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Tonic And Phasic Inhibitory Mechanisms Mediating Sensorimotor Decision-Making In The Goldfish Auditory Startle Circuit

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TONIC AND PHASIC INHIBITORY MECHANISMS MEDIATING SENSORIMOTOR DECISION-MAKING IN THE GOLDFISH AUDITORY STARTLE CIRCUIT

by

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A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment for the degree of Doctor of Philosophy, the City University of New York

2015
This manuscript has been read and accepted for the
Graduate Faculty in Psychology in satisfaction of the
Dissertation requirement for the degree of Doctor of Philosophy

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Abstract

TONIC AND PHASIC INHIBITORY MECHANISMS MEDIATING SENSORIMOTOR PLASTICITY IN THE GOLDFISH AUDITORY STARTLE CIRCUIT

by

Paul C.P. Curtin

Advisor: Professor Thomas Preuss

This work describes related studies of cellular and synaptic signaling mechanisms involved in the balance of excitation and inhibition in the goldfish auditory startle circuit. The general purpose of these experiments was to identify novel mechanisms that contribute to action selection at different stages of the motor control hierarchy. The methods applied to achieve this goal tested the effects of selective antagonists for target receptor systems on sound-evoked excitation and inhibition of startle.

Chapter 2 describes a study of a poorly-understood serotonergic mechanism, the 5-HT$_{5A}$ receptor, that was not previously functionally characterized in native tissues or associated with neural or behavioral processes. Treatment with a selective 5-HT$_{5A}$ antagonist caused a 26.41 ± 3.98% reduction in sound-evoked excitation of startle. Subsequent experiments revealed that the 5-HT$_{5A}$ antagonist significantly reduced post-synaptic excitability in the Mauthner-cell (M-cell) neurons that initiate startle. Despite these effects, prepulse inhibition (PPI) of the startle response remained robustly intact after treatment with the 5-HT$_{5A}$ antagonist. The 5-HT$_{5A}$ receptor is thus not a likely mechanism for PPI, but does act as a selective modulator of startle excitability. A final series of experiments confirmed that the 5-HT$_{5A}$ antagonist reduced M-cell excitability by increasing Cl$^-$ conductance, likely by activating Cl$^-$ channels.
Chapter 3 presents experiments focused on the inhibitory neurotransmitters that directly mediate the phasic inhibitory process elicited during PPI. Strychnine, a glycine receptor (GlyR) antagonist, caused an 87.43 ± 21.53% increase in sound-evoked excitation of startle, but PPI remained robustly intact, despite this. GlyRs thus likely mediate a tonic inhibitory process that was blocked by strychnine treatment, but glycinergic components of sound-evoked inhibition decayed too rapidly (<50 ms) to contribute to the prolonged time-course of PPI.

In a parallel series of experiments, treatment with bicuculline, the GABAAR antagonist, caused similar increases in sound-evoked excitation (by 133.8 ± 10.3%) of startle, but the GABAAR antagonist also significantly reduced auditory PPI at inter-stimulus intervals of 100 ms and less. In sum, these findings indicate that glycine and GABA tonically inhibit the M-cell startle circuit, but GABA is also the primary effector mechanism for inhibitory signaling during PPI.

In summary, three goals were accomplished. First, the thorough functional characterization of 5-HT5A provides a fully integrated serotonergic mechanism, and this appears to provide an ideal tool for selective potentiation of startle. Next, experiments with strychnine emphasize a short-lived role of GlyRs in sound-evoked (feed-forward) inhibition, and also act as mediators of a tonic inhibitory process that controls startle excitability. Last, experiments with bicuculline identify GABA as the inhibitory neurotransmitter that directly mediates PPI.
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CHAPTER 1

1.1. GENERAL INTRODUCTION

Among the very few fundamental principles that can faithfully describe behavior in universal terms is the tenet that multiple decision-making systems control the selection of actions (James, 1890; Gallistel, 1980; Grillner, 1997). The aim of this thesis is to identify novel mechanisms that allow the nervous system to select or otherwise determine the decision-making systems that will control action in a given context. Our experimental approaches applied pharmacological and electrophysiological methods to dissect the contributions of various receptor systems to sensorimotor plasticity in the decision-making neurons that initiate the goldfish startle response. This chapter introduces the basic elements of motor control and the hierarchical organization of action selection, emphasizes the use of startle as a model system for studying these processes, and identifies outstanding research questions that were not previously addressed in prior studies. These topics provide the basis for our Research Aims.

1.2. SENSORIMOTOR INTEGRATION AND DECISION-MAKING

*Origins of the Reflex Arc*

Descartes (1596-1650) developed a philosophical doctrine at the height of the Enlightenment that was enormously influential in the 19th century development of modern psychology and physiological studies (Bennett & Hacker, 2002; Glimcher, 2004). He viewed the nature of human behavior as essentially dualistic, originating either in an immaterial mind or the material body. The mind generated what he termed "voluntary" behaviors, which might be learned or spontaneous, and included all movements a person meant to initiate. The critical component of his perspective may actually be his analysis of the "involuntary" behaviors that he
called reflexes. Reflexes were unlike voluntary acts because reflexes were highly stereotyped, occurred more or less similarly in all peoples, and were triggered by sensation rather than intent (Bennett & Hacker, 2002; Glimcher, 2005). This last point is critical to Descartes' view that the reflexive actor is merely a passive conductor of sensory impulses. This formulation was driven by the compelling ambition that a sufficient understanding of reflexive structures and associated stimulus-response parameters might allow the construction of a deterministic model to perfectly predict actions from only the sensory features the animal encounters. This idea continued to inspire physiologists centuries after it was proposed, and was enormously influential in early schools of psychology that emerged in the late 19th century.

The basic theoretical framework of biology was fundamentally transformed in the 19th century: First, by the development of evolutionary theory (Darwin, 1859), then by the development of methods for physiological studies of the nervous system (Bennett & Hacker, 2002). Flourens pioneered systematic lesioning methods to probe the anatomical functions of the brain; he ultimately advanced a functional perspective that identified three main components of the nervous system devoted to sensory/ perceptive processing, action selection, and motor control (Bennett & Hacker, 2002). Marshall Hall is credited with developing the theory of the reflex arc from physiological experiments in isolated frog-limb preparations, and found it followed a similar 3-part arrangement. He systematically lesioned afferent and efferent nerves to identify mechanisms involved in reflexive contraction of the isolated limb, and identified discrete sensory and motor nerves that were integrated in the spinal cord. This early physiological model thus related the control of reflexive movements to three physiological components: 1) An afferent arc comprised of a sensory receptor excited by stimulation and a sensory nerve that conducts excitation to the spine; 2) the spinal cord that integrated the circuit;
3) And a motor nerve that conveyed excitation to a terminal effector that elicited contractions in the muscles of the limb. Hall's concept of reflexive action emphasized that the reflex arc was a discrete structure, limited to the spinal cord and peripheral tissues, and was a passive conductor of sensory impulses fundamentally unlike the presumably-sophisticated computational processes of the brain (Bennett & Hacker, 2002).

The reflex arc concept nonetheless provided a very influential theoretical perspective for the Functionalist and Associative schools of psychology that emerged in the late 19th century. William James, prominent among the former school's founders, made the reflex arc the focus of the *Principles of Psychology* (1890). He believed the reflex arc provided a critical connection between psychological processes and biological structures and functions. The reflex arc concept was further developed to distinguish proposed "central" arcs from peripheral reflex arcs. This presumed that central circuits integrated more sophisticated cognitive/computational faculties than peripheral arcs through the assemblage of connections to other central arcs. Pavlov enthusiastically endorsed this perspective; he viewed his own studies of associative learning in the mechanistic context of the reflex arc (Backe, 1999). Dewey (1896; Bredo, 2014) famously critiqued the enthusiasm of early psychologists for the assumption, as he saw it, that complex psychological processes could be meaningfully understood in the contexts of physiological mechanisms mediating unrelated processes. The pending publication of Sherrington's (1906) studies of reflex control would profoundly shift early perspectives on reflexes, and reject many assumptions that were incorporated into early perspectives on reflex arc theory. But, Sherrington's work was similarly optimistic that studies of reflexive action would be useful in understanding other neural functions.
Integrating the Arc

The publication of C.S. Sherrington's *The Integrative Action of the Nervous System* (1906) fundamentally transformed theoretical perspectives on reflexive action and motor control (Gallistel, 1980; Bennett & Hacker, 2002; Burke, 2007). The key theoretical developments advanced in his work (Sherrington, 1906) was the rejection of reflexes as passive conductors, or as discrete circuits functioning in isolation from other neural processes. Elements of the reflex arc should instead be viewed as integrated decision-making units functioning in a coordinated system. This section introduces the evidence supporting this perspective on reflex control, and outlines the integrated inhibitory processes that Sherrington characterized in contexts of motor coordination, action selection, and precise control of movement.

The experimental approaches developed by Sherrington (1906) offered systematic, quantitative analyses of information-processing and decision-making in spinal reflex networks. This was achieved by mapping the intensity and timing (relative to eliciting stimuli) of reflexive movements elicited by stimuli of varying kind and intensity (Sherrington, 1906, 1910; reviewed in Gallistel, 1980). Sherrington found that the intensity of the eliciting stimuli needed to surpass a minimal *threshold* to elicit reflexive responses, but response thresholds were highly dynamic. The intensity of motor responses elicited with supra-threshold stimuli was similarly plastic; while very strong stimuli generally elicited stronger responses than very weak stimuli, no fixed input-output relationship could reliably predict the intensity of reflexive responses on the basis of stimulus strength, alone. Sherrington characterized various computational properties of the excitatory pathway that contribute to this plasticity; the integration of inhibitory processes (discussed below) inevitably also contributes. Whatever the postulated mechanism, these findings mean that action is not *determined* in reflex circuits; it is *decided*.
Sherrington identified the site(s) of reflexive decision-making as the *common paths*, referring to the pattern of interneuron pathways recruited by the sensorimotor interface to elicit motor neuron recruitment. Sherrington recognized that the motor neurons would always be limited in number because they innervate muscles which are obviously fixed in number; therefore, they must be shared in all behaviors. For those reasons, the α-motoneurons are called the final common path. Paraphrasing Gallistel (1980, p 51), the α-motoneurons comprising the final common path are analogous to the limited set of keys on a keyboard while higher motor systems (reflexes) are akin to words. The organization of higher units competing to access the final common path is called the lattice structure/organization, and this property of spinal circuits drives competition among adjacent pathways. This competition is the mechanism that determines what motor system will "win" control over the initiation of behavior; thus, a critical element of action selection.

The dynamic decision-making parameters observed in reflexive circuits are driven in part by computational mechanisms at the level of common paths, including various forms of summation. This process was identified experimentally by generating a series of weak stimuli that failed to excite the reflex to threshold; as multiple weak inputs are successively integrated, the reflex activates due to summation of successive inputs over time. Sub-threshold excitation may thus linger after sensory inputs terminate, and this can change the magnitude of the response elicited by a subsequent sensory input; this process is called *temporal summation* (Gallistel, 1980; Burke, 2007). This is a critical computational process that allows the assemblage of sophisticated logical operations. When the response to a succeeding stimulus is amplified to a greater magnitude than direct summation of all preceding stimuli could achieve, summation is said to be *supralinear*; when responses are weaker than expected, the summation is *sublinear*. 
Sublinear summation also provides a computational mechanism that can be used to assemble conditional responses consistent with Boolean logic (i.e., AND/OR statements). For example, sublinear summation in a reflex pathway can act like an "AND" function to establish conditional decision-making parameters, e.g., the reflex is triggered only when visual AND auditory inputs are integrated (Gallistel, 1980). The uni-directional conduction of excitation in reflex pathways, similarly, implicitly allows the construction of Boolean "OR" functions, e.g., fire when visual OR auditory inputs are integrated. These inherent logical and computational information-processing properties of reflex circuits far exceed the presumed faculties attributed by early theorists, e.g., Hall (see prior section) to the simple spinal reflex arc (Backe, 1999; Bennet & Hacker, 2002).

Thus, the evidence and theoretical perspective that Sherrington advanced (1906) fundamentally rejected the basic criteria that were initially used as to define reflexes as a category, e.g., reflexes as simplistic and deterministic mechanisms (Backe, 1999; Bennet & Hacker, 2002). This integrated perspective was extended still further from passive reflex arc models by the identification of a series of inhibitory processes that are functionally integrated into every aspect of reflexive motor control. The reflex thus reflects "The Integrative Action of the Nervous System," Sherrington's (1906) title, in that the activation of even the simplest reflexive movement involves the coordination of multiple excitatory/inhibitory circuits and muscle groups (reviewed in Gallistel, 2008).

A critical step in the establishment of this principle was Sherrington's identification of the inhibitory process, reciprocal inhibition, which prevents opposing reflexive muscle contractions when a reflexive movement is initiated (reviewed in Gallistel, 1980; Burke, 2007). The origins of this process were localized to spinal networks. The necessity of such a system is apparent in the
organization of muscle groups in opposing (agonist/antagonist) pairs. That is, if antagonist muscle groups were not inhibited during agonist contraction, they would counter the force elicited by the muscle command, attenuating or preventing the intended movement. Sherrington showed that the activation of reflexive motor pathways triggers reciprocal inhibition of the motor pathways corresponding to opposing muscles. This framework emphasizes the integration of excitatory and inhibitory processes in motor control systems, generally, and also illustrates the important role of reciprocal inhibition in the coordination of movement (Gallistel, 1980; Burke, 2007).

Two related forms of reciprocal inhibition, distinguished by Gallistel (1980) as recurrent reciprocal inhibition and precurrent reciprocal inhibition, are critical to another general principle of motor control: Competition among motor control systems. This was discussed previously in the context of competing to reach the final common path. Explicitly, reciprocal inhibition of adjacent excitatory competitors is the functional mechanism of this competition, i.e., each circuit competes against others by activating reciprocal inhibition to oppose their excitation. Unlike the process described above, these forms of reciprocal inhibitory mechanisms are primarily critical to action selection. That is, given the likely scenario of simultaneous sensory inputs exciting multiple excitatory pathways, the mechanism by which a motor response is selected (Gallistel, 1980; Burke, 2007).

Recurrent reciprocal inhibition contributes to a winner-take-all form of competition among motor systems, whereby the excitatory pathway that is activated first prevents other excitatory pathways from being activated. It originates in spinal interneurons that are activated when associated excitatory interneurons fire (in that sense, recurrent), and triggers the inhibition of adjacent or opposing excitatory pathways (in that sense, reciprocal). The principle of
competition among parallel motor circuits as the primary mechanism of action selection is recapitulated at every stage of the motor control hierarchy (Gallistel, 1980; Burke, 2007).

