, GABAergic, and glutamatergic systems are dysregulated during conditions such as schizophrenia, epilepsy, depression, substance abuse, bipolar disorder, neuropathic pain, and learning disabilities[6-12]. These conditions only have drugs for suppressing symptoms of the disease and lack curative treatments. This is especially true for conditions requiring glycinergic or glutamatergic ligands. The GABAergic system has a diverse set of allosteric ligands for GABAA receptors but less so for competitive ligands as well as ligands for GABAB receptors. Using ligands of these systems has shown promise as therapies but often fail to meet clinical standards due to poor bioavailability or poor specificity.

Neuroactive Small Molecule Development

Developing small molecule drugs is difficult. *In vivo*, no molecule only binds to a single protein, and the pharmacodynamic and pharmacokinetic properties can prevent or alter target engagement. Developing drugs targeting the nervous system is more difficult. Not only do the typical drug development hurdles apply, but the major organ of the nervous system, the brain, is surrounded by the blood brain barrier. The blood brain barrier is designed to deny the large majority of small molecules while only letting in others in an highly controlled manner, especially those resembling the endogenous neurotransmitters[13]. This protects the nervous system from uncontrolled activation given many receptors are activated by amino acids which are highly abundant in the body and blood. The barrier uses both molecular conformations and chemical properties for selective permeability[14]. While the molecular conformations are less characterized, this barrier is known to be impermeable to molecules with charges at physiological pH such as carboxylic acid functional groups. This keeps out the majority of drugs and endogenous small molecules.

If a molecule makes it to the brain, it is subjected to metabolic processes. Metabolism of small molecules is unique, and the chemistry is optimized to anabolize and catabolize neurotransmitters through amino transferases or monoamine oxidases[15-17] (Figure 2.02a,b). The removal of the amine group prevents the molecules from being recognized by neurological receptors, allowing the products to be secreted from cells without non-specific activation. This biological process of the brain indicates an essential property for molecules to be neuroactive, recognition of amine functionality. This underlying chemistry of amine transferases has been problematic for drugs. Drugs with primary or secondary amines that are able to be recognized by one of the many amine transferases in the brain are subject to their catabolism. Not only can the

products of these reactions have their own effects particularly when the molecule has more than one amine group, the innate metabolism of the brain can also be greatly dysregulated. D-cycloserine, for example, irreversibly binds to GABA amino transferases and blocks their function[17, 18] (Figure 2.02c). These effects can cause the buildup of neurotransmitters in neurons and glia as well as prevent the synthesis of other neurotransmitters. This produces its own changes in neurological communication and causes off target effects. These effects emerge in the clinic after several days of treatment as drowsiness, seizures, psychosis, anxiety, numbness and other central nervous system effects, and they prevent its use outside of last resort tuberculosis treatment.

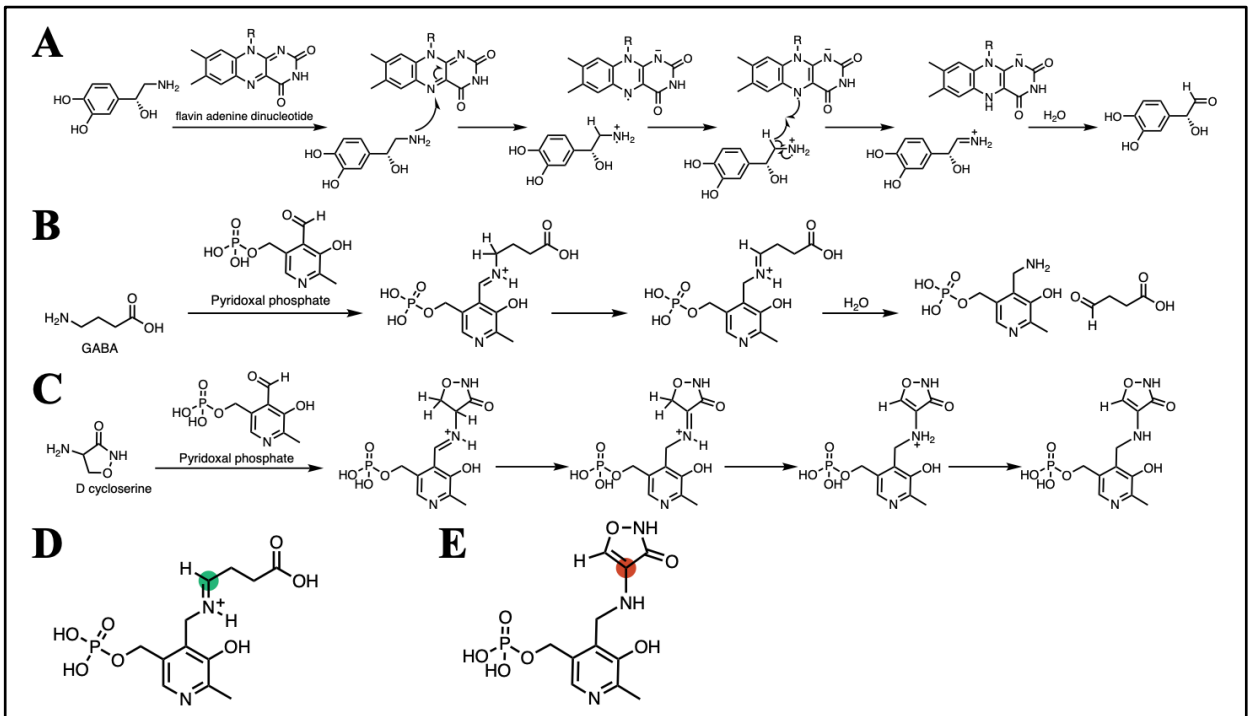

Figure 2.02 Amine Removal From Neurotransmitters. Monoamine oxidases (MAOs) (A) and aminotransferases (B) remove the amine group from neurotransmitters. Drugs with primary and secondary amines can interfere with endogenous metabolism such as DCS (C). Typical aminotransferase complexes of pyridoxal phosphate and neurotransmitters are reversible such as GABA (D), but the complex with DCS is irreversible (E)

Bioavailability of Small Molecules

To further understand the roles of neuroactive receptors in homeostatic and pathological conditions, novel ligands for these receptors have to be synthesized. But first, the challenges must be addressed. The challenges of drug development fall into two main areas: the bioavailability of the molecule and the specificity of the molecule for its desired target and resulting effect. Chemical properties that significantly contribute to bioavailability can be predicted or measured such as the acid dissociation constant (pKa), calculated lipophilicity (cLogP), hydrogen bond donors and acceptors, total polar surface area (tPSA), and molar refractivity[19] (Table 2.01).

Table 2.01 Common Calculated Physiochemical Properties to Predict Bioavailability

Property	**Ideal Range for Bioavailability**	**Ideal Range for Neuroactivity**
molecular weight (g/mol)	<500	<450
lipophilicity (cLogP)	0.5-5	2-5
hydrogen bond donors (n)	5	3
hydrogen bond acceptors (n)	10	7
total polar surface area (A)	<140	<70
molar refractivity (m3/mol)	40-130	40-130

The amino acid receptors engage their ligands by recognizing the two chemical groups which are present on all amino acids, the amine and carboxylic acid groups. These groups are recognized by their charge and/or their hydrogen bonding capabilities. Both of these groups play key roles in the underlying biology as mentioned above and have very poor bioavailability, which makes directly using these groups in ligands unfavorable. Certain groups, biological isosteres, are known to be suitable replacements for problematic groups such as tetrazoles for carboxylic acids[20, 21] (Figure 2.03). These groups retain the chemical properties of the original group, but are often "milder" than the original group. Tetrazoles, for example, have a more neutral pKa than carboxylic acids which results in less of a negative charge on this group in physiological conditions. Tetrazoles also have the ability to delocalize the charge out over more atoms through

tautomerization. In a similar line of reasoning, tertiary dimethyl amines are biological isosteres for other primary and secondary amines. These groups retain a similar but slightly more neutral pKa which generally improves pharmacodynamic properties, and they increase the lipophilicity of the molecule which is desired for reducing the high hydrophilicity of amino acids. Indeed, dimethylglycine binds and activates neurological receptors which use glycine as a ligand[22].