Precurrent reciprocal inhibition, per Gallistel's (1980) terminology, contributes to action selection similarly, i.e., favoring a winner-take-all competition among parallel processes, but is achieved with a different configuration of circuit elements. In this arrangement, the inhibitory interneurons that prevent excitation of adjacent excitatory pathways (the reciprocal component) are excited directly by sensory inputs from the afferent pathway (hence precurrent). Two adjacent circuits receiving equal sensory inputs will thus be equally inhibited by the opposing precurrent inhibition; this push-pull dynamic is analogous to the differential principle in electronics, in that, the overall signal strength (sensory input) no longer determines the outcome, but rather the difference in signal strength between adjacent circuits. Gallistel (1980) proposed this concept to compensate for limited sensitivity at the level of signal transduction (Gallistel, 1980). An alternative functionality is suggested by a similarly organized feed-forward inhibitory process in the Mauthner-cell (M-cell) startle circuit, whereby the disynaptic chemical inhibition acts as a high-pass filter that only allows for excitatory inputs to fire principle neurons (M-cells) if they can saturate and surpass afferent inhibition, or trigger depolarization to threshold before inhibition-onset (Korn & Faber, 2005).

New Units, Old Hierarchy

Few bodies of experimental work can withstand a century of progress. If all aspects of integrative reflex theory (Sherrington, 1906) had since been found faulty, Sherrington would still be famous among physiologists if only for popularizing the systematic use of stimulus-response measures for mechanistic dissections of decision-making processes. These remain the basis of current approaches to neuroscience, including the experimental methods applied in subsequent
chapters of this thesis. Inevitably, some conclusions reached by Sherrington (1906) were
reexamined with new evidence and found lacking; most glaringly, his concept of reflexes as the
fundamental unit of motor control (discussed below). Nonetheless, the basic principles of motor
coordination and hierarchical control that he characterized in spinal reflexes are essentially
recapitulated on multiple levels of the nervous system (Lashley, 1938; reviewed in
Gallistel, 1980). This section introduces essential elements in motor control systems that were not
addressed in his work, and outlines the motor control hierarchy that inevitably developed from
the principles of reflexive decision making, i.e., variable thresholds, logical operators,
competition, potentiation, recruitment, etc., and lattice structure in the common paths.

It is widely accepted, today, that reflexes are not the sole elemental units of motor control
(reviewed in Marder, 2008; also, Büschges et al., 2011), but preliminary evidence of non-
reflexive control units emerged in Sherrington's own work (1906, 1910) and that of his
contemporaries (Brown, 1911). These included the repeated experimental observation
(Sherrington, 1906, 1910) that oscillations in reflexive movements were often independent of the
frequency of eliciting stimuli. In the particular case of decerebated cats, Sherrington (1910) and
Brown (1911) independently observed semi-intact movements of the "step-like" paw flexion.
Sherrington explained these through the mechanism of reflex-chaining; that is, rhythmic and/or
sequential movements were assembled such that an initial reflexive movement is the eliciting
stimulus to subsequent responses (Sherrington, 1910).

Brown (1911) proposed an alternative mechanism to explain these and other cyclical
movement patterns, e.g., chewing, flight, locomotion, swimming, etc. The neural oscillator he
proposed became the fundamental unit of endogenous rhythmic motor commands (Staras et al.,
1998). Oscillators became the fundamental elements of central pattern generators (CPGs),
networks made of *neural oscillator* assemblies, and these comprise a ubiquitous neural control system observed at all stages of the nervous system (Marder, & Bucher, 2001). Brown (1911) proposed a remarkably prescient model, the *half-center model*, to explain how pairs of coupled-neurons, each activating reciprocal inhibition of the other, would generate an endogenous, biphasic rhythm, which could be expanded and varied indefinitely with additional couplings. It wasn't until Wilson (1961) demonstrated the rhythmic activation of wing muscles (fictive movement) in an isolated locust preparation that the basic oscillator element and the CPG became widely accepted components of the motor hierarchy (Marder & Bucher, 2005; Ayali & Lange, 2010).

The widespread early popularity and influence of integrative reflex theory and the relative late acceptance of CPGs has reversed in the past 50 years. The importance of reflexive circuits in postural control, protective functions, and withdrawal/avoidance responses remains as Sherrington (1906) stated it; the putative roles of CPGs, in contrast, continue to expand as novel aspects of endogenous rhythms are identified in different contexts. CPGs are seen now as the common paths in that mediation of processes and structural/functional elements of the CNS as diverse as regulating hunger/thirst via hypothalamic circuits (Buschges et al., 2011), controlling heart rate and breathing (reticular formations), chewing, swallowing, reaching, and diverse other functions involved in coordinated sequential actions (Marder & Bucher, 2001; Marder & Bucher, 2005; Buschges et al., 2011).

But while the role of CPGs has expanded to dominate the elementary control system ruling α-motoneurons, the fundamental organization of motor pathways still obeys the lattice framework identified by Sherrington (Gallistel, 1980; Marder & Bucher, 2005; Buschges et al., 2011). That is, adjacent systems must always compete for a limited pool of α-motoneurons, and
the fundamental mechanisms of that competition are reciprocal inhibitory mechanisms used to interfere with competing motor systems; higher motor systems, of course, selectively inhibit those circuits they can't recruit (Lashley, 1938; Gallistel, 1980). Thus competition among adjacent motor systems remains critical to action selection and the recruitment of integrated lower motor systems, and inhibition of opposing systems to gain access to the final common path drives the assemblage of increasingly complex control processes. Lashley (1938) called the process of recruiting or inhibiting downstream circuits "selective potentiation."

Another concept which has played a significant role in our thinking about motor control has been that of the command neuron. The command neuron concept was developed in the crayfish model system to describe the mechanism that activated the tail-flip escape responses (Weirsma, 1947; Wiersma & Ikeda, 1964; Edwards et al., 1999). Wiersma (1947) identified a sensorimotor interface consisting of four giant interneurons (LGs; lateral giant interneurons) excited by mechanosensory inputs. If afference to the four LG neurons triggered a single action potential in any LG, the escape response was executed in full; if no LG fired, the response did not happen. The logic was that these neurons were necessary and sufficient to cause a full behavioral sequence.

The command neuron concept was tremendously influential, perhaps for its familiar similarity to the well-characterized structure of the reflex circuit. That is, command neurons connected the familiar context of a circuit resembling the final common path to the initiation of entire behavioral sequences (Edwards et al., 1999). This invited the premise that the well-developed methodology used in the investigation of spinal reflexes could also be applied in the richer context of higher-order neural functions. Thus still-nebulous processes like perception,
sociality, communication, aggression, motivation, and memory might come to be expressed in the familiar terms of excitation and inhibition, at the sensorimotor interface.

The term has fallen somewhat out of favor after extensive and prolonged controversy and criticism of the defining criteria (Edwards et al., 1999). Kupperman & Weiss (2001) reviewed all the terms suggested in its place, which include influential-neuron, decision-making neuron, delegate neuron, higher-order neuron (HON), and command-like neuron. What these concepts all shared was the notion that hierarchical decision-making systems comprised of low-level movement coordinators (CPGs) activated in various contexts by higher-level movement initiators (command circuits) enabled fully robust and dynamic behavioral repertoires (Marder & Bucher, 2005; Ayali & Lange, 2010).

Command neurons in the cricket, for example, activate different stridulation patterns (rhythmic mating signals) depending on their own internal state (Hedwig, 2000); this contextual plasticity is a familiar recapitulation of reflexive logic, now in contexts of whole intact behaviors. Similarly emphasizing command-neuron control of behavioral plasticity, Nolen & Hoy (1986) identified a command neuron circuit, INT-1, that initiated a predator-avoidance response during cricket flight. The command neuron, INT-1, received inputs from auditory inputs, but these only elicit responses during flight. This sort of behavioral plasticity in varying contexts is an expected feature of dynamic decision-making systems, and Nolen & Hoy (1986) elegantly demonstrated this was achieved by tonic inhibition of the command neuron when the flight oscillator (CPG controlling wing contraction) was inactive. Additionally, the response was highly selective, reflecting aspects of feature-detection, which Nolen & Hoy (1986) attribute to selective use of Boolean operators to assemble the conditional logic for a "bat detector" sensory pathway.
A fundamental goal in behavioral neuroscience is to identify and explicate mechanistic components of information-processing, motor coordination, and decision-making that are only apparent in decision-making systems that emerge in the brain. How, for example, are processes like learning, attention, memory, and perception integrated at the mechanistic level of cellular and synaptic processes? How are key mechanisms of motor coordination and control in the spine, i.e., selective potentiation of lower systems and competition among adjacent systems (Sherrington, 1905; Lashley, 1938; Gallistel, 1980), among others, integrated with or recruited by cognitive, perceptual, and other higher processes? Or do cognitive and/or other higher processes emerge in the competition over decision-making systems? The basic goal of this thesis is to contribute towards our understanding of such processes, and subsequent sections introduce a model system which may be useful in answering such questions.

1.3. THE ACOUSTIC STARTLE REFLEX

The vertebrate startle response is a highly stereotyped, rapidly-activated contraction of facial and skeletal muscles elicited by the onset of intense and/or abrupt sensory stimuli. Startle shares features associated with reflexive behaviors, e.g., a highly stereotyped motor response elicited by stimuli (Koch, 1999; Fendt & Yeomans, 2001; Yeomans et al., 2006). Contrary to the spinal motor control locus characterized in classical studies (e.g., Sherrington, 1906), the decision-making systems that control the initiation of startle emerge in the reticular networks of the hindbrain (Faber & Korn, 1978). Consequently startle occupies a junction in the motor control hierarchy that is integrated in the sophisticated modulatory processes associated with the brain, but shares functional and organizational properties associated with reflexive control in the spine. These parameters present a model system for mechanistic analyses of psychological processes, e.g., attention, learning, sociality, emotion, memory, etc., in the familiar and
accessible contexts of excitation and inhibition of a decision-making system (Koch, 1999; Yeomans et al., 2006; Medan & Preuss, 2014).

**Startle properties**

The startle response is multi-modal, i.e., it can be initiated by visual, auditory, and/or tactile stimuli; there are limited data indicating startle may also be modulated by olfactory stimuli (Koch, 1999). Nonetheless, the acoustic startle response (ASR) is the predominant focus of mechanistic studies in non-humans for several practical purposes (Koch, 1999; Koch & Fendt, 2003; Medan, V., & Preuss, 2011). First, acoustic stimuli are easily controlled and reproduced under various laboratory conditions. Additionally, the auditory excitatory startle pathways are direct and unambiguous (i.e., mono- or di-synaptic to the VIIIth nerve), and are consequently better-studied and understood than visual and/or tactile startle pathways (Koch, 1999; Koch & Fendt, 2003). Last, the range of acoustic stimulus-parameters (frequency and amplitude) that provide useful measures of startle in experimental studies are also similar across vertebrates, including humans, rodents, and fish, thereby facilitating comparison among studies (Koch, 1999; Koch & Fendt, 2003).

Startle is a common feature of vertebrate behavioral repertoires and its evolutionary origins are thought to be homologous (Medan & Preuss, 2011). The adaptive value of startle is inferred to be a protective function that acts to facilitate collision avoidance/predator-escape (Koch, 1999). The benefit of such a system is critically dependent on the decision-making parameters that determine when the response is triggered. That is, whereas a well-timed flinch or body-bend may protect vulnerable structures from collision or reorient the body to escape predation, a premature, insufficient, or exaggerated response causes an interruption of ongoing motor programs, preempts more adaptive responses, and/or signals vulnerability (Koch, 1999).
Likely for these and/or related reasons, excitation of the startle response is tightly regulated but also highly malleable at the level of the intrinsic excitatory circuit and its extrinsic modulation via multiple central inhibitory circuits (Koch, 1999; Korn & Faber, 2005; Medan & Preuss, 2014).

**Startle physiology**

The physiology of startle decision-making systems has been extensively characterized in fish and mammals, though at different levels of analysis (Koch, 1999; Korn & Faber, 2005; Medan & Preuss, 2014). In mammals associated excitatory networks and descending modulatory pathways were systemically mapped with lesioning experiments (Koch & Schnitzler, 1997; Koch, 1999; Yeomans et al., 2006), but we have only one *in vivo* mechanistic analyses of information-processing in the startle network (Lingelholh & Friauf, 1994). In contrast, the startle network in fish is functionally well characterized with electrophysiological methods, but its integration with higher structures is less clear (Korn & Faber, 2005; Medan & Preuss, 2014). In both prominent vertebrate startle systems, the response is produced by excitation of a primary excitatory pathway, i.e., the so-called hindbrain "startle circuit" that is monosynaptic to the VIIIth (auditory) cranial nerve, but visual and tactile inputs are also integrated in startle decision-making (Koch, 1999; Korn & Faber, 2005). In teleosts, paired, bilaterally symmetrical afferent networks terminate on the dendrites of the Mauthner cell, which functions as a "command neuron" for startle behavior. In mammals, the analogous function is produced by activating a population response in a pool of giant neurons of the caudal pontine reticular nucleus (PnC neurons) (Koch & Schnitzler, 1998; Koch, 1999; Fendt & Yeomans, 2001); these determine the magnitude of startle. Importantly, unlike the teleost equivalent, the mammalian startle response is graded, rather than "all-or-none".
**Startle plasticity**

The plasticity of the startle response, referring to its highly variable stimulus-response conditions in varying sensory and behavioral contexts, is critical to its utility as an experimental model system. Startle provides a rare model system for information-processing and decision-making that can provide a clear behavioral readout of localized processes in the brain. Graham (1975) and Hoffman (1980) developed parallel lines of research that were influential in establishing widespread interest in the topic of startle plasticity, i.e., stimulus- or context-evoked changes in startle excitability. Hoffman (1980) developed stimulus protocols and behavioral methods to study startle plasticity in a rat model system; his work established the use of repeated stimuli to drive changes in startle excitability. He demonstrated that long-lasting *startle habituation* could be reliably evoked at the level of the startle circuit by repeatedly presenting stimuli over many trials; the inhibitory process was quantified in the declining magnitude of startle over time. He also found *startle sensitization*, i.e., enhanced response magnitude, that could be evoked in habituation paradigms by presenting aversive stimuli in another modality, e.g., an electrical stimulus causes subsequent trials to exhibit greater responses than baseline.

Sensitization and habituation are considered critical contributors to dynamic decision-making systems, enabling filtering adjustments that maintain response thresholds relative to environmental contexts (Koch, 1997; 1999). They also comprise a form of non-associative learning, which now were localized to discrete systems (Hoffman, 1980; Koch, 1999). A subsequent series of studies developed the use of behavioral pharmacology to probe information-processing (Kehne & Davis, 1984; Koch & Friauf, 1995). These methods were applied far more commonly than physiological studies of startle, but often yielded contradictory results and inspired lingering uncertainty as to various loci or sites of action relevant to the mechanisms
studied. Prominent among these inconsistencies, behavioral pharmacological studies of glycine, a prominent inhibitory neurotransmitter, yielded inconsistent and sometimes opposing effects on behavior, which were difficult to localize, and then later, failed replication (Kehne & Davis, 1984; Koch & Friauf, 1995; Fendt, 1999; Geis & Schmid, 2011). This may reflect the particular challenge of trying to antagonize critical inhibitory systems near the site of their primary action (for glycine, the spine).

Graham (1975) developed a stimulus protocol to elicit a fast-activating transient inhibition, that was recruited, integrated, and decayed all in less than a second. This measure, now called prepulse inhibition (PPI) has since become a primary measure of sensorimotor gating. PPI describes a stimulus-elicited reduction in startle excitability, i.e., inhibition of startle (evoked by a relatively strong stimulus, the pulse) briefly (50-1000 ms) following a weak sensory stimulus (the prepulse). PPI is thought to prevent temporal summation, in a mechanistic sense, of weak inputs to the startle network, which might summate and disrupt the stimulus-processing in more-appropriate decision-making systems than startle. Consistent with this notion, subsequent investigations in rodents (Koch & Schnitzler, 1997; Yeomans et al., 2006) applied lesioning methods to identify the source of inhibition, and indeed found midbrain nuclei triggering the descending inhibition. Thus this gating process reflects a dynamic selective (de)potentiation of startle in contexts where a sudden movement will distract from decision-making processes in higher circuits.
1.4. PREPULSE INHIBITION

Prepulse Inhibition

Following Graham's (1975) development of the prepulse inhibition (PPI) paradigm, the PPI protocol was widely adopted by a fast-growing body of researchers, with early studies focusing on its relevance in contexts of sensorimotor plasticity and time-linked information processing (Filion et al., 1998). PPI also became important to cognitive measures, offering an easily-quantified assay of pre-attentive filtering processes to contrast with other, slower assays. Consistent with the notion that PPI is a relevant filtering mechanism, or measure, clinical studies identified consistent PPI deficits in many kinds of information-processing disorders (Parwani et al., 2000; Braff, Swerdlow, & Geyer, 1999). PPI thus became a prominent diagnostic tool for clinical neuropsychology. The "filter hypothesis of schizophrenia" (Broberg et al., 2010) proposes that pathological deficits in selective inhibitory functions, e.g., PPI, contribute to the etiology of schizophrenia and other pathologies.

Investigations in rodent model systems found that measures of PPI were quite stable over different species and even types of startle; that is, PPI of the human eye blink response (measured in EMG of the orbicularis oculi muscle) follows a similar magnitude and time-course as PPI of the whole-body flinch of rodents (Filion et al., 1998; Koch, 1999). Though PPI is measured in startle behavior and physiology, it is thought to be produced independently of the startle circuit; that is, the time-course of startle attenuation relative to prepulse onset indicates the output of central inhibitory mechanisms acting on startle circuits (PnC neurons/ M-cells). The explicit notion emphasized by Graham (1975) is that the startle circuit and the putative PPI circuit (the inhibitory circuit activated by prepulse stimuli) are independent of each other, except that the PPI circuit acts to inhibit startle.
Prepulse inhibition is observed in all species studied thus far; this includes mollusks, zebrafish, cichlids, goldfish, mice, rats, monkeys, and humans (Braff, Swerdlow, & Geyer, 1999; Frost, 2005; Burgess & Granato, 2007). The principle defining characteristics of the PPI process are a reduction in startle excitability elicited by presenting a sub-threshold stimulus shortly (20-500 ms) prior to startling stimuli. This is measured differently in various paradigms, but most commonly contrast the magnitude of startle on baseline trials against startle magnitude following prepulses at various inter-stimulus intervals (ISIs). The magnitude of PPI is reliably predicted by prepulse-pulse inter-stimulus intervals (ISIs) (Koch, 1999).

Properties of prepulse inhibition

Graham (1975) identified the following properties of prepulse inhibition: It occurs without prior exposure to stimuli or testing paradigm, and is thus not a learned response to prepulse or a conditioned inhibition to pulse stimuli. PPI is, like startle, multi-modal, i.e., auditory prepulses attenuate visual startles; PPI is thus not a phenomenon of habituation of afferent pathways. PPI occurs independently of attention (in sleeping and in decerebrated animals) but is modified by attention and other factors (Braff et al., 2001; Fendt & Yeomans, 2001). PPI is not a function of sensory masking, refractoriness, or cochlear activity generated by the lead stimulus because prepulse stimuli are either identical or of lower intensities relative to pulse stimuli, or of a different sensory modality, entirely. PPI is also different from non-associative learning, e.g., sensitization and/or habituation, because it occurs on the first trial with artificial stimuli, does not appreciably attenuate over time, and is reliably predicted by the inter-stimulus interval (Koch & Schnitzler, 1997; Koch, 1999). PPI is modulated by fear-conditioning, attention, and prepulse-stimulus salience (Liang et al., 1992; Roskam & Koch, 2006; Du et al., 2010). The time-scale of PPI also yields insight into the interaction of pre-attentive (< 150 ms)
and attentive control of information processing (Graham, 1975). That is, the time-course of PPI may reflect the recruitment of mechanisms activated prior to the coordination of attention, but is modulated by attention at longer time scales (> 150 ms).

**Mechanistic substrates of prepulse inhibition**

The role of midbrain (and forebrain) circuits in the control of PPI is well-characterized anatomically in rodent models (Koch & Schnitzler, 1997; Koch, 1999; Fendt et al., 2001; Yeomans et al., 2006) and, more recently, *in vitro* brain slice preparations derived from rat embryos (e.g., Bosch & Schmid, 2006, 2008; Yeomans et al., 2010). A consistent finding among behavioral pharmacology and *in vitro* studies is that different neurotransmitter receptor systems are linked to different temporal components of associated inhibition. Bosch & Schmid (2008), for example, tied time-specific modulations of startle excitability to cholinergic inhibition; similarly, Yeomans et al. (2010) found that the GABA<sub>A</sub> receptor contributed to inhibition produced by brief inter-stimulus intervals, whereas the GABA<sub>B</sub> receptor contributed to longer-lasting inhibition. Neuromodulatory processes are also critical in control of PPI, in particular serotonin and dopamine. PPI is disrupted after treating rats with serotonergic (5-HT) agonists of the 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, and 5-HT<sub>2A</sub> receptors (Swerdlow & Geyer, 1998), for example, though in some behavioral studies opposing effects of 5-HT<sub>1A</sub> & 5-HT<sub>1B</sub> receptors on PPI were described (Feifel et al., 2003). As in other instances, here the necessity for *in vivo* functional measures is emphasized. In the connection of PPI to schizophrenia and the current serotonin-dopamine pathological theory that attributes processing deficits to interactions among those systems, the roles of 5-HT and DA receptors are prominent targets (Braff et al., 1999; Braff, 2010). But present methods are largely limited to behavior and pharmacology, and those advances are hard to interpret both in terms of associated locus of action, and the nature of the effect. Substantive
advances thus depend on developing new model systems that can identify neuronal mechanisms that act directly on PPI, not associated pathways, and link deficits induced in experimental conditions to the cellular/synaptic mechanisms that cause it.

*Theoretical perspectives on prepulse inhibition*

Graham (1975) theorized that PPI provides an elementary sensory filter that attenuates secondary inputs in order to preserve ongoing information processing. Implicit to this notion is that PPI is a "top-down" function whereby hindbrain circuits are modulated via mid- and forebrain circuits (Koch & Schnitzer, 1997; Koch, 1999; Yeomans et al., 2006). The observation that PPI is observed in invertebrate species and neural networks seems incompatible with this notion, e.g., the *tritonia* mollusk system characterized by Frost (2003). Further, it is unclear to what extent PPI reflects an integrative, computational process, or is rather derived from biophysical parameters and intrinsic properties of neural circuits; or, emerges from the integration of both. For example, Schicatano et al. (2000) discovered that changes in reflex excitability associated with Parkinson’s disease effectively modulated PPI of the eye blink reflex; similarly, in a rodent model, Blumenthal (1997) found that habituation of the startle reflex modulated PPI. Sandner & Canal (2007), emphasizing the problematic complexity of dissecting discrete mechanisms that operate on a common pathway, developed a model of PPI wherein startle excitability and PPI were related. On the other hand, extrinsic regulation of startle excitability during PPI by midbrain (and forebrain) circuits is well-characterized anatomically (Koch & Schnitzer, 1997; Koch, 1999; Fendt et al., 2001; Yeomans et al., 2006) and physiologically (e.g., Bosch & Schmid, 2006, 2008; Yeomans et al., 2010). Theoretical perspectives on PPI are thus somewhat conflicted as to the explicit origins of inhibition: Does PPI emerge from the exclusive activation of "extrinsic" mechanisms, i.e., a pre-synaptic
inhibitory circuit, as proposed by Graham (1975), or does PPI emerge from the intrinsic properties of startle, or some integration of intrinsic/ extrinsic factors. To some extent these issues present a bottleneck to progress, as neither of the most popular models used in studying startle, i.e., humans and rodents, are particularly accessible to the in vivo measures necessary to resolve them.

1.5. THE MAUTHNER CELL STARTLE CIRCUIT

The goldfish startle response, a characteristic "C"-shaped bend of the body (hence called the "C-start"), shares many features in common with startle and related modulatory processes in other vertebrates (e.g., PPI, and rodent models) (Korn & Faber, 2005). These include similar stimulus-response properties, response latencies (10-15 ms from auditory stimuli onset), and the anatomical localization of hindbrain decision-making ("startle circuit") networks. Teleost fish appear unique among vertebrates in that the startle response is triggered by a single identifiable neuron, the Mauthner-cell (M-cell) that is readily accessible for in vivo electrophysiology (Zottoli, 1977; Eaton et al., 2001). Consequently, this presents an appropriate model system to characterize cellular and synaptic mechanisms contributing to startle plasticity.

The Mauthner-cell is a "command neuron" in that, when it is depolarized to threshold, it activates the entire startle behavioral response by firing a single action potential (AP) (Zottoli, 1977; Eaton et al., 2001). The M-cell is thus unambiguously the sensorimotor interface of the startle circuit and the site where excitatory/ inhibitory networks converge (Faber & Korn, 1978; 1986; Korn & Faber, 2005). The direct and unambiguous connection between M-cell properties and startle allows for complementary investigations of physiology and behavior that can be used to confirm the consistency and validity of conclusions drawn from each.
Properties of the M-cell circuit

The Mauthner-cell (M-cell) system is composed of two bilaterally symmetrical reticulospinal M-cells, each receiving monosynaptic inputs from the auditory-vestibular VIIIth nerve (Furukawa & Ishi, 1967; Faber & Korn, 1978; 1986; Eaton et al., 2001). Each M-cell is the focus of two well-characterized inhibitory networks that control feed-forward inhibition that is activated by auditory afferences, and a feedback inhibitory process activated when the M-cell fires (Furukawa, 1966; Faber & Korn, 1986). Both inhibitory networks act on glycinergic processes, but GABAergic inhibition is thought to be important as well, given expression of GABA_A receptors, but its role is less clear (Furukawa & Ishi, 1967; Korn & Faber, 2005).

The intrinsic properties of the M-cells also drive characteristic stimulus-response properties. Atypically, the M-cell membrane is passively inexcitable, meaning there are no voltage-gated sodium channels expressed, or other mechanisms of active signal propagation. However, the M-cell's response function, e.g., membrane excitability, is non-linear at rest, and when depolarized almost to threshold. These nonlinear functions of membrane excitability are driven by inward rectifying K+ channels that organize current-voltage relationships during depolarization of the M-cell (Faber & Korn, 1986; Mintz & Korn, 1991). The latter inward rectification is transiently activated by 5-HT but reverses 1-15 minutes after activation; its physiological function is unclear, being unassociated with any outward current and activated by strong hyperpolarizations unlikely to occur in physiological contexts.

Neurotransmitter functions in the M-cell

The M-cell is innervated by 5-HT projections of at least two types (Gotow et al., 1990; Whittaker et al., 2011). Mintz & Korn (1991) demonstrated serotonergic modulation of the pre-synaptic inhibitory network as well as post-synaptic modulation of the M-cell, itself.
Importantly, Whittaker et al. (2011) showed that only 5-HT$_{5A}$ and 5-HT$_{6}$ receptors are expressed in the M-cell of cichlids. Medan & Preuss (2011) found that dopamine, another monoamine neurotransmitter, regulates time-specific inhibition of the M-cell during sensorimotor gating. Other prominent neurotransmitters associated with the M-cell system include glutamate, which acts as the primary excitatory effector at chemical synapses (Furukawa, 1966; Faber & Korn, 2005). Chemical inhibition of the M-cell is primarily mediated via GABAergic and glycineergic neurotransmission (Korn & Faber, 2005).

1.6. RESEARCH GOALS

A. General

The preceding review emphasizes four critical points. First, broadly, multiple decision-making systems control action-selection at higher and lower stages of processing, but relatively few are accessible at a given moment due to reciprocal inhibition of adjacent circuits, and selective inhibition of lower units (e.g., Lashley, 1951; Gallistel, 1980). The selection of available decision-making circuits in a given context is driven by competition among adjacent circuits, and by the selective potentiation (discrete increases or decreases in excitability) of lower circuits, as in many forms of startle plasticity, including sensitization/ habituation and PPI. Second, the acoustic startle response is commonly used as a behavioral readout of information-processing in a low-level, reticulospinal "command-like" circuit, the "startle circuit" (Koch & Fendt, 2003). By this logic, plasticity in startle response parameters can provide an assay for multiple processes that contribute to information-processing at this level, as well as processes involved in hierarchical coordination with other decision-making systems, including sensitization/ habituation, sensorimotor gating, associative learning, etc. Among those well-
characterized components of startle plasticity, the common conceptualization of prepulse inhibition (PPI) offers the clearest model of selective potentiation because it is conceived as purely a product of inputs from higher (midbrain) networks to lower command circuits (the PnC "startle circuit") in sensory/behavioral contexts (briefly following weak stimuli) where decisions from the startle network are disruptive. This is likely also how other processes are organized, for example, fear potentiation of startle likely involves amygdala inputs, but associative learning may also occur among local networks, as in Oda et al. (1998). Last, recent studies of the M-cell startle circuit (Medan & Preuss, 2011) indicate that this model system can be used for in vivo mechanistic PPI studies at the level of the M-cell command neuron that initiates startle. This presents an opportunity to address outstanding mechanistic questions that are inaccessible to behavioral approaches or ex vivo physiological methods.

The points summarized above provide the basis for the goals addressed in this thesis. In a general sense, these are twofold. First, to advance studies in the M-cell system that can provide direct in vivo evidence at the cellular level to resolve outstanding discrepancies in the rodent behavioral pharmacology literature. Two problems, in particular, are amenable to this approach. First, although there is unambiguous evidence that neuromodulatory effects of dopaminergic and serotonergic signaling drive dynamic shifts in startle plasticity and PPI, it is not clear if those effects are mediated at the level of the startle circuit, or in associated upstream networks. As Medan & Preuss (2011) localized dopaminergic effects to the startle circuit, a major focus of this thesis was to connect serotonergic modulation of startle plasticity and PPI to the post-synaptic properties of M-cells. Similarly, with respect to direct inhibitory neurotransmission, the nature of the inhibitory signaling mechanisms directly mediating PPI remain ambiguous. Thus, a second
line of experiments will examine the roles of glycine and GABA receptors in startle excitability and PPI and attempt to resolve the contributions of each.