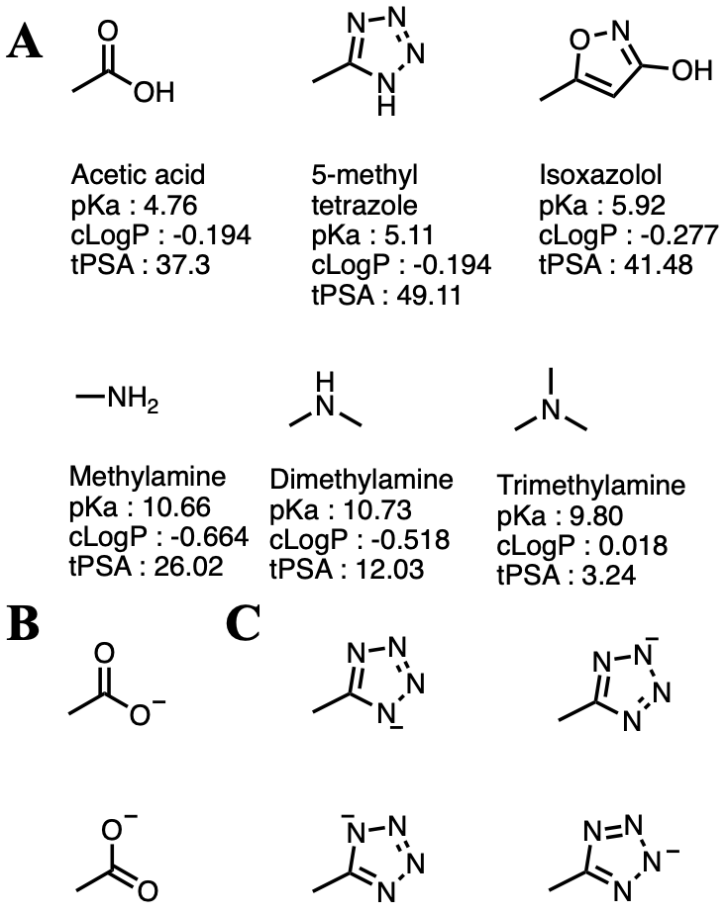

Figure 2.03 Biological Isosteres of Carboxylic Acids and Amines. Shown are isosteres with calculated chemical properties (A) and the tautomerization of carboxylic acid (B) and tetrazole (C)

In a demonstration of these biological isosteres on the molecule, the physiochemical properties of progressively replacing the amine and carboxylic acid groups for amino acids were calculated. To represent these properties and bioavailability in general, lipophilicity as the cLogP and total polar surface area were calculated for a series of progressively replacing amino acids

with biological isosteres (Figure 2.04). cLogP is used to predict how the molecule will be distributed throughout the body and total polar surface area is used to predict if a molecule will pass the blood brain barrier. As guidelines from the literature, the cLogP of a molecule should be between 2 and 5, and the total polar surface area should be less than 90A[19, 23, 24]. With these calculations, the progressive replacement of the primary amine and carboxylic acid produce molecules with more favorable calculated properties.

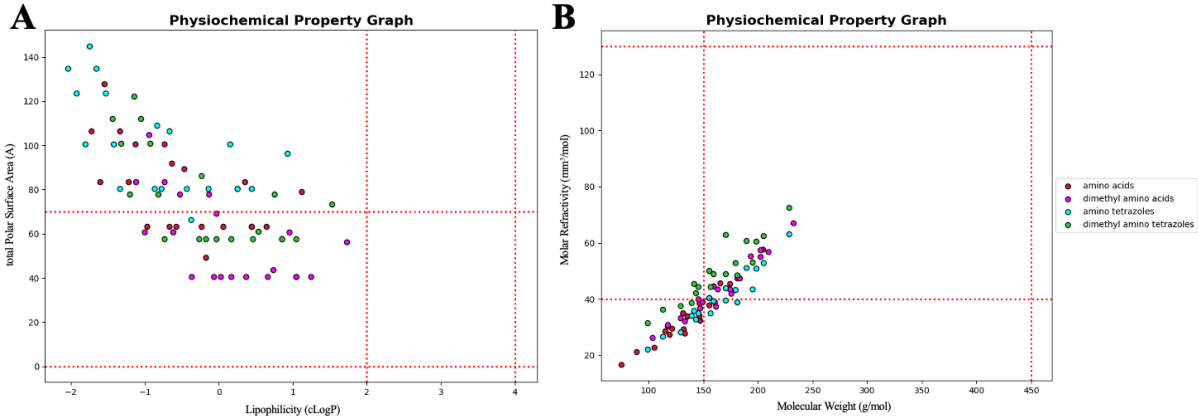

Figure 2.04 Calculated Physiochemical Properties of Amino Acids and Derivates. Lipophilicity vs total polar surface area (A) and molecular weight vs molar refractivity (B) calculated for all 20 amino acids (red), demethylated amino acids (magenta), amino tetrazoles (cyan), and dimethyl amino tetrazoles (green).

New Neuroactive Small Molecules

Given the endogenous neuroactivity of amino acids, the amino acid scaffold is ideal for the development of novel neuroactive small molecules. The challenge of using this scaffold is the carboxylic acid and primary amine groups which have poor bioavailability and metabolic properties. If these groups could be improved, the scaffold could be diversified to interact with a wide range of neurological receptors. Biological isosteres exist for both of these groups, but they have yet to be evaluated on the amino acid scaffold as potential neuroactive molecules. To evaluate this scaffold, amino acid like molecules with the carboxylic acid isostere, tetrazole, and secondary amines are first synthesized, named synthetic utility derivatives of amino acids (SUDAA).

Results

Multicomponent Reactions for Amino Acid Scaffolds

One of the first known reactions to synthesize amino acids was the Strecker reaction[25]. The Strecker reaction is a multicomponent reaction where first an amine and carbonyl are mixed to form an imine followed by the addition of cyanide (Figure 2.05a). These products are initially carboxamides, but the carboxylic acid can be produced from acidic solvolysis conditions. While this reaction is useful for producing amino acids, it is limited to producing carboxamides and carboxylic acids.

A closely related reaction is the Ugi reaction[26, 27]. The first step of this reaction is the same as the Strecker; the mixing of an amino and carbonyl to make an imine (Figure 2.05b). The second step differs with the addition of an isocyanide and a carboxyl acid. The isocyanide adds to the imine to produce an iminium intermediate which can be attacked by the carboxylic acid. This intermediate then undergoes a Mumm rearrangement by an acyl transfer of the carbonyl group of the previous carboxyl acid to the amine. Thus, the amine substrate of this reaction is limited to ammonia and primary amines as secondary amines are unable to perform the Mumm rearrangement for the final product. These Ugi products are close to having the desired amino acid scaffold but would require several more transformations to produce the desired amine and carboxylic acid functionality.

A derivative of the Ugi reaction is the Ugi tetrazole reaction[28-31]. The difference is the use of an azide instead of a carboxylic acid in the second step of the reaction (Figure 2.05c). The attack of the azide on the iminium intermediate produces a 1,5 substituted tetrazole, and there is no Mumm rearrangement. This allows secondary amines to be used in the production of tetrazoles on an amino acid like scaffold. The final step to produce an amino acid-like scaffold is synthesizing

a 5 substituted tetrazole from the 1,5 product of the Ugi tetrazole reaction. This can be done by using a reversible isocyanide in the reactions. Reversible isocyanides, such as Walborsky's isocyanide, can be used as an isocyanide, and then functional group attached to the cyanide in the isocyanide can be removed[28, 31]. The use of Walborsky's isocyanide in the Ugi tetrazole allows 5 substituted tetrazoles to be produced by treating the Ugi products with acid. This Ugi tetrazole reaction with Walborsky's isocyanide synthesizes amino acid like molecules in two steps.

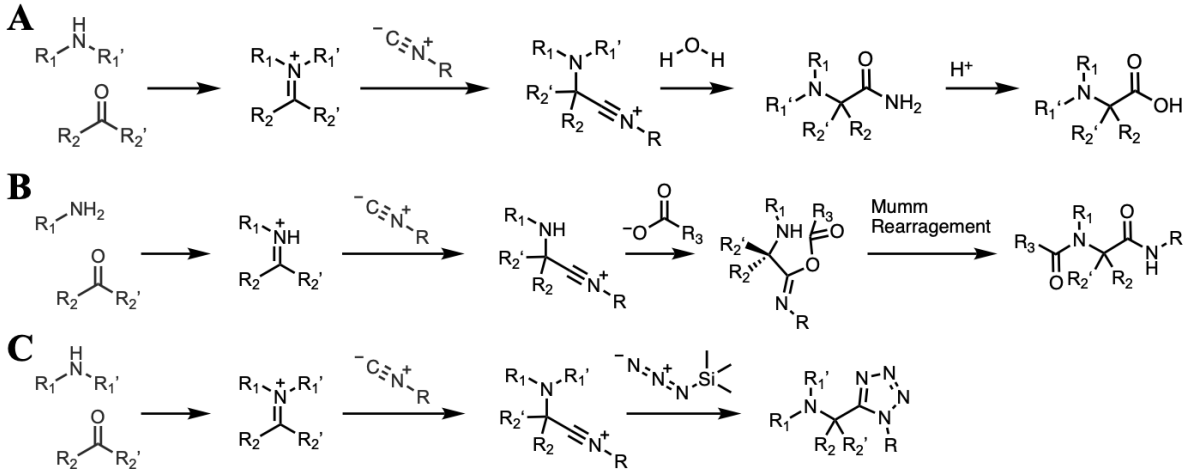

Figure 2.05 Muticomponent Reaction Mechanisms. The Strecker (A), Ugi (B), and Ugi tetrazole (C) reaction mechanisms are shown with their shared initial step.

Synthesis of Amino Acid Tetrazoles

To make the Ugi tetrazole products, Walborsky's isocyanide first needed to be synthesized. This was done by first formylating 1,1,3,3-tetramethylbutylamine with a mixed anhydride, and then followed by dehydration of the formyl amine with phosphorus oxychloride (Figure 2.06a,b). The final product was purified by vacuum distillation to afford Walborsky's isocyanide in 70% yield over two steps on mole scale. With the reversible isocyanide in hand, the Ugi tetrazole reaction could then be performed.

For the initial Ugi tetrazole reaction, dimethylamine and cyclopentanone were chosen for the amine and carbonyl components respectively. Cyclopentyl for the carbonyl component results in this group at the alpha position of the amino acid scaffold, and the full amino acid counterpart, cycloleucine, is neuroactive. This first reaction with the addition of Walborsky's isocyanide and trimethylsilyl azide proceeded well in good yield (Figure 2.06c). This product purified though column chromatography was then deprotected with 50% HCl in EtOH for an 82% yield over two steps(Figure 2.06d).