B. Specific Aims

**Specific Aim 1:** To characterize the functionality of the 5-HT$_{5A}$ receptor in startle plasticity and prepulse inhibition. This study followed two advances: first, the discovery that the 5-HT$_{5A}$ receptor was expressed in the M-cell (Whittaker et al., 2011); second, the recent development/availability of selective 5-HT$_{5A}$ antagonists. These presented an opportunity to study a 5-HT receptor system that had eluded all efforts toward functional characterization since its identification in 1996. These experiments, presented in Chapter 2, tested the general hypotheses that (H1) the 5-HT$_{5A}$ receptor modulates sound-evoked excitation in the M-cell, persistently or during PPI-only; (H2) the 5-HT$_{5A}$ receptor modulates post-synaptic excitability in the M-cell; (H3) changes in post-synaptic excitability (e.g., conductance) can be connected to changes in Cl-permeability. The explicit background relevant to these hypotheses is introduced in that context, in Chapter 2.

**Specific Aims 2 and 3:** To identify glycine receptor (GlyRs) and GABA$_{A}$ receptor dependent components of sensorimotor plasticity and PPI. These Aims are addressed in two series of pharmacological experiments reported in a single manuscript in Chapter 3. These investigated the neurotransmitters, as opposed neuromodulatory mechanisms identified in Chapter 2, involved in the direct mediation of prepulse inhibition (PPI). This study tested the hypotheses that (H1) GlyRs and GABA$_{A}$Rs mediate the magnitude and time-course of sound-evoked excitation; (H2) PPI is reduced after treatment with GlyR and GABA$_{A}$R antagonists; (H3) the effects of GlyR
and GABA\textsubscript{A}R antagonists characterized in \textit{H1} and \textit{H2} are mediated by post-synaptic changes in M-cell excitability.
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*Current Opinion in Neurobiology, 663–669.*


CHAPTER 2

previously published as

"The 5-HT$_{5A}$ receptor regulates excitability in the auditory startle circuit: functional implications for sensorimotor gating"

by

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ABSTRACT

Here we applied behavioral testing, pharmacology, and *in vivo* electrophysiology to determine the function of the 5-HT$_{5A}$ receptor in goldfish startle plasticity and sensorimotor gating. In an initial series of behavioral experiments we characterized the effects of a selective 5-HT$_{5A}$ antagonist, *SB-699551*, on prepulse inhibition of the acoustic startle response. Those experiments showed a dose-dependent decline in startle rates in prepulse conditions. Subsequent behavioral experiments showed that *SB-699551* also reduced baseline startle rates (i.e., without prepulse). To determine the cellular mechanisms underlying these behaviors, we tested the effects of two distinct selective 5-HT$_{5A}$ antagonists, *SB-699551* and *A-843277*, on the intrinsic membrane properties and synaptic sound-response of the Mauthner-cell (M-cell), the decision-making neuron of the startle circuit. Auditory-evoked post-synaptic potentials (PSPs) recorded in the M-cell were similarly attenuated following treatment with either 5-HT$_{5A}$ antagonist (*SB-699551*: 26.41 ± 3.98 % reduction; *A-843277*: 17.52 ± 6.24 % reduction). This attenuation was produced by a tonic (intrinsic) reduction in M-cell input resistance, likely mediated by a Cl$^-$ conductance, that added to the extrinsic inhibition produced by an auditory prepulse. Interestingly, the effector mechanisms underlying neural PPI itself were unaffected by antagonist treatment. In sum, these results provide an *in vivo* electrophysiological characterization of the 5-HT$_{5A}$ receptor and its behavioral relevance, and provide a new perspective on the interaction of intrinsic and extrinsic modulatory mechanisms in startle plasticity and sensorimotor gating.
INTRODUCTION

Serotonin (5-HT) contributes to sensorimotor integration and decision-making by directly and indirectly regulating excitability. The functional plasticity of 5-HT is facilitated by 14 discrete receptor subtypes comprising 7 homologous receptor families (5-HT\textsubscript{1-7}) (Barnes, 1999; Filip & Bader, 2009). Among these, the 5-HT\textsubscript{5A} receptor has proven remarkably challenging to characterize due to the limited availability of selective ligands (Nelson, 2004; Thomas, 2006; Kessai et al., 2012). The 5-HT\textsubscript{5A} receptor is nonetheless broadly distributed in the CNS and was recently functionally characterized in native rodent tissues \textit{ex vivo} (Goodfellow et al., 2012), emphasizing the importance of resolving its functionality \textit{in vivo}. Here we studied the function of the 5-HT\textsubscript{5A} receptor in the startle circuit and behavior of goldfish.

Startle is a common tool for neuropharmacological studies because it offers an easily quantified indicator of neural excitability and is the final common path for multiple modulatory processes (Koch, 1999; Koch & Fendt, 2003). Startle plasticity is commonly studied with the prepulse inhibition (PPI) paradigm, a measure of sensorimotor gating evoked by weak stimuli that attenuate the startle response elicited by subsequent stronger stimuli (Graham, 1974; Hoffman & Ison, 1980; Koch, 1999). Deficits in PPI are associated with several information processing disorders, notably schizophrenia (Braff, Geyer, & Swerdlow, 2001; Braff, 2010). Importantly, schizophrenic populations also exhibit an abnormality in 5-HT\textsubscript{5A} coding sequences, making this receptor a potential clinical target (Arias et al., 2001; Iwata et al., 2001; Thomas, 2006).

The Mauthner-cell (M-cell) system of fish presents a unique opportunity to characterize the functionality of 5-HT\textsubscript{5A} in the context of a vertebrate circuit accessible for \textit{in vivo} electrophysiology. The two reticulospinal M-cells integrate excitatory and inhibitory multimodal
inputs; most prominently, a direct, monosynaptic excitation from the auditory vestibular (VIII\textsuperscript{th}) nerve (Furukawa & Ishi, 1967; Korn & Faber, 2005). A single action potential (AP) in one M-cell initiates the characteristic startle response, the C-start (Zottoli, 1977; Weiss et al., 2006). The M-cells therefore provide the sensorimotor interface of the startle circuit and have proven to be ideally suited to study the mechanisms underlying startle plasticity and sensorimotor gating in goldfish (Neumeister et al., 2008; Medan & Preuss, 2011), African cichlids (Neumeister et al., 2011; Whitaker et al., 2011), and zebrafish (Burgess & Granato, 2007).

The M-cell is innervated by 5-HT projections of at least two types (Gotow et al., 1990; Whittaker et al., 2011); further, Mintz & Korn (1991) demonstrated serotonergic modulation of the pre-synaptic inhibitory network as well as post-synaptic modulation of the M-cell, itself. Importantly, Whitaker et al. (2011) showed that only 5-HT\textsubscript{5} and 5-HT\textsubscript{6} receptors are expressed in the M-cell. These studies provided the rationale for investigating the functional role of 5-HT\textsubscript{5A} receptors in the vertebrate startle circuit with complementary electrophysiological and behavioral experiments in goldfish. Our results indicate that 5-HT\textsubscript{5A} regulates M-cell excitability by modulation of a membrane conductance, which in turn influences the magnitude of sensorimotor gating and behavioral PPI.

**METHODS**

**Subjects**

Sixty goldfish (*Carassius auratus*) of either sex, 7-13 cm in standard body length, purchased from Billy Bland Fisheries (Taylor, AR), Hunting Creek Fisheries (Thurmont, MD), or Ozark Fisheries (Stoutland, MO) were used in the behavior (\(n = 28\)) and physiology experiments (\(n = 32\)). Fish were allowed to acclimate for three weeks after transport in rectangular plexiglass holding tanks (30x30x60 cm; 95 l). Tanks were supplied with recirculating
conditioned water maintained at 18°C. Water was conditioned as described in detail by Szabo et al. (2006). Ambient light was set to a 12 hr light/dark photoperiod.

**Behavioral Experiments**

Experiments were conducted in a circular acrylic tank (76 cm diameter, 20 cm water height) mounted on an anti-vibration table to reduce external mechanosensory cues. To minimize visual cues, the top cover and sides of the tank were rendered opaque. Conditioned water circulating through the tank and connecting to an external reservoir was maintained at 18°C. Single goldfish were transferred using a small plastic container from the holding tank into the center of the experimental tank. Animals were given a 10 minutes acclimation time to the tank before they were injected either for drug or sham (saline) treatment, followed by another 10 minutes acclimation period before the first experimental trial (see below).

As previously described in Neumeister et al. (2008), ventral views of the freely swimming fish were recorded via a mirror placed below the tank at a 45° angle, using a high-speed video camera (Olympus iSpeed2, Newton, MA). Recordings were saved to a hard drive. Two underwater loudspeakers located at opposite sides inside the experimental tank were used to deliver sound stimuli consisting of 200 Hz sound pulses (5 ms duration) created as single-cycle sine waves with Igor Pro software (WaveMetrics, Portland, OR) and amplified with a Servo 120 amplifier (Samson, Soysset, NY). For prepulse inhibition (PPI) trials, a non-startling acoustic pulse (prepulse) ranging from 128.8-137.32 dB preceded a startle pulse of 151.93-169.29 dB (SPL relative to 1 µPa in water, which translates to about 62 dB less in air relative to 20 µPa, i.e., relative to the human hearing threshold). The prepulse-pulse inter-stimulus interval (ISI) was measured from onset of stimuli. ISIs of 50 or 500 ms were used to characterize short- and long-lasting PPI effects. Pulses without preceding prepulses were used to elicit baseline startle.
Startle rates (evoked startles/trials) were determined in 3 different stimulus conditions presented in random order; each fish was exposed to 14 pulse-only trials, 5 PPI trials with an inter-stimulus interval (ISI) of 50 ms, and 5 PPI trials with an ISI of 500 ms. Speakers (left or right) were randomly alternated between each trial, and the time between trials varied from 1 to 8 minutes to avoid habituation.

Startle escape responses were recorded and visually examined at a time resolution of ±1 ms to determine startle rate and response latency. Responses with latencies greater than 18 ms were excluded from the analysis (mean = 2.1 trials/fish) since they cannot unambiguously be associated with M-cell activity (Zottoli, 1977). Escapes in response to a prepulse stimulus were also excluded (mean = 3.5 trials/fish). The assessment of invalid trials was done during the experiments and compensated by adding respective trials to the ongoing experiment to reach a consistent number of trials (24).

Behavioral Pharmacology

SB-699551 (Tocris Biosciences), a selective 5-HT$_{5A}$ antagonist (Corbett et al., 2005) was dissolved in saline and administered via intraperitoneal (i.p.) injections in treatment conditions, and saline vehicle in control conditions. Fish were briefly removed from the experimental tank for injection; this procedure did not last longer than a few seconds and the fish typically immediately resumed swimming when released. Volumes injected did not exceed 200 µl. In an initial series of experiments, we used a within-subjects design with a saline control and three different dosages of SB-699551 (0.5, 0.75, and 0.9 mg/kg b.w.) in subsequent experimental sessions. Each experimental session was 15-20 days (mean = 16.58 days) apart.
A second series of experiments was conducted with a between-subjects design, wherein subjects randomly received injections either of saline or SB-699551 at the 0.90 mg/kg dosage. 18 goldfish were randomly assigned to either the saline or SB-699551 (0.90 mg/kg) treatment condition. Acoustic startle stimuli were presented at three intensities, with a similar range as above; each fish was exposed to 24 pulse-only trials. Speakers (left or right) and stimulus intensity was randomly alternated between each trial. The time between trials varied randomly from 1 to 8 minutes (to avoid habituation), but the total duration of behavioral testing for each animal was 108 minutes. The experimenters were blind to the subject’s treatment condition during experimentation and analysis.

Electrophysiology

We employed previously described in vivo surgical and electrophysiological recording techniques (Preuss & Faber, 2003; Medan & Preuss, 2011). Subjects were immersed in ice water for 10-15 minutes, and then treated with topical anesthetic (20% benzocaine gel; Ultradent) at incision sites and pressure points (pin placement) 5 minutes prior to surgical procedures. Fish were then placed in the recording chamber, stabilized with one steel pin on each side of the head, and ventilated through the mouth with recirculating, aerated conditioned water at 18°C. The general anesthetic MS-222 was dissolved in the recirculating water at a dosage (20 mg/l) that does not interfere with auditory processing (Palmer & Mensinger, 2004; Cordova & Braun, 2007). The recording chamber was mounted inside an opaque, thin walled tank filled with temperature controlled (18°C) water covering the fish body up to the midline.

Next, the spinal cord was exposed with a small lateral incision at the caudal midbody. Bipolar electrodes were placed on the unopened spinal cord to transmit low-intensity (5-8V) electrical stimulation generated by an isolated stimulator (Digitimer Ltd., Welwyn Garden City,
UK). This allowed antidromic activation of the Mauthner cell (M-cell) axons, confirmed by a visible muscular contraction (twitch). Subjects were then injected intramuscularly with d-tubocurarine ($1 \mu g/g$ b.w.; Abbot, Chicago, IL), and a small craniotomy exposed the medulla for intracellular recordings. In anticipation of later experimental drug treatments an injection needle connected with tubing to a syringe was inserted intramuscularly prior to placement of recording electrodes.

Antidromic stimulation produces a negative potential in the M-cell axon cap (typically 15-20 mV), which unambiguously identifies the axon hillock and allows intracellular recordings from defined locations along the M-cell soma-dendritic membrane (Furukawa 1966; Faber et al., 1989). Intracellular recording of M-cell responses to sound stimuli were acquired using an Axoprobe-1A amplifier (Axon Instruments, Foster City, CA) in current clamp mode with sharp electrodes (3-8 MΩ) filled with 5 M potassium acetate (KAc) or 5M potassium chloride (KCl). Recordings were stored on-line with a Macintosh G5 using a data acquisition card (PCI-E, National Instruments, Austin, TX) sampling at 25KHz.

Sound stimuli consisted of single-cycle sound pips (200 Hz) produced by a function generator (Agilent 33210A, Santa Clara, CA) connected to a shielded subwoofer (SA-WN250, Sony) located at 30 cm distance to the recording chamber; however, due to transfer loss through the media of the recording chamber, maximum underwater sound intensity was limited to 147 dB relative to 1 µPa in water. These limitations, however, did not hinder physiology assessment of sub-threshold pre-pulse effects because those intensities resemble the pre-pulse intensities used in the behavioral experiments (see also Neumeister et al., 2008). Sound stimuli were recorded with a microphone placed 10 cm over the fish's head and stored together with the recordings. A
hydrophone (SQ01, Sensor, Collingwood, ON, Canada) was used for sound calibration but was removed during experiments.

PPI of the M-cell synaptic response was measured by presenting sound pulses separated by 20, 50, 150, or 500 ms ISIs (as in Medan & Preuss, 2011). The peak amplitude of the post-synaptic potential (PSP) activated by the leading sound pulse ($PSP_{\text{pre-pulse}}$) was compared to the peak amplitude of the PSP activated by the latter sound pulse ($PSP_{\text{pulse}}$) to provide a measure of PPI. The PPI effect was calculated as $(100 - \frac{PSP_{\text{pulse}}}{PSP_{\text{pre-pulse}}} \times 100)$, the implication being that higher percentages reflect greater PPI. Average values were computed from measures in 5-10 traces and were used for analysis.