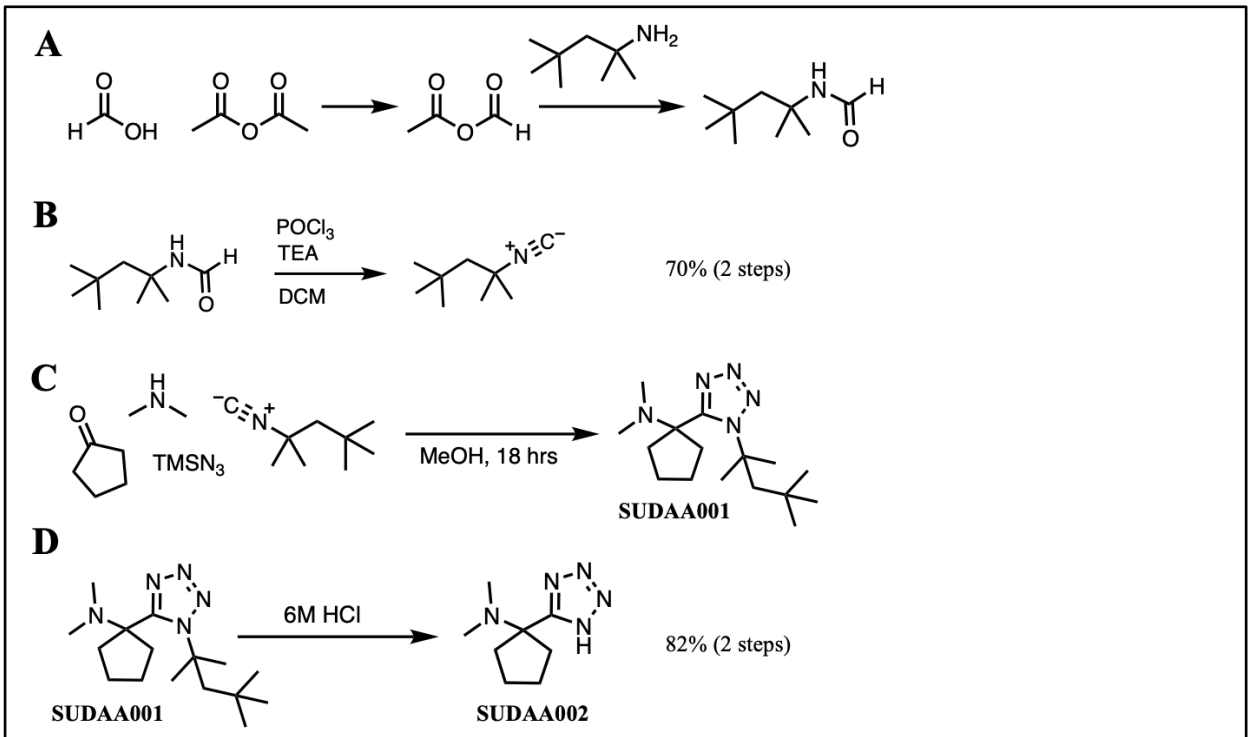

Figure 2.06 Synthesis of Ugi Tetrazole Products. The formylation (A) and dehydration (B) of 1,1, 3,3-tetramethylamine to synthesize Walborsky's isocyanide and the Ugi tetrazole reaction (C) with removal of the tert-octyl group (D) are shown.

With this success, a range of carbonyl compounds were used in the Ugi tetrazole reaction with many having neuroactive amino acid correlates such as amino cyclobutyl carboxylic acid (Figure 2.07). One molecule was made to evaluate steric bulk at the amino position with diethyl amine used for the reaction instead of dimethylamine. This larger group may block the engagement of the amino group with some neurotransmitter receptors.

Finally, the equivalent of glycine for this scaffold was synthesized. Using the general Ugi tetrazole reaction with formaldehyde, the desired product was not formed in any yield. The major product appeared to be a polymerization product largely formed from dimethylamine and formaldehyde. With the goal of reducing the probability of this product formation, the order of addition for the Ugi reaction was changed. Dimethylamine, Walborsky's isocyanide, and trimethylsilyl azide was mixed in methanol followed by the cooling of the reaction to 0 C and dropwise addition of aqueous formaldehyde. This produced the desired Ugi tetrazole product purified by column chromatography in 57% yield. Treating the product with 6 N HCl in dioxane produced the desired dimethyl glycine tetrazole.

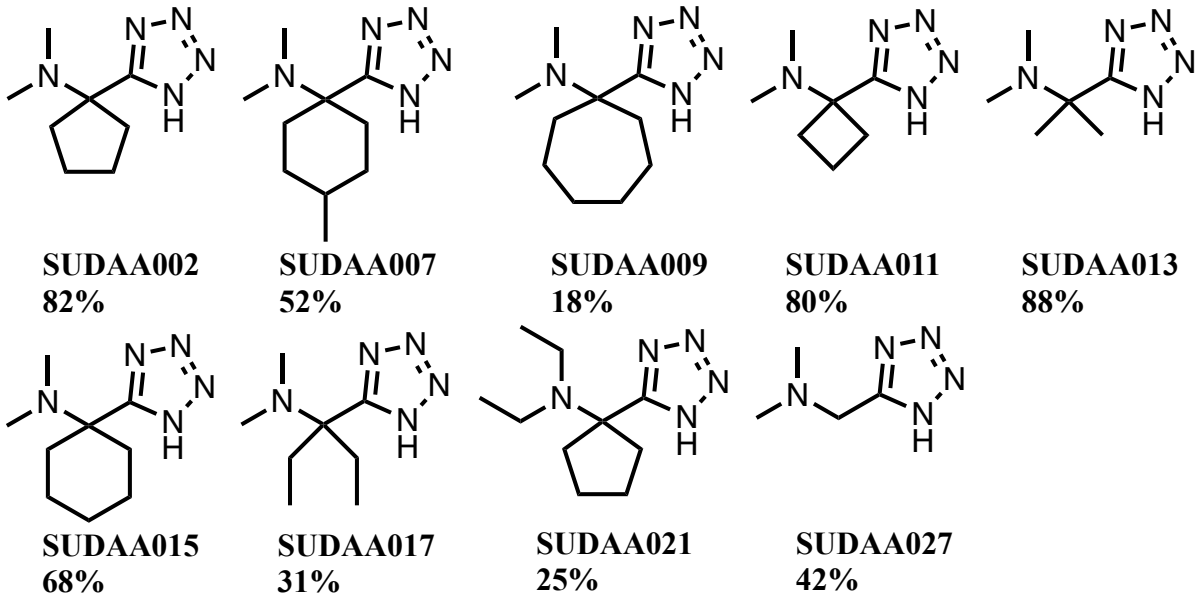

Figure 2.07 Yield of Final Products. Yields shown are over two steps.

Discussion

Modular Synthesis Platform for the Amino Acid Scaffold

The advantage of using the Ugi tetrazole reaction is the diversity of components that can be used and the ease of installing desired functionality at each position in a single step. This in combination with the ability to synthesize biological isosteres makes it ideal for the synthesis of

neuroactive molecules. Despite this promise, these types of molecules have yet to be synthesized for the purpose of neuroactivity. In this aim, several molecules were synthesized from the Ugi tetrazole in moderate to good yield for evaluation in following behavioral experiments.

Experimental

Formylation of 1,1,3,3-tetramethylbutylamine

Acetic anhydride (60 mmol) was added to a round bottom flask mixed with a stir bar followed by the addition of formic acid (70 mmol), and the solution was stirred at room temperature. After 1 hour, the solution was cooled to 0 C and 1,1,3,3-tetramethylamine (10 mmol) was added dropwise. After the addition, the solution was allowed to warm to room temperature overnight. At completion of the reaction, the solution was diluted with dichloromethane (DCM) (50 mL), washed with water (x3), sodium bicarbonate (x3), brine (x1), and extracted into DCM (x3). The solution was dried with sodium sulfate, and the solvent removed by rotary evaporation to yield 1,1,3,3-tetramethylbutylformamide (clear odorless oil) in quantitative yield. When needed, the oil was azeotroped with toluene to remove any remaining formic acid.

Dehydration of 1,1,3,3-tetramethylbutylformamide

To an oven dried round bottom flask with a stir bar, 1,1,3,3-tetramethylbutylformamide (10 mmol) was added and the atmosphere was purged with argon. DCM (2M) and triethylamine (TEA) (50 mmol) was added, and the solution was cooled to 0 C in an ice bath. Phosphorous oxychloride ($POCl_3$) (10 mmol) was added dropwise, and the reaction was allowed to warm to room temperature overnight. At completion of the reaction, water (20 mL) was added, and the mixture was extracted into DCM and washed with aqueous sodium bicarbonate. The solution was

dried with sodium sulfate and the solvent was removed with rotary evaporation. The resulting brown oil was purified though vacuum distillation (5 mbar, 30C) to yield 1,1,3,3-tetramethylisocyanide in 70% yield.

General Ugi Tetrazole

Dimethylamine (2M in MeOH, 1 mmol) was added to a round bottom flask with a stir bar followed by methanol (MeOH) (1M) and ketone (1 mmol). The solution was allowed to stir for 30 minutes before the addition of 1,1,3,3-tetramethylisocyanide (1.1 mmol) and trimethylsilyl-azide (TMS-azide) (1.1 mmol). The reaction was monitored by TLC. At completion, the reaction mixture was concentrated by rotary evaporation and purified by column chromatography (3 hexane : 1 ethyl acetate).

Modified Ugi Tetrazole

Dimethylamine (2M in MeOH, 1 mmol) was added to a round bottom flask with a stir bar followed by MeOH (1M), 1,1,3,3-tetramethylisocyanide (1.1 mmol) and trimethylsilyl-azide (TMS-azide) (1.1 mmol). The solution was cooled to 0 C and aqueous formaldehyde (1.0 mmol) was added dropwise. The reaction was allowed to warm to room temperature and monitored by TLC. After 4 hours, the reaction mixture was concentrated by rotary evaporation and purified by column chromatography (1 hexane : 1 ethyl acetate).

General Deprotection of Ugi Tetrazole Products

Ugi tetrazole product (0.5 mmol) was added to a scintillation vial with a stir bar. A 50% solution of HCl : EtOH (2 mL) was added, and the solution was stirred for 1 hour. At completion

of the reaction, the solvent was removed by rotary evaporation and the product was washed with ethanol. Reaction yields are typically quantitative.

Molecule Characterization

SUDAA002 : 1H NMR (400 MHz, CDCl3) 2.784 (s, 6H), 1.852 (complex, 8H)

MS (ESI) : 181.2 (181.13 expected)

SUDAA007 : 1H NMR (400 MHz, CDCl3) 2.165 (complex, 6H), 1.87 (complex, 5H), 1.79 (complex, 5H), 1.036 (complex, 2H)

MS (ESI) : 209.4 (209.16 expected)

SUDAA009 : 1H NMR (400 MHz, CDCl3) 2.300 (complex, 6H), 2.300 (complex, 6H), 2.300 (complex, 6H)

MS (ESI) : 209.7 (209.16 expected)

SUDAA011 : 1H NMR (400 MHz, CDCl3) 2.694 (complex, 6H), 1.805 (s, 6H)

MS (ESI) : 167.2 (167.12 expected)

SUDAA013 : 1H NMR (400 MHz, CDCl3) 2.202 (complex, 6H), 1.598, (s, 6H)

MS (ESI) : 155.3 (155.12 expected)

SUDAA015 : 1H NMR (400 MHz, CDCl3) 2.175 (complex, 6H), 1.88 (s, 8H), 1.72 (complex, 2H)

MS (ESI) : 195.8 (195.15 expected)

SUDAA017 : 1H NMR (400 MHz, CDCl3) 2.404 (s, 6H), 1.892 (s, 4H), 0.810 (complex, 6H)

MS (ESI) : 183.5 (183.15 expected)

SUDAA021 : 1H NMR (400 MHz, CDCl3) 2.484 (s, 8H), 1.620 (s, 6H), 1.503 (s, 2H)

MS (ESI) : 209.7 (209.16 expected)

SUDAA027 : 1H NMR (400 MHz, CDCl3) 3.81 (s, 2H), 2.302 (s, 6H)

MS (ESI) : 127.7 (127.09 expected)

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CHAPTER 3 : QUANTIFICATION AND EVALUATION OF NEUROACTIVITY IN THE FAST C BEND STARTLE RESPONSE FOR AMINO ACID LIKE MOLECULES

Abstract

The identification of specific behavior effect of molecules has been essential to characterizing neuroactives, but few of these discoveries have been made in recent years. This is in part due to the challenges of evaluating behavior in large animals like rodents. In a systematic and high throughput approach to overcoming these challenges, a larval zebrafish behavioral profiling system was developed at UCSF. Larval zebrafish are surprisingly similar to humans with many analogous genes, brain structures, and behaviors. Using a collection of characterized neuroactive molecules and newly synthesized molecules designed to be more bioavailable neuroactive amino acids, the behavioral profiling system identified perturbations in a simple behavior, the fast C bend startle response. These novel molecules were neuroactive, often at low concentrations, and resembled the activity of their amino acid counterpart when one was tested. These results highlight the use of the phenotypes of a single behavior to evaluate the neuroactivity of both known and unknown molecules.

Introduction

Evaluating Behavioral Effects of Neuroactive Molecules

The large majority of neuroactive drugs were discovered because of their behavioral effects. Indeed, some of these behavioral effects were discovered initially in humans, proving an immediate relationship between the molecule activity and human behavior, but non-incidental discovery of neuroactive molecules requires the use of animals and drawing conclusions between the animal and humans. The most prominent animal is the rodent. Rodents have a wide variety of behaviors whose molecular causes have been identified, and these have been used to identify molecular mechanisms of neuroactive molecules. Pethidine was identified as muscle relaxant due

to its anti-spasmodic effects on colons isolated from guinea pigs, but its true mechanism of opioid receptor engagement was revealed when pethidine caused a characteristic straub tail in rodents[1]. Pethidine went on to be the first synthetic opioid to be used for analgesia in humans.

High Throughput Evaluation of Behavioral Phenotypes

These early types of accidental discovery prompted more systematic approaches into the use of behavior to characterize molecules. Propofol, an anesthetic, was discovered by evaluating a library of phenols in rodents for their ability to induce sedation and the loss of righting reflex[2]. While successful, the experiment was extremely laborious. Five rodents were used for each condition, and each molecule in the 51-compound library was evaluated in six to eight different concentrations for approximately 1500 rodents used for the study. Each rodent required manual injection of the molecule followed by characterizing the immediate effects and duration of effects. Furthermore, this does not include the follow up studies in rabbits.

While this was successful for the discovery of propofol, not many other drugs have been discovered with this approach in rodents. This is likely due to the time consuming and laborious nature of behavioral experimentation with these animals. An animal model that swims to solve these problems is the larval zebrafish. The larval zebrafish is small which allows easy manipulation and is produced in high numbers, typically 30 to 50 embryos from a single breeding. This allows relatively few adult zebrafish to produce thousands of larvae which can be pipetted into 96-well plates for experiments. Dosing these animals with drugs is as simple as pipetting drug solutions to the water of the animals. The drugs reach the fish through their skin, mouth, gills, stomach, and other tissues contacting the environment, and many times produce effects analogous to those in humans[3-7]. The analogies are not limited to the effects of drugs; Zebrafish share a high gene

homology with humans and an even higher homology of disease-causing genes[8]. Zebrafish also have all the same neurotransmitters as humans and many homologous brain regions[9]. These facts in combination with larval zebrafish developing rapidly and displaying a wide range of behaviors by seven days post-fertilization make them an excellent animal model to investigate behaviors and the effect of molecules on those behaviors.

In using larval zebrafish to study behavior, a larval zebrafish behavioral profiling system was developed at UCSF[10, 11]. This system holds a 96-well plate on a stage underneath a high-speed camera and records the motion of larval zebrafish while a variety of stimuli including lights and sounds are played to the animals (Figure 1.05). When pipetting six fish per well in a 96-well plate, the behavior of 480 animals can be evaluated in a single experiment, and when performing experiments in three biological replicates, the number of animals used nears that of the propofol discovery with a fraction of the labor involved. Thus, the behavioral profiling system enables rapid and high throughput characterization of different conditions on specific behaviors.

Novel Neuroactive Molecule Evaluation

Prime candidates for neuroactivity are amino acids. The nervous system uses amino acids and close derivates as neurotransmitters, and as a result similar molecules are neuroactive (Figure 2.01). Synthetic and noncanonical amino acids were discovered to be highly effective probes of specific neurotransmitter receptors, and much of our understanding of molecular mechanisms in the nervous system depends on these molecules[12-15]. While useful, amino acids themselves often cannot be used to investigate behavior due to their poor bioavailability. Problematic primary amine and carboxylic acid functional groups on these molecules are both highly polar and charged in physiological conditions. This results in the molecules being unable to pass the blood brain barrier,

and their often-rapid excretion from the body through the kidneys. To investigate molecular mechanisms in behavior without injecting molecules directly into the brain, amino acid like molecules with improved pharmacodynamic and pharmacokinetic properties must be synthesized.

A series of amino acid like molecules were synthesized with the aim of producing molecules with improved bioavailability (Figure 2.07). The carboxylic acid of the scaffold was replaced with an isostere of a tetrazole. Tetrazoles maintain similar but muted properties of carboxylic acid due to the reduced electronegativity of nitrogen compared to oxygen and the resonance structures which spread the negative charge over four atoms compared to two atoms. The primary amine group was replaced with a dimethylamine group which displays similar properties with a more neutral pKa and reduced hydrophilicity. The dimethylamine group does lose hydrogen bond donors compared to the original but is still able to maintain many of the same interactions through the charge and accepting a hydrogen bond. This is showcased by dimethylglycine and glycine sharing ligand engagement sites in the body such as in the NMDA receptor[16]. These amino acid-like molecules have a high probability of neuroactivity.

The Fast C Bend Startle Response as a Measure of Neuroactivity

The UCSF behavioral profiling system is an excellent method to characterize the activity of these molecules. This system has been used to characterize a wide range of behaviors and effects of small molecules including the effect of etomidate on the fast C bend startle response. Etomidate appears to cause a loss of responsiveness like other sedatives except in response to sounds which still elicit the fast C bend startle response[17]. These etomidate-treated animals make a great model of studying a chemically isolated behavior.