To examine the effect of 5-HT$_{5A}$ antagonists on membrane properties previously studied in the M-cell, such as AP-thresholds, input resistance, and linearity (Neumeister et al., 2008; Medan & Preuss, 2011), we injected current ramps via a second intrasomatic electrode (KAc, 3-5 MΩ) while maintaining the voltage-recordings. A function generator (Wavetek, 39, Norwich, UK) was used to regulate current injection, producing a positive current ramp (0-200 nA / 20 ms). A compensation circuit built in the Axoprobe-1A amplifier eliminated cross-talk between the electrodes. Current-voltage relationships were measured without sensory stimulation or with an auditory prepulse (200 Hz, 147dB) preceding current injection by 20, 50, 150, or 500 ms.

Following assessment of baseline conditions subjects were injected with 5-HT$_{5A}$ antagonists. In experiments with $SB-699551$, the drug was dissolved in saline at a dosage of 0.90 mg/kg b.w., and measures taken in the baseline condition were repeated 10-30 minutes after injection. Resting membrane potential (RMP) was continuously monitored to ensure stable recording conditions and/or possible effects of the drug on this parameter. A typical experiment lasted 3-4 hours. In another subset of electrophysiology experiments, we used an alternative
selective 5-HT<sub>5A</sub> antagonist, *A-843277*, which was kindly provided by R.L. Gannon (personal communication; see also Gannon et al., 2009). As per the instructions provided, and, similar to the method reported by Kessai et al. (2012), the drug was dissolved in distilled water with 15 µL of Tween 80 (Sigma), for a final dosage concentration of 10 mg/kg b.w., as was applied by Gannon et al. (2009) and Kessai et al. (2012). Experiments with *A-843277* were otherwise identical to those where *SB-699551* was administered, as the intent was to compare the consistency of each antagonist’s effect.

All experiments were conducted according to the guidelines and approved protocols of the Hunter College (CUNY) Institutional Animal Care and Use Committee.

*Statistical Analysis*

Data were analyzed with JMP 8.0.2 (SAS Institute, Inc) and figures were created in Graphpad (Prism, v.5.0) or Igor Pro (Wavemetrics, v.5.03). Data presented in figures describe mean values and error bars illustrate standard error of the mean (SEM). D’Agostino and Pearson omnibus normality tests were used to confirm that datasets met assumptions of normality. Given the parameters of our data, we tested inferential statistical hypotheses with generalized linear mixed models (GLMMs). The GLMM is the appropriate statistical model for this dataset because it allows comparison of continuous conditions (e.g. ISI), can accommodate unequal sample sizes, and allows inferential tests of 2-way interactions and effects. In all analyses, subjects were treated as random effects (thus, repeated measures) and stimulus (no prepulse, ISI<sub>20</sub>, ISI<sub>50</sub>, ISI<sub>150</sub>, ISI<sub>500</sub>) and dosage (saline, 0.50, 0.75, 0.90 mg/kg b.w.) conditions were treated as fixed effects. Dependent variables tested in these models included startle probability, threshold voltage, threshold current, and input resistance. Note that the peak magnitude of post-synaptic potentials (PSPs) and the latency to peak magnitude of the PSP were the only effects not tested in a GLMM.
because those tests did not need to consider multiple factors and levels; accordingly, simple
matched T-tests were applied for these direct tests.

RESULTS

Behavior

The initial behavioral experiments tested the effect of three different dosages (0.50, 0.75,
0.90 mg/g b.w.) of the 5-HT5A antagonist, SB-699551, on the acoustic startle rate of fish in three
stimulus conditions: with no prepulse or with prepulses at inter-stimulus intervals (ISIs) of 50 ms
or 500 ms. The results of those experiments, presented in Fig.1A, showed a decline in mean
startle rates that was most pronounced in prepulse trials with the two highest dosages of the 5-
HT5A antagonist. Specifically, we found that startle rates in the ISI50 and ISI500 stimulus
conditions were most strongly affected by the drug at the 0.75 mg/kg (ISI50: 74.07 ± 22.07 %
reduction; ISI500: 80 ± 15.28 % reduction) and 0.90 mg/kg (ISI50: 87.5 ± 15.28% reduction;
ISI500: 70 ± 24.94% reduction) dosages relative to saline controls. Startle rates in the no prepulse
stimulus condition were less sensitive to drug treatment (black line, Fig. 1A; maximum dosage
effect: 14.01 % reduction at 0.90 mg/kg dosage) than startle rates in prepulse conditions (Fig.
1A). We tested the significance of dosage- and stimulus-condition effects on startle rates in a
generalized linear mixed model (GLMM). Our analysis identified significant main effects of
antagonist dosage (F3,99 = 11.78, n = 10, p < 0.0001) and stimulus conditions (F8,99 = 13.9494, n =
10, p < 0.0001) on startle rates. Post-hoc analyses (Tukey's HSD) found no significant effects of
dosage conditions on startle rates in no-prepulse stimulus conditions, but startle rates were
significantly reduced in prepulse trials for ISI50 at the 0.90 mg/g dosage (p = 0.0214) and for
ISI500 at both the 0.75 (p = 0.0071) and 0.90 mg/g (p = 0.0316) dosages as compared to saline
controls (Fig. 1A). The magnitude of pre-pulse inhibition (PPI) is typically quantified by comparing startle rates in no prepulse trials with prepulse trials for the same dosage. Figure 1B plots this quantification of PPI across dosage conditions to illustrate two important effects. First, treatment with the antagonist seemingly enhanced PPI, particularly for ISI500 (light gray line, Fig. 1B). Secondly, these facilitations of PPI also produced a convergence of PPI magnitude for ISI50 and ISI500 stimulus conditions, essentially eliminating ISI dependencies. This lack of ISI-dependency might indicate a drug-induced saturation of PPI. Alternatively, the apparent facilitation of PPI could indicate a generalized reduction of excitability in the startle circuit that is added to the inhibition evoked by the prepulse; indeed, the 14.01% reduction in startle responsiveness in pulse-only conditions (Fig. 1A black line, saline vs. 0.75 and 0.90 mg/kg dosages) provides some support for this interpretation.

To directly test the 5-HT5A antagonist’s effect on startle sensitivity independent of the prepulse inhibitory network, we tested subjects (n = 18) in a between-groups design using startle stimuli in the same intensity range as above. We found that subjects in the SB-699551 (0.90 mg/kg) group (Fig. 1C, gray line) had significantly lower startle rates than subjects in the saline-injection group (Fig. 1C, black line) (F1,48 = 13.13, p = 0.0007, n = 18), but neither stimulus intensity (F2,48 = 1.022, p = 0.3675, n = 18) nor the interaction of drug x intensity (F2,48 = 0.3804, p = 0.6856, n = 18) had any significant effect on startle rate. The general depression of the startle stimulus-response curve in the SB-699551 condition indicates that the 5-HT5A antagonist reduced startle rates over the whole range of stimulus intensities used. Additionally, an analysis of startle rates across trials indicated that there was no significant change in startle rate over the course of the experiment (F1,46 = 1.0163, p = 0.4432). Thus, the available data indicate that the drug effectively and consistently reduced startle over the 108 minutes of behavioral testing.
Physiology

The goal of the physiology experiments was to identify the effector mechanisms that the 5-HT$_{5A}$ antagonist acted on to reduce startle sensitivity in behavioral tests. Given that the behavioral results indicated a strong effect of SB-699551 at 0.90 mg/kg, and the challenges of in vivo electrophysiology, all physiological experiments with SB-699551 were conducted at that dosage. An additional 5-HT$_{5A}$ selective antagonist, A-843277 (10 mg/kg b.w.), was also applied in some physiological experiments to confirm that the effects observed were consistent and not specific to a distinct antagonist.

5-HT$_{5A}$ antagonist attenuates synaptic response

We first recorded the synaptic response of the M-cell to sound pips (without prepulse) before and after drug application. We found that SB-699551 reduced sound-evoked post-synaptic potentials (PSPs) in the M-cell (Fig. 2A). To quantify this effect, we measured the peak amplitude of M-cell PSPs (control: $n=14$, mean = 5.98 ± 0.63 mV) and found a 26.41 ± 3.98% decrease in peak depolarization after treatment with SB-699551 (paired t-test, $n=14$, $t=4.176$, $p=0.0011$; Fig. 2B). The latency to peak depolarization from the onset of sound (latency$\text{control} = 4.24 ± 0.14$ ms, $n=14$), however, was unaffected by treatment with SB-699551 (paired t-test, $n=14$, $p=0.2342$). These findings indicate a generalized drug-induced reduction in sound-evoked excitation that did not impact the temporal characteristics of the sound-evoked PSP.

Next, we administered a different 5-HT$_{5A}$ selective antagonist, A-843277, to test if the effects produced by the selective antagonist SB-699551 could be reproduced by an alternative selective 5-HT$_{5A}$ antagonist. The effects of A-843277 on the sound-evoked PSP were, in fact, similar to the effects of SB-699551 (compare Fig. 2A, black trace vs. gray trace, and Fig. 2C, black trace vs. gray trace); that is, A-843277 caused a significant reduction (17.52 ± 6.24 %
reduction in peak magnitude; paired t-test, \( n = 7, t = 2.629, p = 0.03 \) in the peak amplitude of sound-evoked PSPs (\( \text{PSP}_{\text{control}} = 6.203 \pm 0.59 \text{ mV}; \text{PSP}_{\text{A-843277}} = 5.26 \pm 0.78 \text{ mV} \); see Fig. 2D, black vs gray bars). As with \( SB-699551 \), the latency to peak depolarization from the onset of sound stimuli was unaffected (paired t-test, \( n = 7, t = 0.1586, p = 0.1586 \)) by treatment with \( A-843277 \) (latency\(_{\text{control}} = 4.76 \pm 0.18 \text{ ms}, n = 7 \); latency\(_{\text{A-843277}} = 5.35 \pm 0.59 \text{ ms}, n = 7 \)).

We then asked how 5-HT\(_{5A} \) antagonists affected synaptic PPI by measuring the amplitude of M-cell PSPs following a preceding sound pulse at 4 inter-stimulus intervals (ISIs; 20, 50, 150, 500 ms). Figure 3A shows sample recordings at ISI 50 ms illustrating that a prepulse decreased the overall magnitude of the pulse PSP (black trace vs. red trace). Repeating the experiment 10-25 minutes after drug application showed, as expected, a reduction in the\( \text{PSP}_{\text{pulse}} \) compared to non-drug controls (compare Fig. 3A black trace vs. blue trace) with an added attenuation of the PSP following a prepulse (Fig. 3A black and blue traces vs red traces). In other words, with drug treatment, the PPI effect is superimposed onto a tonic inhibition (gray double-arrowhead line in Fig. 3A); however, the PPI effect itself appears largely unchanged (indicated by brackets in Fig. 3A). To test this notion, we compared the PPI effect (100- \( \text{PSP}_{\text{prepulse}}/\text{PSP}_{\text{pulse}} \times 100 \)) on the PSP peak amplitude between control and drug conditions at different ISIs. The results showed that the duration of ISIs determined the magnitude of prepulse-pulse attenuation (\( F_{3,32} = 35.59, p < 0.0001, n = 9 \)), but \( SB-699551 \) caused no significant change in the PPI effect itself (\( F_{1,32} = 0.95, p = 0.3367, n = 9 \); Fig. 3B). Further, we found no significant interaction between ISI and drug treatment factors (\( F_{3,32} = 0.59, p = 0.6270, n = 9 \)). Importantly, this measure (% PPI) reflects the reduction in\( \text{PSP}_{\text{pulse}} \) relative to\( \text{PSP}_{\text{prepulse}} \) in the same treatment condition; meaning, the relative consistency of prepulse-pulse relationships are tested but the context in which they occur (i.e., significantly reduced excitation) are not. We replicated these stimulus conditions with
application of \textit{A-843277} and found, as before, similar results (see Fig. 3C). That is, whereas ISI is a significant determinant of \% PPI (F_{3,24} = 51.58, p < 0.0001, n = 7), neither \textit{A-843277} (F_{1,24} = 0.06, p = 0.6224, n = 7) nor the interaction of ISI and \textit{A-843277} (F_{3,24} = 0.16, p = 0.9208, n = 7) caused any significant change in PPI. Taken together, these results indicate that 5-HT_{5A} antagonists reduced the synaptic response to sound pips in the M-cell but do not affect synaptic PPI.

\textit{Effects of 5-HT_{5A} on M-cell membrane properties}

As noted, we previously showed that the 5-HT_{5A} receptor is expressed in the M-cell (Whittaker et al., 2011). Hypothesizing that the receptor is functional, we next asked if \textit{SB-699551} reduces the synaptic response through a post-synaptic mechanism. To test this we measured drug- and PPI-evoked changes in M-cell input resistance by injecting a current ramp into the M-cell lateral proximal dendrite while recording membrane voltage with a second electrode in the soma (see Methods). The rationale of using a current ramp was to assess drug effects on different M-cell properties (e.g., threshold current and voltage-dependent conductances) over the full range of membrane depolarizations in a standardized fashion (Neumeister et al., 2008). Figure 4A shows sample recordings of such an experiment in different stimulus conditions before and after drug treatment. The M-cell exhibits a well-characterized (Faber & Korn, 1986; Neumeister et al., 2008) membrane non-linearity that dynamically increases resistance (thus, excitability) when membrane depolarization exceeds 5 mV (Faber & Korn, 1986; Neumeister et al., 2008). This non-linearity can be characterized by measuring initial-state input resistance (slope 1) defined as the I/V slope 0-2 ms from onset of current injection (see Fig. 4B) and at a depolarized-state input resistance, slope 2 (I/V slope measured 1-3 ms prior to the onset of the AP; see Fig. 4B). Neumeister et al. (2008) and Medan & Preuss
(2011) showed that pre-pulse stimuli reduce input resistance differently in the initial-state and depolarized-state, providing two distinct cellular mechanisms that contribute to PPI (Fig. 4B, black vs. red plots; see also Neumeister et al., 2008; Medan & Preuss, 2011). Accordingly, we analyzed the putative effect of SB-699551 on initial-state and depolarized-state input resistance after acoustic pre-pulses at four distinct lead times (20, 50, 150, 500 ms).

Our results indicate that pre-pulse stimuli and the 5-HT$_{5A}$ antagonist activate independent but additive post-synaptic mechanisms that contribute to startle inhibition. Namely, we identified significant variability of slope 1 across different prepulse/pulse ISIs ($F_{4, 81} = 15.11, p < 0.001$; Fig 4C, no-prepulse vs. ISIs, compare numbers) and drug treatment ($F_{1, 81} = 32.81, p < 0.0001$; Fig 4C, black vs. blue lines, compare letters) conditions. Post-hoc tests revealed that input resistance was significantly reduced for ISI$_{20}$ ($p < 0.0001$), ISI$_{50}$ ($p < 0.0478$), and ISI$_{150}$ ($p < 0.0094$) stimulus conditions relative to no-prepulse controls. Post-hoc tests also revealed that treatment with the antagonist caused a significant reduction in input resistance ($t = -5.728, p < 0.0001$), but there was no significant interaction between the effects of pre-pulses and the effect of the drug ($F_{4, 81} = 0.2054, p = 0.9347$). These findings distinguish between a cellular mechanism that contributes to short-lasting (20-150 ms) PPI and a general inhibitory shunt (see below) of M-cell excitability after treatment with the antagonist. Importantly, these effects are cumulative, e.g. the initial-state membrane is least excitable in PPI conditions after treatment with the antagonist.