The fast C bend startle response has a rich history involved in the discovery of neuroscience and was discovered to be an ethological fixed action pattern[18-23] (Figure 1.03). Fixed action patterns meet several behavioral characteristics that result from the underlying organization of the neural circuitry. The modulation of these characteristics is tied to the activity in specific parts of the neural circuitry. The first of these characteristics is the detection of startling stimuli such as certain sounds. This starts with the detection of sound by hair cells in the inner ear[24-27]. The location of the hair cells within the inner ear determines which frequences of sound the hair cell can detect, and when sound reaches the hair cells, they release glutamate to stimulate AMPA receptors on primary afferents[28-32]. The primary afferents which are a part of the fast C bend startle response are unique. Instead of making connections with hair cells in one specific area like those that detect specific frequences, these afferents make connections with hair cells at a wide range of locations. This enables these afferents to detect the shared characteristic of sound across different frequencies, the intensity of sound. The primary afferents are activated once an intensity threshold is surpassed and stimulate the Mauthner cells at the center of the circuit[23, 33-35]. These primary afferents are also connected to and stimulate intermediate inhibitory glycinergic and GABAergic neurons which then connect to the Mauthner cells[35-42]. These neurons play a critical role in setting the threshold for detecting a "startling" stimulus by requiring the Mauthner cell to be sufficiently activated by the primary afferents before their activation. This inhibitory component determines how much activation of the Mauthner cell is required before the Mauthner is inhibited from firing. In other words, the primary afferents and interneurons determine how loud a noise must be within a certain time to be considered startling. These molecular mechanisms of the circuit emerge in the responsiveness and pre-pulse inhibition phenotypes of the fast C bend startle response.

A third phenotype of the fast C bend startle response is habituation or the learning to anticipate an upcoming startling stimulus when the stimulus is repeated rhythmically[43-50]. Habituation is a form of neural plasticity in which the strengths between neurons are strengthen or weakened, and these molecular mechanisms are characterized in the circuit as well. The Mauthner cell and interneurons receiving input from the primary afferents have NMDA receptors at these synapses[43-47]. When the NMDA receptor is activated, the influx of calcium through its central pore leads to synaptic changes such as changes in the number and ratio of neurotransmitter receptors expressed at synapses. This causes synapses to become more or less sensitive to activation of the presynaptic neuron. These changes occur both at the synapses with the Mauthner cell and the intermediate inhibitory neurons, and the result of habituation is from changes in both of these synapses.

Changes in these phenotypes is a result in the change in the described neural circuitry. Small molecules targeting receptors underlying these phenotypes are established to indeed modulate the phenotype accordingly. Glycine and GABA receptor ligands modulate different phases of pre-pulse inhibition[51], and NMDA receptor ligands modulate habituation[47]. Thus, these three behavioral phenotypes of the fast C bend startle can be used to identify novel molecules engaging with these receptors. These connections between the neural circuitry and behavioral phenotypes in combination with etomidate isolating the behavior for measurement by the behavioral profiling system makes the fast C bend startle response an excellent behavioral model to characterize the activity of neuroactive amino acid like molecules.

Results

A Behavioral Battery to Evaluate the Fast C Bend Startle Response

To evaluate the fast C bend startle response, a custom battery was designed to capture the three phenotypes, responsiveness, pre-pulse inhibition, and habituation, with three types of assays. The first assay, acoustic responsiveness, is measured with four soft solenoid taps played every 30 seconds to evaluate how the animals are responding to the acoustic stimulus. This soft solenoid tap produces an acoustic stimulus capable of inducing the fast C bend startle response. The pre-pulse inhibition is measured by the ability of a non-behavior inducing stimulus to inhibit a triggering stimulus. In this case, a soft speaker noise which the fish do not respond was played at a range of 100 ms intervals before a soft tap stimulus: simultaneous, 100 ms, 200 ms, 300 ms, 400 ms, 500 ms, 600 ms, 700 ms, 800 ms, and 900 ms. For the habituation, several assays were designed playing a soft solenoid tap at various intervals to induce habituation of the fast C bend startle response. The fish begin habituating when taps are played every 10s and habituate quicker as the rate of tapping increases. Thus, three habituation assays of different intervals, 10s, 5s, and 2.5s were used to visualized changes in habituation.

Evaluating a Collection of Neuroactive Molecules

The amino acid like molecules were evaluated in a seven-point threefold dilution series in three technical replicates per experiment and at least two biological replicates of experiments. Three biological replicates of SUDAA002, SUDAA007, SUDAA009, and SUDAA021 are analyzed, but the third biological replicates of the other molecules were not collected due to instrument error. In addition to these molecules, several known molecules were also evaluated: D-cycloserine, HA-966, NMDA, aminocyclobutyl carboxylic acid (ACBC), glycine, and

dimethylglycine (Figure 3.01). D-cycloserine (DCS) is a partial agonist of NMDA receptors and an inhibitor of GABA transaminase[52-54]. HA-966 is a partial agonist of NMDA receptors and likely also binds to GABA-B receptors[55, 56]. NMDA is a full agonist of NMDA receptors as well as a full agonist of AMPA and kainate receptors albeit at reduced potency compared to NMDA receptors[57]. ACBC is known to be a partial agonist of NMDA receptors, but its activity at other receptors has not been evaluated[58]. Glycine is an agonist of glycine receptors and NMDA receptors, while dimethylglycine is only known to be an agonist of NMDA receptors[57]. It is not clear if dimethylglycine has been evaluated for activity at other receptors. This lack of specificity in neuroactive molecules is standard with no established molecules being active at only a single receptor. Despite this, their characterized effects are expected to produce corresponding changes in behavioral phenotypes.

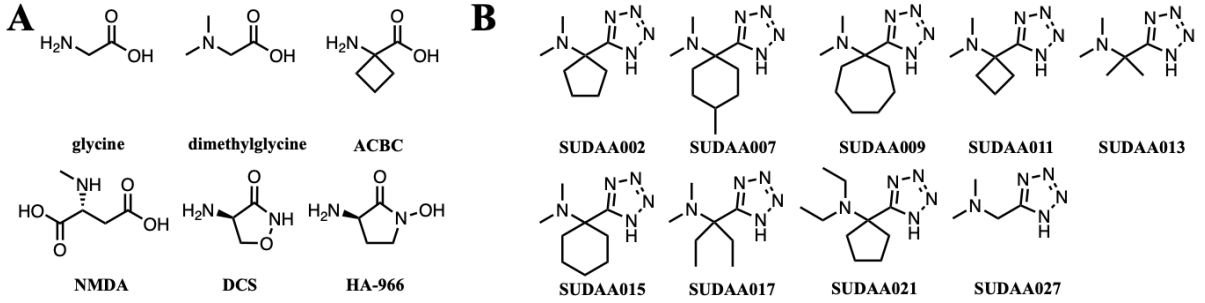

Figure 3.01 Molecules Tested. Known neuroactives (A) and novel molecules (B) for behavioral evaluation

Changes in Fast C Bend Startle Responsiveness

As mentioned, the soft tap baseline assay evaluates the baseline responsiveness of the animals to the soft tap stimulus. The four responses to the four taps in the assay are averaged together into a single value for each condition to quantify the responsiveness. Untreated larval zebrafish have a responsiveness of approximately 1600 (Figure 3.02a). The molecules ACBC, HA-966, and DCS, did not appear to produce any meaningful changes with similar values across their titrations. On the contrary, NMDA, glycine, and dimethylglycine all greatly enhanced the

responsiveness (Figure 3.02b). In the new amino acid like molecules, most produced no changed in responsiveness. SUDAA011 and SUDAA017 did show an enhanced responsiveness but only at low and few concentrations (Figure 3.02c). One molecule, SUDAA027, did show a greatly enhanced responsiveness (Figure 3.02d). SUDAA027 is also dimethyl aminotetrazole, a chemically closely related molecule to glycine and dimethylglycine.