We identified a similar convergence of inhibitory mechanisms active in the depolarized membrane, i.e., a significant reduction in slope 2 ($F_{4, 81} = 7.181, p < 0.0001$; Fig. 4D, no-prepulse vs. ISIs) for all ISIs (ISI$_{20}$: $p < 0.0001$; ISI$_{50}$: $p = 0.0004$; ISI$_{150}$: $p = 0.0105$; ISI$_{500}$: $p = 0.0098$) relative to no-prepulse controls, and drug treatment ($F_{1, 81} = 31.644, p < 0.0001$; Fig. 4D, black vs
blue lines; post-hoc t=-5.625, p< 0.0001). As for slope 1, there was no significant interaction between drug treatment and stimulus condition effects (F4,81 = 1.8199, p = 0.133).

**SB-699551** had no effect on resting membrane potential (RMP) (RMP<sub>control</sub> = -80.91 ± 0.95 mV, n = 13; RMP<sub>SB-699551</sub> = -81.61 ± 1.414 mV, n = 9; student's t = 0.4223, p = 0.676). RMP was similarly unaffected by treatment with **A-843277** (RMP<sub>control</sub> = -82.44 ± 1.09 mV; RMP<sub>A-843277</sub> = -80.74 ±1.83 mV; paired t-test, n = 7, t = 0.9212, p = 0.3925). Consistent with the observed decrease in input resistance the drug increased threshold current indicated by the observation that current injections (limited to 200 nA by the amplifier) elicited action potentials only in 3 of 9 fish tested after drug application.

To confirm these results with parallel methods, we compared the amplitude of antidromically-evoked M-cell action potentials (APs) after treatment with both 5-HT<sub>5A</sub> antagonists. Since the M-cell membrane is inexcitable (i.e., non-regenerative), the magnitude of a passively-conducted APs provides an indirect measure of input resistance. We found that both selective 5-HT<sub>5A</sub> antagonists caused a reduction in the peak amplitude of M-cells APs (**SB-699551**: 10.93 ± 3.41 % reduction, see Fig. 5A; **A-843277**: 10.94 ± 1.41% reduction, see Fig. 5C). These reductions were significant following treatment with **SB-699551** (AP<sub>control</sub> = 33.39 ± 2.46 mV, AP<sub>drug</sub> = 29.87 ± 2.78 mV; paired t-test, n = 6, t = 3.608, p = 0.0154, see Fig. 5B) and with **A-843277** (AP<sub>control</sub> = 35.2984 ± 1.55 mV, AP<sub>drug</sub> = 31.4414 ± 1.53 mV; paired t-test, n = 5, t = 7.7566, p = 0.0015, see Fig. 5D). We also compared the drugs’ effects on the width (duration) of APs, measured at 1/3 of peak depolarization. Neither **SB-699551** nor **A-843277** produced any significant change in AP duration (**SB-699551**: paired t-test, n = 6, t = 0.1253, p = 0.9052; **A-843277**: paired t-test, n = 5, t = 0.1625, p = 0.8788). In sum, these findings provide further confirmation that antagonizing the 5-HT<sub>5A</sub> receptor produces a depression of post-synaptic
excitability. Additionally, the consistency of these effects provides evidence that each antagonist acts selectively on the 5-HT$_{5A}$ receptor.

**SB-699551 enhances Cl$^{-}$ conductance**

Tonic inhibition in the M-cell is linked to changes in Cl$^{-}$ conductance (Korn et al., 1987; Hatta & Korn, 1991; reviewed in Korn & Faber, 2005). Accordingly, in these experiments we tested if changes in Cl$^{-}$ conductance were related to the tonic changes in M-cell excitability we observed in prior experiments (see above). Because the M-cell RMP is near the Cl$^{-}$ equilibrium potential, changes in Cl$^{-}$ conductance do not produce changes in M-cell membrane potential when recordings are made with cationic solutions (e.g., KAc), as were used in all prior experiments. Intracellular recordings made with anionic recording solutions, however, can reveal Cl$^{-}$ currents as frank membrane depolarizations by altering the local Cl$^{-}$ concentration, and thereby driving force (Fukawi et al., 1965; Diamond, 1968; Diamond et al., 1973). Consequently, if the reduced excitability we found in previous experiments was driven by an increase in Cl$^{-}$ conductance, then the 5-HT$_{5A}$ antagonist should evoke an increase in depolarization with an anionic recording solution.

We tested this notion by recording intracellular responses to sound stimuli in control and drug conditions as in prior experiments, but using electrodes filled with a KCl$^{-}$ (5M) recording solution. The M-cell PSPs include purely excitatory and mixed excitatory/inhibitory components that can be dissected by the time-course of the response (see Szabo et al, 2006; Weiss et al., 2008). Whereas within 5 ms of the onset of sound stimuli the M-cell receives only electrical and chemical excitatory inputs (see Fig. 6A EPSP), the latter part of the response (see Fig. 6A PSP) integrates mixed excitatory and inhibitory inputs from associated feed-forward circuits (see Pereda et al., 1994; reviewed in Korn & Faber, 2005). Accordingly, we analyzed the peak
response to sound stimuli at those two intervals. Our results show that the EPSP and PSP components of the sound-response were equally enhanced by treatment with SB-699551 (Fig. 6A).

Within 5 ms of stimulus onset we found a significant increase in depolarization following treatment with SB-699551 (Fig. 6B; paired t, n = 9; t = 3.583, p = 0.0089). Similarly, peak responses > 5 ms from stimulus onset showed a significant increase in depolarization (Fig. 6C; paired t-test, n = 9, t = 4.062, p = 0.0048). The fact that the 5-HT<sub>5A</sub> antagonist increased sound-evoked depolarization without changing the time-course of the response suggests an underlying tonic enhancement of Cl<sup>-</sup> conductance. We also considered, however, that the antagonist could produce a conductance change associated with inhibitory networks. Changes in inhibitory inputs should be apparent in the latter components of the PSP that are not present in the initial EPSP. To test this, we compared the percentage change in EPSPs to PSPs after treatment with SB-699551, but found no significant difference in the drug’s effect over the time-course of the sound response (Fig. 6D; paired t-test, n = 9, t = 0.9241, p = 0.3825). Taken together, these results consistently suggest that the 5-HT<sub>5A</sub> antagonist produces a tonic increase in M-cell Cl<sup>-</sup> conductance.

DISCUSSION

The aim of this study was to determine the functional contribution of the 5-HT<sub>5A</sub> receptor in startle plasticity and sensorimotor gating. Our methodology linked the effect of a selective 5-HT<sub>5A</sub> antagonist on startle behavior to the underlying neural circuit that controls the behavioral response. Here we report the two main conclusions that can be drawn from our findings, and follow with more detailed examination of the evidence in favor of each. First, the 5-HT<sub>5A</sub> receptor regulates excitability of the startle circuit through a modulation of input resistance,
likely through a Cl⁻ current. Secondly, antagonizing the 5-HT₅A receptor leads to a reduction in startle rate during behavioral PPI (i.e., to an apparent increase in PPI); however, our electrophysiological experiments demonstrate that this is due to an additive interaction of drug-induced intrinsic and PPI-induced extrinsic inhibitory mechanisms. We believe these findings are important and of broad interest since they provide a new perspective on the modulation of PPI by intrinsic vs. extrinsic factors, an ongoing controversy in the field of sensorimotor gating (see below).

*Effects of SB-699551 on the M-cell*

The significant reductions in M-cell input resistance after treatments with two distinct 5-HT₅A antagonists (Figs. 4, 5) offer independent evidence that the 5-HT₅A receptor regulates intrinsic excitability of the M-cell, the decision-making neuron of the startle circuit. Consistent with this, we found significant reductions in the magnitude of sound-evoked M-cell PSPs (Fig. 2) and an attenuation of startle responsiveness. The 5-HT₅A receptor was known to be expressed in the M-cell (Whittaker et al., 2011), but our current findings confirm that this receptor is functional and plays an important role in modulating startle responsiveness in goldfish. These results are consistent with past studies of serotonergic modulation in the M-cell system and other startle circuits. Mintz & Korn (1991) found that 5-HT modulates a voltage-dependent conductance in the M-cell. Similarly, 5-HT increases input resistance in the lateral-giant escape neurons of the crayfish (Antonsen & Edwards, 2007). Thus, broadly, 5-HT plays an important role in modulating the excitability of startle-escape circuits and behavior in both vertebrates and invertebrates.

Our results show that a 5-HT₅A antagonist decreases M-cell membrane resistance by modulating a M-cell membrane conductance. The effects of *SB-699551* were almost equally
strong in a membrane close to RMP and close to threshold (Fig. 4), suggesting that the affected conductance shows no voltage-dependence within this physiological range of membrane depolarization. Previous studies of the 5-HT$_{5A}$ receptor (Hurley et al., 1998; Franken et al., 1998; Thomas et al., 2000; Noda et al., 2003, 2004) indicate that this receptor is negatively coupled to (i.e., suppresses) adenylyl cyclase formation, which in turn suppresses the cAMP second messenger system. In the M-cell, accumulation of cAMP enhances glycine-mediated inhibitory Cl$^-$ currents (Wolzen & Faber, 1989) without corresponding changes in RMP, as observed in the present study. Indeed, our results did show an apparent activation of a Cl$^-$ conductance with SB-699551 treatment (Fig. 6), and thus provide an important step in identifying the underlying mechanism/s through which 5-HT regulates M-cell excitability. Electrophysiological studies in ex vivo preparations, however, have also linked the 5-HT$_{5A}$ receptor to an inward-rectifying K$^+$ current (Noda et al., 2004; Goodfellow et al., 2012). Indeed, in the M-cell, a membrane non-linearity, linked to an inward rectifier, dynamically increases input resistance during depolarization, and the elimination of this non-linearity by a prepulse mediates PPI (Faber & Korn, 1986; Neumeister et al., 2008). The noted voltage-independency of drug effects in the present study, however, together with the lack of clear drug effect on PPI, suggest that a different effector mechanism is regulated by 5-HT$_{5A}$ in the M-cell, probably a cAMP-regulated Cl$^-$ conductance (see above) (Wolzen & Faber, 1989; Noda et al., 2004).

We also considered the possibility that the 5-HT$_{5A}$ antagonists we applied may act non-selectively in the goldfish brain, or may act on pre-synaptic circuits that modulate the M-cell. Several lines of evidence, however, suggest otherwise. First, two distinct selective antagonists independently produced near-identical effects on the synaptic response and membrane properties of the M-cell. Second, based on its binding affinity, the most likely course of a non-selective
The effect for SB-6995551 is to act on the 5-HT transporter (5-HTT) (Corbett et al., 2005). Mintz & Korn (1991), however, showed that antagonizing the M-cell 5-HTT produces different post-synaptic effects than were observed with the selective 5-HT\textsubscript{5A} antagonists used here; thus, these antagonists likely did not act on the 5-HTT. Third, although other 5-HT receptors are expressed in the fish brain, the only 5-HT receptors expressed in the M-cell are 5-HT\textsubscript{5A} and 5-HT\textsubscript{6} (Whittaker et al., 2011). Consistent with a putative post-synaptic action of the 5-HT\textsubscript{5A} receptor, we observed changes in M-cell resistance following treatment with either 5-HT\textsubscript{5A} antagonist (Figs 4,5). Finally, dosage-driven non-selective effects were an important concern in our experimental design. For that reason we chose dosages for the drugs that were amongst the lowest previously reported in the literature with intact animals (Gannon et al., 2008; Kessai et al., 2012). Altogether, we believe the most parsimonious interpretation of the available evidence is that the 5-HT\textsubscript{5A} receptor plays a modulatory role in the M-cell.

**Effects of SB-6995551 on startle and PPI**

As PPI is traditionally quantified in behavior, our results could be interpreted as a drug induced enhancement of PPI, attributable to increased activity in prepulse inhibitory circuit/s. Our electrophysiological analysis of PPI at the level of the M-cell (Figs. 3,4), however, revealed that the relative magnitude of PPI was unchanged by the application of the 5-HT\textsubscript{5A} antagonist. Instead, our findings suggest that the antagonist induced a tonic inhibition in the startle circuit that was superimposed upon the inhibition evoked by prepulses. In other words, although each of these separate events individually reduced M-cell input resistance, it is their concerted action that effectively pushes the M-cell out of threshold range and consequently reduces startle rate close to zero, manifested as an apparent enhancement of PPI. These separate effects are not easily distinguished at the behavioral level, although the convergence in the magnitude of PPI at
different ISIs following drug application (Fig. 1B), (i.e., the elimination of ISI dependencies) can be seen as an indicator of a drug-induced generalized reduction in excitability. This interpretation was further substantiated in a follow up experiments that directly showed an attenuation in startle rate in non-PPI trials (Fig. 1C).

Importantly, these findings demonstrate that intrinsic properties of the startle circuit can influence the emergence of PPI at the behavioral level. The intrinsic excitability of the startle circuit and the extrinsic mechanisms that produce PPI are commonly interpreted as independent, but our findings are not the first evidence suggesting otherwise. Schicatano et al. (2000) reported that changes in reflex excitability associated with Parkinson’s disease effectively modulated PPI of the eye blink reflex; similarly, in a rodent model, Blumenthal (1997) found that habituation of the startle reflex modulated PPI.

On the other hand, extrinsic regulation of startle excitability during PPI by midbrain (and forebrain) circuits is well-characterized anatomically (Koch & Schnitzler, 1997; Koch, 1999; Fendt et al., 2001; Yeomans et al., 2006) and physiologically (e.g., Bosch & Schmid, 2006, 2008; Yeomans et al., 2010). A consistent finding that has emerged from studies in ex vivo rodent preparations is that different neurotransmitters and receptor systems can be linked to discrete components of the PPI time course (Bosch & Schmid, 2006, 2008; Yeomans et al., 2010). Similarly, in vivo studies in the M-cell system characterized a time-specific disruption of auditory PPI caused by activation of dopamine receptors (Medan & Preuss, 2011). The present study shows a phasic post-synaptic inhibition that is likely activated by descending (extrinsic) PPI circuits. This phasic inhibition adds to the intrinsic inhibitory tone of the startle circuit. Importantly, it shows that such linear interactions at the synaptic level (Fig 4C) can produce apparently supralinear behavioral changes (e.g., Fig 1A black vs. gray lines), particularly in an
all-or-none startle system such as the M-cell where a single action potential initiates the behavioral response. We believe these results provide a new perspective to resolve an apparent controversy in the field regarding the significance of intrinsic and extrinsic inhibitory mechanism underlying PPI (Blumenthal, 1997; Schicatano et al., 2000; Sandner & Canal, 2007).