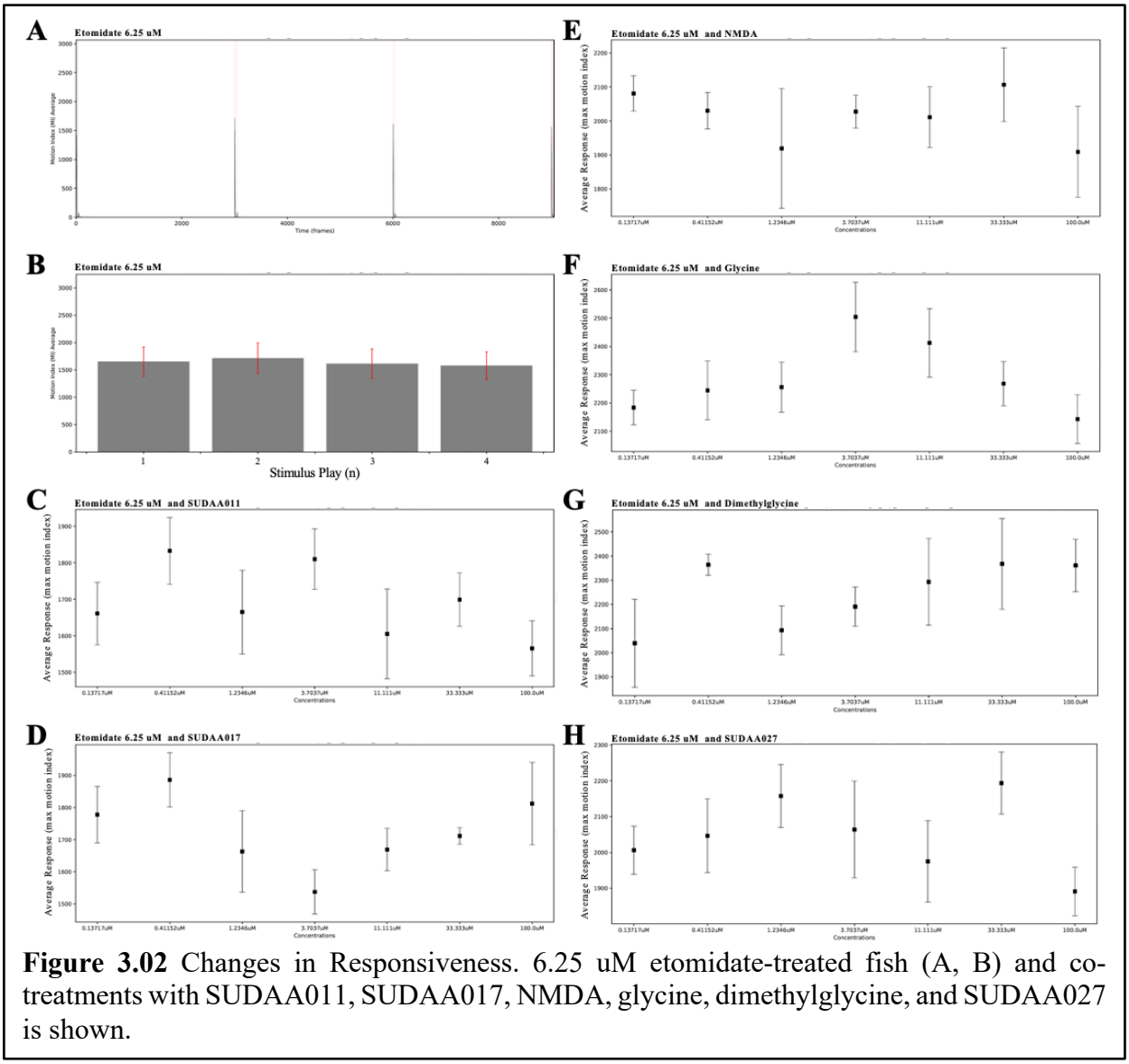

Figure 3.02 Changes in Responsiveness. 6.25 uM etomidate-treated fish (A, B) and co-treatments with SUDAA011, SUDAA017, NMDA, glycine, dimethylglycine, and SUDAA027 is shown.

Changes in Fast C Bend Startle Pre-Pulse Inhibition

The pre-pulse inhibition assay probes the ability of a non-startling stimulus to inhibit a following startling stimulus (Figure 1.18). In untreated fish, the response is inhibited when the non-startling stimulus is played 200-400ms before the startling stimulus. In general, most molecules had no effect on the pre-pulse inhibition responses (Figure 3.03a). The molecules that

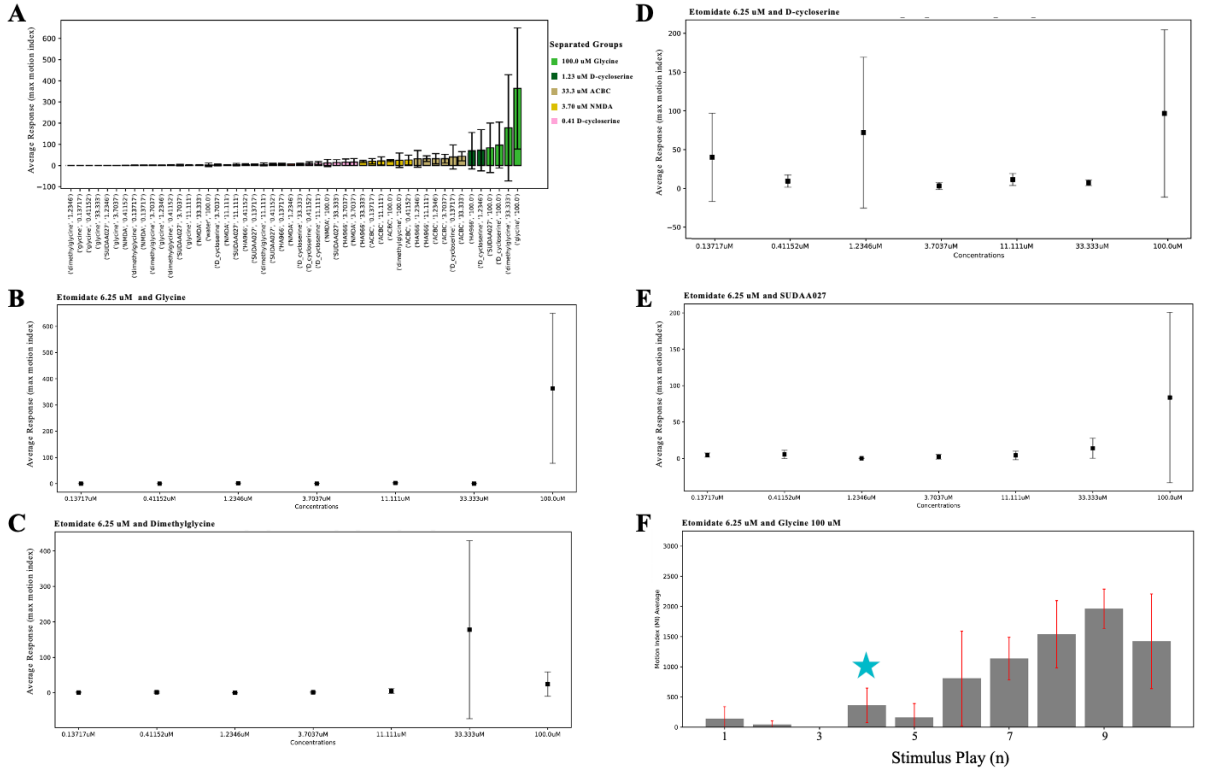

Figure 3.03 Changes in Pre-Pulse Inhibition. Full condition results (A) and co-treatments with glycine(B), dimethylglycine(C), DCS (D), SUDAA027 (E) are shown. Increased responses during inhibited intervals (blue star) for the co-treatment of 6.25 uM etomidate and 100 uM glycine (F)

were able to disrupt this phenotype and allow fish to respond to values of at least 150 during this timeframe were glycine, dimethylglycine, DCS, HA-966 and SUDAA027 (Figure 3.03b,c,d,e,f). While these responses are not as resolved as the other assays, these molecules do all share a similarity in engaging the glycinergic or GABAergic inhibitory systems.

An unexpected effect of some of the molecules, was the increase in responsiveness to other stimuli (Figure 3.04a). Some of these molecules caused the fish to respond to the non-startling

sound of which NMDA was the greatest . This phenotype was not expected and to the best of my knowledge has not been characterized previously. These conditions in which fish responded to the non-startling stimulus are not accurate measures of pre-pulse inhibition. It should be noted that for these molecules at concentrations where the fish did not respond to the non-startling stimulus, there was no effect on pre-pulse inhibition (Figure 3.04b).

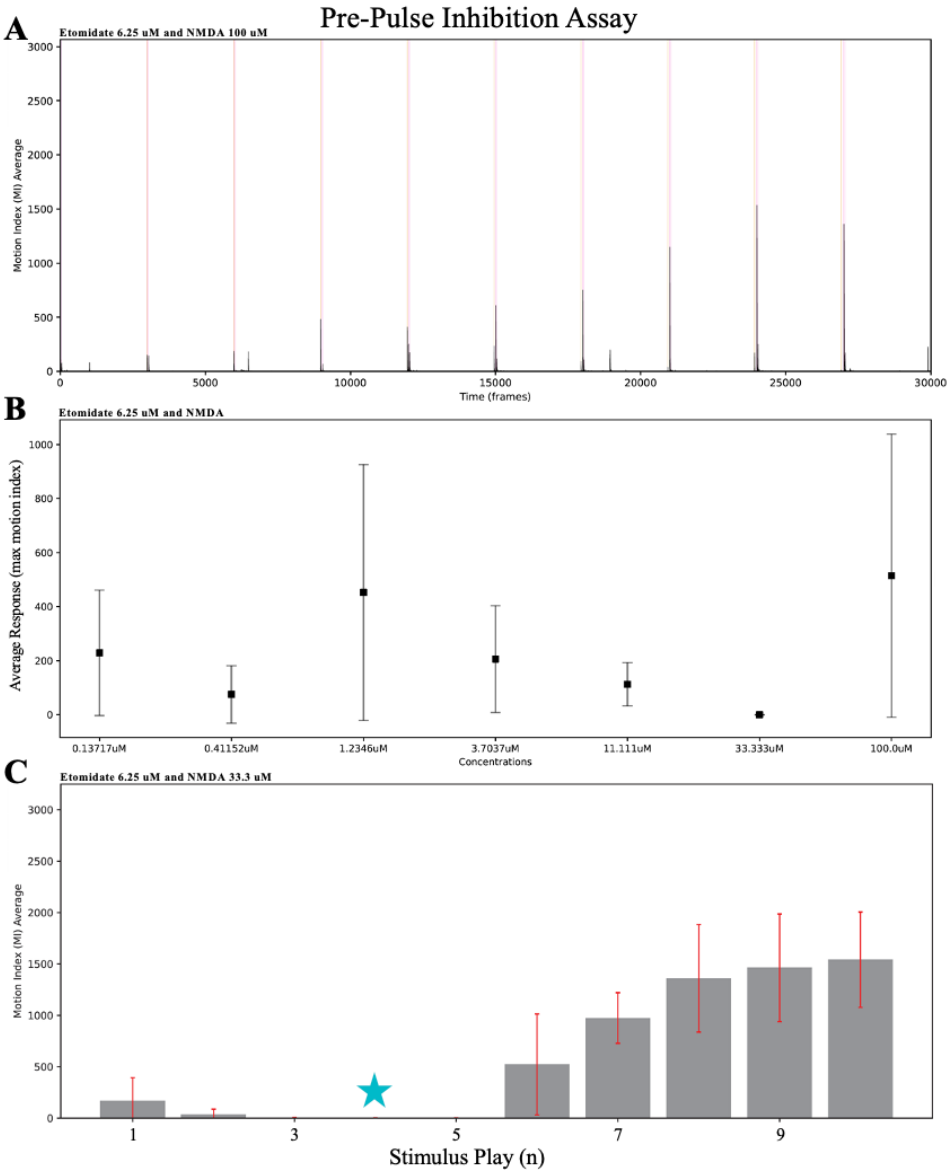

Figure 3.04 Increased Responses to MP3 Speaker Noise. The motion indices of the pre-pulse inhibition assay for 6.25 uM etomidate and 100 uM NMDA (A) and the concentration-dependent effects on MP3 responses (B). A concentration not inducing MP3 responsiveness, 33.3 uM

Changes in Fast C Bend Startle Habituation

The habituation of the fast C bend startle response is measured in three different assays. In the 10s soft tap assay, fish habituate minimally, and the corresponding molecular machinery is minimally activated (Figure 1.16a). In the 5s and 2.5s soft tap assay, the fish habituate at increasing rates (Figure 1.16b,c). During the 10s assay, many molecules did not significantly influence the rate of habituation (Figure 3.05). Molecules that were able to increase habituation during this time

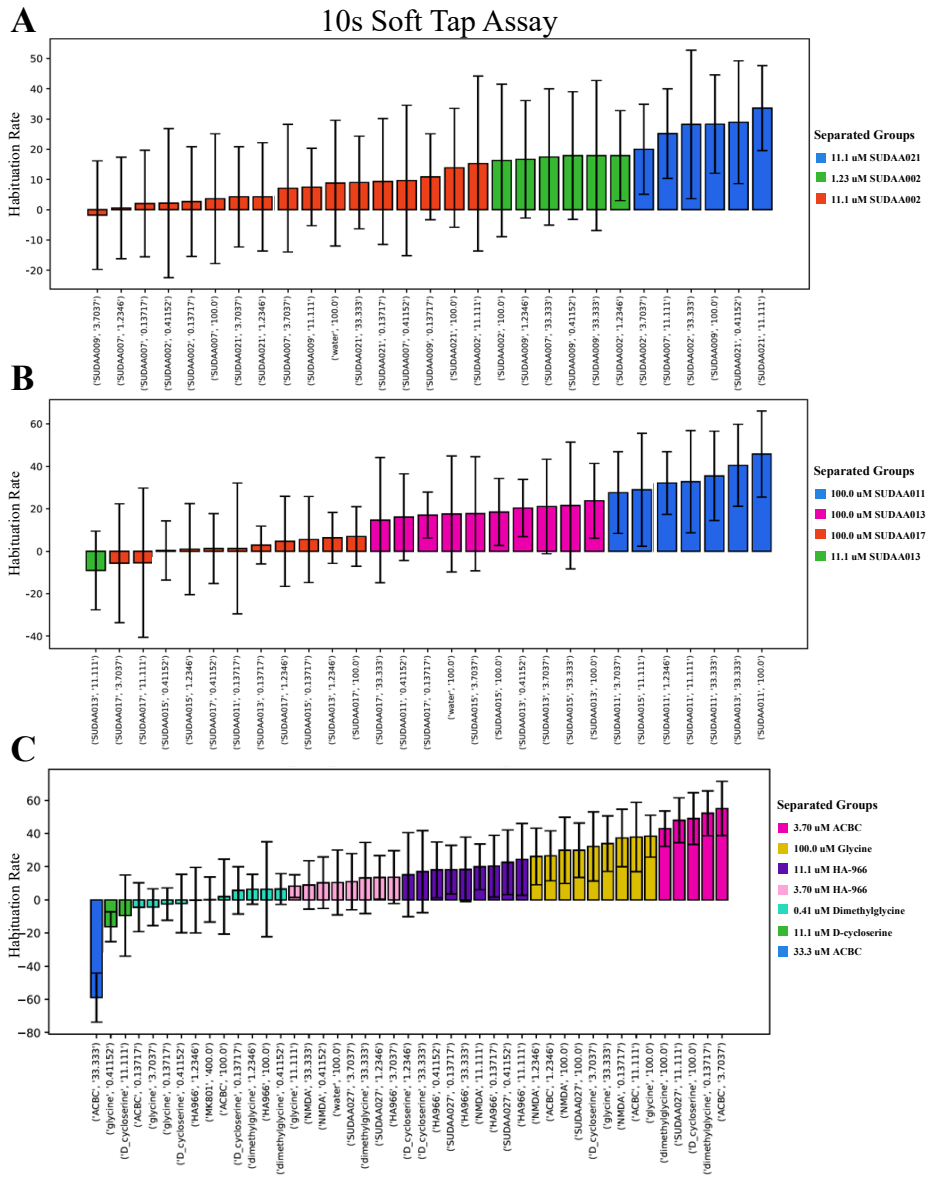

Figure 3.05 Changes in Habituation for the 10s Soft Tap Assay. Calculated habituation values for all conditions over three graphs (A, B, C).

included NMDA, ACBC, glycine, dimethylglycine, DCS, SUDAA011, SUDAA013, and SUDAA021. Only one molecule, ACBC, was able to inhibit/reverse habituation. The molecules increasing habituation during the 5s and 2.5s habituation assay included all of the same molecules as those in the 10s assay in addition to HA-966, SUDAA002, SUDAA009, SUDAA007, and SUDAA017 (Figure 3.06, 3.07). The molecules able to inhibit/reverse habituation also grew

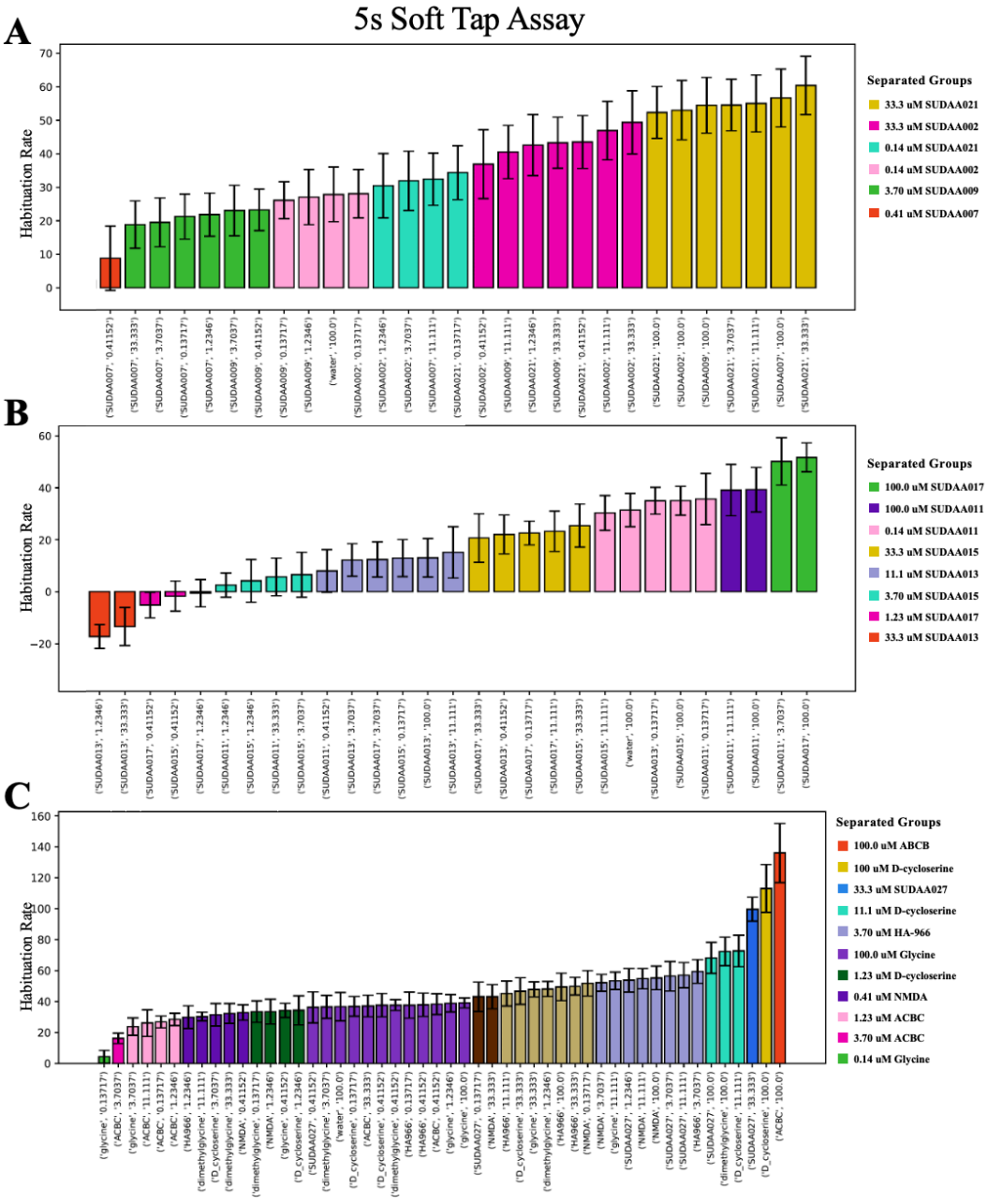

Figure 3.06 Changes in Habituation for the 5s Soft Tap Assay. Calculated habituation values for all conditions over three graphs (A, B, C).

compared to the 10s assay. ACBC again showed reduced habituation at several concentrations along with glycine, SUDAA007, SUDAA011, SUDAA013, SUDAA015, SUDAA017.