Moreover, taken together these findings broadly fit the notion that dopaminergic modulation regulates the time-course and magnitude of PPI (Medan & Preuss, 2011) while 5-HT regulates tonic excitability. This conception may be relevant to the generalized serotonin-dopamine hypothesis that has been advanced to conceptualize neurotransmitter interactions contributing to schizophrenia, and the associated deficits in PPI common to schizophrenic populations (Parwani et al., 2000; Braff, Geyer, & Swerdlow, 2001; Braff, 2010). More generally, our findings also yield a notable tool for future studies of PPI. Specifically, the convergence of PPI-magnitude at different ISIs may indicate, as we found, that modulation of PPI is attributable to a non-specific modulation of startle excitability. Our results emphasize the importance of studying the emergence of PPI on multiple levels, and the M-cell system provides an appropriate model system for such studies.

Acknowledgements

We thank R.L. Gannon for providing the sample of A-843277 that was used in these experiments. We thank S. Deitrick, J.A. Ficek Torres, and N. Joseph for help with the behavioral experiments, and members of the Preuss laboratory for discussion. We also thank the reviewers for constructive feedback and suggestions for improvement, and Drs. H. P. Zeigler and K. Khodakhah for critical reading of this manuscript. This work was supported by National Science Foundation Grants (IOS 0946637, IOS 11471172) and by a grant from the Professional Staff
Congress (PSC)-CUNY Research Award Program, with additional support from Hunter College and the Graduate Center, CUNY. The authors declare no competing financial interests. V. Medan is currently affiliated with Universidad de Buenos Aires, Buenos Aires, Argentina.
Figure Legends

Figure 2.1. The 5-HT$_{5A}$ antagonist SB-699551 reduces startle rate during prepulse inhibition.

A. Mean startle rates (± SEM, $n = 10$) at different drug dosages (x-axis) to startling sound stimuli with no prepulse (black) and with preceding acoustic prepulse stimuli at two inter-stimulus intervals (ISIs) (gray lines). Different letters and numbers (e.g., A,1, or B,2) indicate significant differences among drug (letters) and stimulus (numbers) conditions, respectively (post-hoc Tukey’s HSD; $p < 0.05$).

B. Plots of the calculated mean PPI effect for ISI$_{50}$ (dark gray) and ISI$_{500}$ (light gray) across dosages (± SEM, $n = 10$). Note that the PPI effect was calculated from the data shown in (A).

C. Mean startle rates (± SEM) in response to pulse-only (no prepulse) acoustic stimuli for naïve subjects in saline ($n = 9$, black line) and SB-699551 ($n = 9$, gray line) treatment conditions for three different startle stimulus intensities (X-axis, dB re 1 μPa in water). Note there was no significant difference across stimulus intensities but treatment conditions (black vs blue lines) were significantly different (GLMM, $p = 0.0007$).

Figure 2.2. 5-HT$_{5A}$ antagonists attenuate the M-cell synaptic sound response.

A. Exemplar traces (KAc electrodes) showing sound-evoked post-synaptic potentials (PSPs) recorded in the M-cell before (black) and after (gray) treatment with the 5-HT$_{5A}$ antagonist SB-699551. Lower trace indicates sound stimuli (200 Hz, pips at 147 dB relative to 1 μPa in water).

B. Plots of mean peak amplitudes of sound-evoked PSPs (± SEM, $n = 14$) for control and SB-699551 treatment. Paired t-test, $p = 0.0011$. 

C. Exemplar traces, as in A (KAc electrodes), but here subjects were treated with the 5-HT\textsubscript{5A} antagonist \textit{A-843277} (10 mg/kg b.w.). Black trace shows control conditions and gray trace shows sound-responses following treatment with \textit{A-843277}.

D. Plots of mean peak amplitudes of sound-evoked PSPs (± SEM, \(n = 7\)) for control and \textit{A-843277} treatment conditions. Paired-t, \(p = 0.03\). Note both antagonists produced similar effects.

Figure 2.3. Convergence of discrete inhibitory mechanisms

A. Exemplar traces (KAc electrodes) showing the sound-evoked post-synaptic potentials (PSPs) with no prepulse (black) and with prepulse (ISI\textsubscript{50}, red) in drug-control (left traces) and in \textit{SB-699551} conditions (right traces). The sound stimulus was identical in all traces (200 Hz, pips at 147 dB relative to 1 μPa in water). Note that prepulse-evoked inhibition (indicated in brackets) is similar in control and drug conditions, but is superimposed (indicated by double-arrowed line) on a tonic inhibition after treatment with \textit{SB-699551}.

B. Plots of mean synaptic PPI effect (± SEM, \(n = 9\)) (see 3A) at varying inter-stimulus intervals in control (black bars) and \textit{SB-699551} (blue bars) treatment conditions. ISI is a significant determinant of PPI intensity (2-Way RM-ANOVA, \(p < 0.0001\)), but \textit{SB-699551} caused no significant change in the PPI effect itself (\(p = 0.3367\)), nor was there any interaction between drug and ISI conditions (\(p = 0.6270\)).

C. Plots of mean synaptic PPI effect (± SEM, \(n = 7\)), as in Fig B, at varying inter-stimulus intervals in control (open bars) and \textit{A-843277}(green bars) treatment conditions. As in Fig B, ISI is a significant determinant of PPI intensity (2-way RM-ANOVA, \(p < 0.0001\)), but there was no significant change in PPI intensity due to drug treatment (\(p = 0.6224\)) or the interaction of ISI and drug treatment (\(p = 0.9208\)).
Figure 2.4. 5-HT$_{5A}$ antagonist evokes post-synaptic reduction in M-cell input resistance

A. At top, voltage traces recorded during current injection experiments in control conditions with no prepulse (black), with 50 ms prepulse (red), and with 50 ms prepulse after treatment with SB-699551 (blue). Middle trace shows sound stimulus used for prepulse. Lower trace shows time course of current injection.

B. Voltage/current (V/I) traces show M-cell depolarization during current injection with no prepulse (black) and 50 ms prepulse (red) in control conditions, and no prepulse (blue) and with 50 ms prepulse (green) after SB-699551 treatment. The depicted range shows depolarization from RMP to action potential threshold (dashed line). Slope 1 and slope 2 (grey boxes) indicate where linear fits were applied to V/I plots to quantify slope (input resistance).

C. Plots of mean input resistance (± SEM) for the initial-state (Slope 1) of M-cell depolarization (control $n = 12$, black line; SB-699551, $n = 9$, blue line) with no prepulse and prepulses at varying ISIs in drug-control and SB-699551-treatment conditions. Letters and numbers indicate significant differences (GLMM, post-hoc, $\alpha=0.05$) between stimulus conditions (numbers) and drug treatment (letters), respectively.

D. Plots of mean input resistance (± SEM) for the depolarized-state M-cell (control $n = 12$, black line; SB-699551, $n = 9$, blue line) during PPI and drug-treatment conditions. Letters and numbers (e.g., A1, B2) indicate significant differences (post-hoc, $\alpha=0.05$) between stimulus (numbers) and treatment (letters) conditions as in (C).
Figure 2.5. 5-HT$_{5A}$ antagonists reduce the amplitude of M-cell action potentials

A. Traces show an antidromically-evoked action potential (AP) recorded in the soma in control (black trace) and SB-699551 (gray trace) treatment conditions. Recordings were made with KAc electrodes. Note the recording electrode was not moved before/after drug application.

B. Plots of mean peak depolarization (± SEM) of APs in control and SB-699551 treatment conditions ($n = 6$, paired-t, $p = 0.0154$).

C. Traces show antidromically-evoked APs in control conditions (black trace) and after treatment with A-843277 (gray trace). Recordings made with KAc electrodes. Note the recording electrode was not moved before/after drug application.

D. Plots of mean peak depolarization (± SEM) of APs in control and A-843277 treatment conditions ($n = 5$, paired-t, $p = 0.0015$). Recordings made with KAc electrodes.

Figure 2.6. 5-HT$_{5A}$ antagonist increases Cl$^-$ conductance

A. Sample traces showing M-cell sound-responses recorded with KCl$^-$ (5M) electrodes. Note that an enhancement of depolarization in these conditions reflects an increase in outward Cl$^-$ conductance due to Cl$^-$ loading of the cell (see Results). Black trace shows a recording in control conditions, whereas the gray trace shows a recording after treatment with SB-699551. Dotted line indicates 5 ms latency following stimulus onset; to the left, the sound response can be interpreted as a pure EPSP (i.e., only excitatory components), whereas to the right of the line the response is a mixed excitatory/inhibitory PSP.

B. Plots of mean peak depolarization (± SEM, $n = 9$) (KCl$^-$ electrodes) of the EPSP; that is, the sound response within < 5 ms of stimulus onset. Black bar plots control conditions and gray bar indicates measures after treatment with SB-699551.
C. Plots of mean peak depolarization (± SEM, n = 9) (KCl\textsuperscript{–} electrodes) of the sound-evoked PSP > 5 ms following stimulus onset. Black bar plots control conditions and gray bar indicates measures after treatment with \textit{SB-699551}.

D. Plots of mean percentage change (± SEM, n = 9) in sound-evoked depolarization (KCl\textsuperscript{–} electrodes) during the initial (EPSP) and latter (PSP) components of the sound response after treatment with \textit{SB-699551}. Note that there was no significant difference in the drug’s effect across the time-course of the response (paired t-test, \( p = 0.3825 \)).
FIGURE 2.1. 5-HT$_{5A}$ ANTAGONIST REDUCES STARTLE RATE DURING PREPULSE INHIBITION

A.

B.

C.
FIGURE 2.2. 5-HT<sub>5A</sub> ANTAGONISTS ATTENUATE THE M-CELL SYNAPTIC SOUND RESPONSE

A. 5 ms
2 mV
200 Hz 147 dB

B. Peak PSP amplitude (mV)

C. 5 ms
2 mV
200 Hz 147 dB

D. Peak PSP amplitude (mV)
FIGURE 2.3. CONVERGENCE OF DISCRETE INHIBITORY MECHANISMS
FIGURE 2.4. 5-HT\textsubscript{5A} ANTAGONIST EVOKE POST-SYNAPTIC REDUCTION IN M-CELL INPUT RESISTANCE
FIGURE 2.5. 5-HT<sub>5A</sub> ANTAGONISTS REDUCE THE AMPLITUDE OF M-CELL ACTION POTENTIALS
FIGURE 2.6. 5-HT$_{5A}$ ANTAGONIST INCREASES CL- CONDUCTANCE

A.

B.

C.

D.

<table>
<thead>
<tr>
<th>Treatment effect</th>
<th>EPSP</th>
<th>PSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>SB-699551</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

** indicates statistical significance.
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CHAPTER 3

previously submitted for publication as

"Glycine and GABA_A receptors mediate tonic and phasic inhibitory processes that contribute to prepulse inhibition in the goldfish startle network "

by

Curtin, P.C.P. & Preuss, T.
ABSTRACT

Prepulse inhibition (PPI) is understood as an inhibitory process that attenuates sensory flow during early stages (20-1000 ms) of information processing. Here, we applied in vivo electrophysiology and pharmacology to determine if prepulse inhibition (PPI) is mediated by glycine receptors (GlyRs) and/or GABA$_A$ receptors (GABA$_A$Rs) in the goldfish auditory startle circuit. Specifically, we used selective antagonists to dissect the contributions of target receptors on sound-evoked postsynaptic potentials (PSPs) recorded in the neurons that initiate startle, the Mauthner-cells (M-cells). We found that strychnine, a GlyR antagonist, disrupted a fast-activated (5 ms) and rapidly (< 50 ms) decaying (feed-forward) inhibitory process that disrupts PPI at 20 ms prepulse/pulse inter-stimulus intervals (ISI). Additionally we observed increases of the evoked postsynaptic potential (PSP) peak amplitude (+87.43 ± 21.53%; N=9) and duration (+204 ± 48.91%, N=9). In contrast, treatment with bicuculline, a GABA$_A$R antagonist, caused a general reduction in PPI across all tested ISIs (20-500 ms), essentially eliminating PPI at ISIs from 20-100 ms. Bicuculline also increased PSP peak amplitude (+133.8 ± 10.3%, N=5) and PSP duration (+284.95 ± 65.64%, N=5). Treatment with either antagonist also tonically increased post-synaptic excitability in the M-cells, reflected by an increase in the magnitude of antidromically-evoked action potentials (APs) by 15.07 ± 3.21%, N=7 and 16.23 ± 7.08%, N=5 for strychnine and bicuculline, respectively. These results suggest that GABA$_A$Rs and GlyRs are functionally segregated to short- and longer-lasting sound-evoked (phasic) inhibitory processes that contribute to PPI, with the mediation of tonic inhibition by both receptor systems being critical for gain control within the M-cell startle circuit.
INTRODUCTION

Startle is a rapid, massive contraction of facial and skeletal muscles that is triggered by the onset of intense and/or abrupt visual, auditory, or tactile stimuli. Startle is thought to function as a protective mechanism that minimizes impacts to vulnerable areas (e.g., the eyes) and/or facilitates collision avoidance or escape (Eaton et al., 1981; Bennett, 1984; Koch, 1999; Yeomans et al., 2002; Yeomans et al., 2006). The startle response is relatively stereotyped and predictably elicited, but is also the target of multiple modulatory mechanisms that enable dynamic adjustments to stimulus-response parameters in varying sensory and behavioral contexts. Prepulse inhibition (PPI) is a central inhibitory process that contributes to startle plasticity by briefly (20-1000 ms) reducing startle excitability while non-startling stimuli (prepulses) are processed (Graham, 1975; Hoffman & Ison, 1980; Koch, 1999). This sensorimotor gating mechanism is thought to preserve sensory processing and action selection by midbrain and forebrain processes that are activated by prepulses and would be disrupted by the subsequent initiation of startle (Graham, 1975). Consistent with this notion, information-processing disorders, including schizophrenia, Tourette’s syndrome, and obsessive-compulsive disorder are associated with diminished or disordered PPI (Swerdlow, 1992; Parwani et al., 2000; Braff et al., 2001). Consequently, identifying the neural processes underlying PPI presents an important goal for basic and translational neuroscience.

Anatomical and pharmacological studies indicate that PPI is mediated by multiple midbrain and forebrain circuits that modulate the time-course of inhibition in the startle circuit via multiple neurotransmitter systems. In mammals, startle is initiated by the firing of a population of giant hindbrain neurons in the ventrocaudal pontine reticular formation (PnC) (Koch, 1999; Fendt et al., 1999; Geis & Schmid, 2011; Yeomans et al., 2002, 2006). Anatomical
studies indicate that PPI is produced by the excitation of midbrain circuits that project inhibitory terminals to PnC neurons; these include nuclei in the inferior colliculus, pedunculopontine tegmental nucleus, superior colliculus, and laterodorsal tegmental nucleus (Koch & Schnitzler, 1997; Fendt et al., 1999; Yeomans et al., 2010). Pharmacological studies in rodents emphasize that these inputs mediate PPI by multiple neurotransmitters that contribute discrete components toward the time-course of inhibition. Muscarinic receptors, for example, contribute to inhibition mediated at longer intervals, i.e., 100-1000 ms from prepulse onsets (Jones & Shannon, 2000; Ukai et al., 2004). GABA receptors are also critical mediators of PPI, with GABA_ARs contributing during the peak inhibitory response, and GABA_B receptors adding to the longer lasting inhibition mediated by muscarinic receptors (Yeomans et al., 2010). These lines of evidence derive from behavioral pharmacology studies in adult animals, or ex vivo slice preparations derived from immature tissue (e.g., Yeomans et al., 2010; Geis & Schmid, 2011).