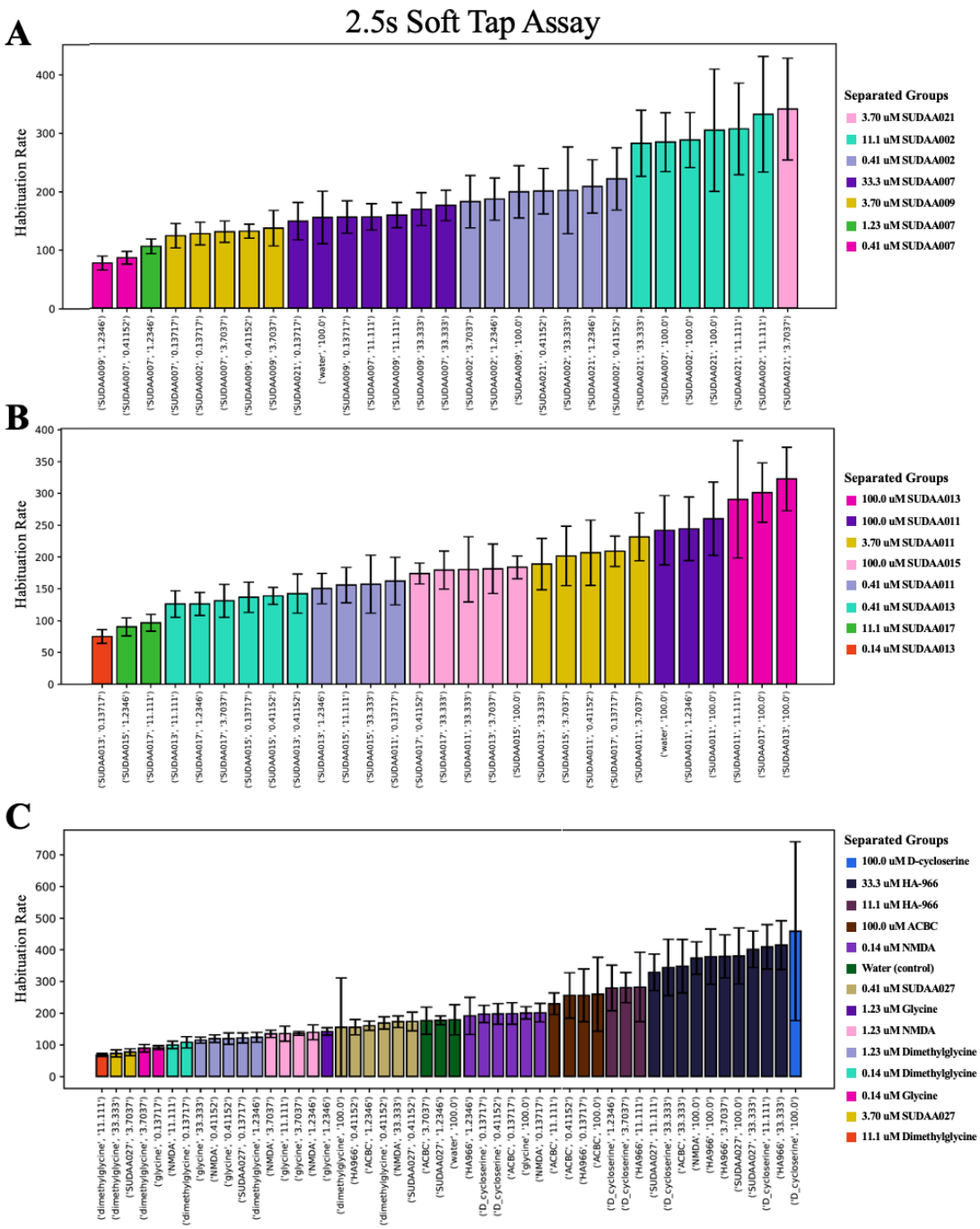

Figure 3.07 Changes in Habituation for the 2.5s Soft Tap Assay. Calculated habituation values for all conditions over three graphs (A, B, C).

Correlations in Activity

From these results, several conclusions can be drawn about the molecules evaluated and their neuroactivity. First, in the overall fast C bend startle responsiveness assay, the full agonists NMDA, glycine, and dimethylglycine were able to increase responsiveness. SUDAA027, a more bioavailable version of glycine and dimethylglycine, mimicked this phenotype. This trend held for the pre-pulse inhibition and habituation assays as well, indicating a shared mechanism for these molecules. SUDAA011, a more bioavailable version of ACBC, mimicked its activity as well. SUDAA011 and ACBC were some of the few compounds able to reduce the rate of habituation. In this group of compounds reducing habituation were SUDAA013 and SUDAA017, close structural analogs of SUDAA011. SUDAA013 and SUDAA017 did not produce the pre-pulse inhibition disruption or increased responsiveness which suggests the small size of the substituents at the alpha position is necessary for these phenotypes.

The molecules SUDAA007, SUDAA009, and SUDAA015 each differ from each other by a single carbon atom, and as expected, their activity is similar as well. These molecules did not alter the phenotypes of responsiveness, pre-pulse inhibition, or habituation during the 10s soft tap assay. During the 2.5s soft tap assay, each of these molecules reduced the rate of habituation in a similar concentration-dependent manner. For the 5s soft tap assay, the activity of these molecules differed slightly. SUDAA015 only decreased the rate of habituation at some concentration. SUDAA007 has a cyclohexane ring with a four-position methyl group at the alpha position of the scaffold, and this molecule decreased the rate of habituation at low concentrations before enhancing the rate of habituation at high concentrations. Increasing the size of the cyclohexane ring to a cycloheptane ring in SUDAA009 caused habituation to only be slightly reduced at low

concentrations before increasing the rate of habituation at high concentrations. Again, these similarities in behavioral phenotypes suggest shared mechanisms of engagement.

Discussion

Effects of Biological Isosteres on the Amino Acid Scaffold

The newly synthesized amino acid like molecules with biological isosteres were neuroactive, and their activity resembled that of their amino acid counterpart when tested. While their mechanisms of action remain unknown, it is strongly suggested they are shared from the similarities in effects on phenotypes with chemically similar molecules. These results strongly support the use of tetrazole and dimethylamine to maintain a similar but more bioavailable binding mode than carboxylic acids and primary amines. For a clear picture on the effects of these groups on pharmacodynamics and pharmacokinetics, similar experimentation in rodents with a single route of administration should be performed. The larval zebrafish results are promising, but as mentioned, the molecules reach the brain of the larval zebrafish through multiple routes including the skin, gills, mouth, and stomach. How these molecules are reaching the brains of the fish is not clear. It is clear the molecules are able to pass the blood brain barrier of these animals at this developmental stage, and engage receptors in a similar manner to their amino acid counterparts.

Specificity in the Nervous System

The evaluation of the neuroactivity of small molecules is complex. Many neurological receptors share the same or very similar ligand binding domains, and as a result, there are no small molecules selective for a single receptor. All FDA approved neuroactives engage multiple receptors at clinically relevant concentrations to produce their desired therapeutic effect. Attempts

to improve neuroactives by increasing their specificity have largely failed. Adding to this complexity, many neuroactives also show concentration-dependent mechanisms of target engagement. For example, DCS at low concentrations acts a partial agonist at the NMDA receptor, but at high concentrations, it acts as an antagonist at the same receptor[58, 59]. These challenges in resolving specificity greatly limit our understanding of neuroscience and the action of neuroactive molecules. Following from this, it is highly likely the newly synthesized amino acid like molecules also engage multiple receptors. Many of the molecules displayed biphasic effects on the phenotypes which is likely to be real given they were reproducible. Ideal follow up experiments to resolve the mechanism of target engagement probe the different components of fast C bend startle response circuit with molecular approaches such as electrophysiology[30-32, 40-46, 48, 51].

Experimental

Zebrafish Husbandry

Up to 10,000 fertilized zebrafish embryos were collected per day from group matings of wild-type zebrafish. Larvae were raised on a 14/10 h light/dark cycle at 28 °C until 7 dpf. Larvae were anesthetized with cold egg water and distributed into square 96-well plates. Plates were incubated at room temperature for 30 min for animals to become active. Molecule stock solutions were applied directly to the egg water, and larvae were incubated with the drug at room temperature for 30 minutes before behavioral analysis. The zebrafish-related procedures were conducted according to established protocols approved by UCSF's Institutional Animal Care and Use Committee (IACUC) and in accordance with the Guide to Care and Use of Laboratory Animals (National Institutes of Health 1996).

Molecules

Etomidate was dissolved in DMSO and diluted to the fish from 300X stock solutions for a final concentration of 0.3% final concentration of DMSO. DMSO was diluted accordingly for comparisons between drug treatments. DMSO did not produce any noticeable behavioral effects upon comparison to untreated fish (data not shown). DCS, HA-966, the amino acids, and SUDAA molecules were dissolved in water.

Fish Spectacles Source Code

The full open-source code and README documentation are available on GitHub. https://github.com/grantburley/SelloLab_Fish_Spectacles

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