The Mauthner-cell (M-cell) circuit in teleost fish presents an alternative model system for studying PPI and startle plasticity that is accessible to in vivo electrophysiology. The M-cells are a pair of large reticulospinal neurons, bilaterally opposed, that integrate excitatory and inhibitory inputs elicited by visual, auditory, and/or tactile stimulation (reviewed in Eaton et al., 2001; Korn & Faber, 2005). A single action-potential (AP) in either M-cell is sufficient to trigger a startle response (the C-start), and inhibition of APs is sufficient to prevent startle; thus, the M-cells are the decision-making sensorimotor interface for startle (Eaton et al., 1981). The M-cells are the focus of two well-characterized inhibitory networks that control startle excitability; these being, a collateral (feedback) inhibitory network that is bilaterally activated by cranial relay neurons when the M-cell fires, and a commissural (feed-forward) inhibitory network activated by parallel VIIIth nerve afferents to counter sound-evoked excitation in the M-cell and thereby regulate
startle response properties (Eaton et al., 2001; Korn & Faber, 2005). Glycine receptor (GlyR) antagonists disrupt feed-forward and feedback inhibition (Faber & Korn, 1978; Faber, 1987; Korn & Faber, 2005), but GABA$_A$ receptors (GABA$_A$Rs) also mediate M-cell excitability and are thought to be involved in auditory processing (Diamond, 1971). These inhibitory networks mediate two distinct types of processes: **phasic inhibition**, that includes transiently activated or stimulus-dependent inhibitory inputs, including the feed-forward and feedback inhibitory processes described, and **tonic inhibition**, that is, persistent inhibitory synaptic noise that arises from spontaneous quantal neurotransmitter release and intermittent firing at inhibitory synaptic terminals on the M-cell (Faber et al., 1989; Hatta et al., 2001; Marti et al., 2008).

A growing number of studies indicate that PPI in the M-cell system is modulated by multiple pre- and post-synaptic mechanisms. Neumeister et al. (2008) showed that PPI in goldfish is mediated by post-synaptic conductance changes activated in the M-cells. Burgess & Granato (2007) showed that dopaminergic agonists disrupt behavioral PPI in zebrafish, while Medan & Preuss (2011) showed dopaminergic mechanisms modulating time-specific components of PPI in the M-cell membrane, likely reflecting control of upstream networks involved in PPI. Furthermore, Curtin et al. (2013) showed that 5-HT$_{5A}$ receptors modulate the excitability of goldfish M-cells, and linked these effects to changes in startle plasticity. Given these advances in our understanding of neuromodulatory processes contributing to startle plasticity, here we investigated the signaling mechanisms directly mediating PPI at the level of the M-cell.

This study focused on the roles of GlyRs and GABA$_A$Rs in the mediation of PPI in the M-cells, the decision-making neurons of the goldfish auditory startle circuit. We targeted these receptor systems because they are densely expressed in the M-cell membrane (Triller et al.,
1985; Seitanidou et al., 1988; Petrov et al., 1991; Lee et al., 1993; Sue et al., 1994) and their involvement in a diverse array of tonic and phasic inhibitory processes is well characterized (discussed above; see also Korn & Faber, 2005). We thus sought to identify the effector mechanisms for auditory PPI in the context of co-activated tonic and phasic inhibitory processes that are typically inaccessible in other model systems. Our findings indicate that GABA_ARs directly mediate the peak inhibitory components of PPI, while GlyRs indirectly contribute to the onset of PPI by the mediation of a feed-forward inhibitory process that overlaps with the earliest components of PPI.

METHODS

Subjects

Sixteen common goldfish (*Carassius auratus*) of either sex were used in these experiments. Adult fish 7-13 cm in standard body length were purchased from Hunting Creek Fisheries (Thurmont, MD). Fish were socially housed, with 5-6 fish per 60 l aquaria, in recirculating conditioned water (7.5 pH; 335 µS; 18°C) with a 12:12 light/dark photoperiod. Animals were housed and treated in accord with protocols established by the Institutional Animal Care and Use Committee (IACUC) of Hunter College, City University of New York.

Electrophysiology

The surgical techniques and methods used for electrophysiological recordings were described previously (Medan & Preuss, 2011; Curtin et al., 2013). Fish were immersed in ice water for 10-15 minutes to induce immobility, then placed in a recording chamber. Two steel pins were placed on each side of the head to stabilize the fish and a tube was placed in the mouth to provide recirculating aerated water containing the general anesthetic, MS-222 (20 mg/l). This
anesthetic dosage was chosen because prior studies have shown it does not interfere with auditory processing (Palmer & Mensinger, 2004; Cordova and Braun, 2007). Recirculating water in the recording chamber was initially near 0°C (when fish were removed from ice to begin procedures) but was gradually heated to 18°C before recordings were taken. Water conditions in all other measures were consistent with conditions in holding tanks.

A small lateral incision was made to expose the spinal cord at the caudal midbody, and a bipolar electrode was placed on the unopened spine to transmit low intensity (5-10 V) electrical stimulation generated by an isolated stimulator (Digitimer, Ltd, Wewyn Garden City, UK). A visible muscular contraction (twitch) was elicited with spinal stimulation to confirm proper placement of the spinal electrode, then d-tubocurarine (1 µg/g b.w.; Abbot, Chicago, IL) was administered intramuscularly. When the twitch response was abolished, typically within 0-3 minutes of injecting the tubocurarine, a craniotomy was performed to expose the medulla for microelectrode placement and recordings.

The M-cell was localized by a characteristic negative extracellular potential (15-20 mV) generated in the axon cap during antidromic stimulation, which provides an unambiguous indicator of electrodes' placement relative to the soma and axon cap (Faber & Korn, 1978). In these experiments, M-cells were impaled somatically with sharp electrodes (3-8 MΩ) filled with 5 M potassium acetate (KAc). An Axoprobe-1A amplifier (Molecular Devices, Foster City, CA) in current-clamp mode measured intracellular potentials, and a data acquisition card (PCI-E, National Instruments, Austin, TX) sampling at 25 KHz in a Macintosh G5 collected and recorded data.
Pharmacology

Our experimental design required the use of GlyR and GABA$\alpha$R antagonists. We chose to use strychnine (Sigma-Aldrich), the classical GlyR antagonist, and bicuculline (Tocris Biosciences), the classical GABA$\alpha$R antagonist, because both drugs have long and well-documented histories of use in the M-cell system. Drugs were dissolved in a 500 µL solution of physiological saline (in mM: 124.0 NaCl, 5.1 KCl, 2.8 NaH$_2$PO$_4$, 0.9 MgSO$_4$, 1.6 CaCl$_2$, 5.6 glucose, and 20.0 HEPES, buffered to pH 7.2) and superfused directly to the exposed medulla, as in past studies (Diamond, 1973; Faber & Korn, 1991; Hatta et al., 2001). This route of administration was chosen over others because it allowed direct reference to past studies and published dose-response curves. Given those studies, strychnine solutions (5 mM) were prepared as per Diamond (1973), and bicuculline solutions (10 mM) were made as per Hatta et al (2001). Physiological measures confirmed these concentrations were sufficient to achieve clear experimental effects (see Results). All solutions were prepared on the day of experiments and were warmed to the temperature of the fish before application.

 Stimulus protocols

Sound (pulse) stimuli were used to activate orthodromic inputs to the M-cells with or without preceding sound stimuli (prepulse), the latter at multiple inter-stimulus intervals (ISIs) ranging between 20-500 ms. Sound stimuli were 200 Hz single-cycle "pips" produced at 80 dB re: 20 µPa in air. This stimulus intensity was chosen to elicit sub-threshold responses in the M-cell because PPI is by definition elicited by sub-threshold sounds, and the use of an identical conditioning (prepulse) and test (pulse) stimuli allows a within-subjects comparison of prepulse-pulse relationships on a trial-by-trial basis that is less sensitive to changes in baseline excitability. Stimuli were generated by a function generator (Agilent 33210A, Santa Clara,
CA), and output to a shielded subwoofer (SA-WN250, Sony) placed 30 cm from the recording chamber. A microphone placed 10 cm above the fish's head recorded acoustic waveforms and encoded these in parallel with intracellular recordings. A hydrophone (SQ01, Sensor, Collingwood, ON, Canada) was also used for sound calibration but was removed during experiments. In testing conditions that measured prepulse inhibition (PPI), sound stimuli were produced at 6 inter-stimulus intervals (ISIs: 20, 50, 75, 150, 300, 500 ms) measured from the onset of each stimulus.

*Waveform analysis of evoked synaptic responses*

Intracellular recordings were analyzed offline with custom and commercial software (Igor Pro; Wavemetrics, Lake Oswego, OR). To analyze the contribution of distinct inhibitory networks on sensory processing, we measured the peak depolarization and duration of sound-evoked post-synaptic potentials (PSPs) for comparison across treatment conditions. PSP duration was defined as the time between peak depolarization and the decline of excitation to 37% of peak, as per the calculation of tau (τ). We also compared peak depolarizations of early and later components of sound-evoked PSPs in order to compare how consistently and/or selectively each treatment condition acted on discrete temporal components of the sound response (see Results for details).

PPI was measured by comparing the peak depolarization evoked by an initial sound stimulus (prepulse) presented 20-500 ms prior to an identical stimulus (pulse), as in Lingenhohl & Friauf (1994), Neumeister et al. (2008), Medan & Preuss (2011), and Curtin et al. (2013). This method allows synaptic PPI effects to be quantified according to a commonly used formula 100 - (PSP_{PULSE}/PSP_{PREPULSE} * 100), i.e., as the normalized percentage change of the pulse response by a prepulse. Thus, higher PPI values reflect greater inhibition. Importantly, this
relative measure allows the consistency of prepulse-pulse relationships to be tested across
treatment conditions independently of possible changes in M-cell excitability (Medan & Preuss,
2011; Curtin et al., 2013).

Statistical Analyses

Data were analyzed with JMP 10.0.0 (SAS) or Graphpad v5.0 (Prism). All data reported
in figures and text reflect mean values and error bars illustrate SEM. All datasets were tested
with the D'Agostino & Pearson Omnibus or K-S tests to confirm assumptions of normality were
met for parametric statistical tests. In almost all cases, simple paired-\(t\) analyses were appropriate
for the within-subjects design of these experiments. The exception to this was the analysis of the
pharmacological effects on PPI, which by design considered three potential main effects (drug,
ISI, and drug X ISI interaction), include two dimensions of repeated measures (ISI and drug
treatment), and two axes that are better measured on continuous rather than categorical scales.
Given those parameters, general linear mixed-models (GLMM) were used for those analyses. In
these models subjects were treated as random effects (i.e., repeated measures) while ISI and
drug-treatment were treated as fixed effects, and the dependent variable was the magnitude of
PPI. Student’s t-tests were used for post-hoc analysis where appropriate.

RESULTS

Glycine receptors mediate inhibition contributing to the onset of PPI

These experiments tested if treatment with strychnine, a glycine receptor (GlyR)
antagonist, affects auditory prepulse inhibition (PPI) in the Mauthner cells (M-cells), the
decision-making neurons of the goldfish startle circuit. Figure 1 A-C shows somatically recorded
PSPs in response to prepulse/pulse sound stimuli (identical subthreshold 200 Hz "pips" at 80 dB;
see methods) for ISIs ranging from 20-75 ms in control (black traces) and strychnine (red traces) treatment conditions. The results show an overall attenuation of the PSP to the secondary stimulus (pulse) when compared to the PSP evoked by the lead stimulus (prepulse) in control conditions, in short, synaptic PPI (Fig. 1, A1-C1). After application of strychnine synaptic PPI magnitude remained largely unchanged for all but the shortest ISI, despite the fact that the drug changed the overall PSP waveform (Fig. 1 A-C; black vs. red traces, see also below). Figure 1D plots the quantification of PPI across control (black line) and strychnine (red line) treatment conditions. Although PPI remained robustly intact after treatment with the GlyR antagonist for all ISIs > 20 ms (Fig.1B-C, black vs. red traces; Fig.1D, black vs. red lines), we observed an ISI-specific reduction of PPI at the shortest ISI tested (20 ms; Fig. 1A, black vs. red traces).

Supporting these results, we found that strychnine had no significant main effect on the magnitude of PPI (F(1,86.87)=2.98, P=0.088, N=9), but our analysis identified a significant ISI X strychnine interaction (F(6,83.68)=5.7276, P<0.0001, N=9). Post-hoc tests (Tukey's HSD) confirmed this effect was due to an ISI-specific reduction in the PPI effect at the 20 ms ISI (P<0.0001). These findings indicate that GlyRs mediate inhibition that contributes to PPI for as long as 20 ms, but this glycinergic component decays within 50 ms of the onset of prepulse stimuli.

_Glycine receptors shape auditory processing via multiple mechanisms_

As noted above, strychnine also affected the waveform of sound evoked M-cell PSPs, i.e., auditory processing. In order to analyze this more directly, we tested the effects of strychnine on M-cell sound-evoked PSPs evoked by a single sound pip (i.e., independent of PPI). Figure 2A shows sample sound responses recorded in control (black trace) and strychnine (red trace) treatment conditions. The overall peak of PSPs increased on average by 87.43 ± 21.53%
(N=9; see Fig. 2B) after treatment with strychnine. We confirmed this increase was statistically significant by paired-t test (t(8)=6.08, P=0.0003, N=9). The duration of sound-evoked PSPs (defined as per the calculation of τ; see methods) was also significantly greater (204.0 ± 48.91% increase; t(8)=6.11, P=0.0003, N=9; Fig. 2C) after treatment with strychnine.

We next analyzed how the GlyR antagonist acted on different components of the overall sound response. The M-cell PSP reflects the integration of multiple excitatory and inhibitory inputs activated by primary auditory afferences. These include electrotonic and chemical excitation via mixed VIIIth nerve synapses at the M-cell lateral dendrite (Furshpan, 1964; Faber & Korn, 1975; Lin & Faber, 1988; Curti & Pereda, 2004). This excitation is counteracted by chemical inhibition (onset of about 5 ms relative to stimulus onset; see Preuss & Faber, 2003; Medan & Preuss, 2011) that peaks at 10-12 ms, mediated by a feed-forward network that is also activated by VIIIth nerve afferents (Korn & Faber, 2005; Szabo et al., 2006; Weiss et al., 2008; see also Introduction). In other words, the monosynaptic excitatory pathway and disynaptic inhibitory pathway allow a brief interval within the first 5 ms of the postsynaptic response when sound-evoked depolarization reflects largely excitatory inputs (i.e., an EPSP), whereas latter components of the sound-response represent the integration of excitatory and inhibitory inputs (i.e., a mixed PSP). Figure 2A (inset) shows the onset of the sound-response in an expanded time scale to emphasizes the effects of strychnine (compare black vs. red traces) on the EPSP (light gray area, 0-5 ms from stimulus onset) and the PSP (dark grey area, > 5 ms from stimulus onset). We found that strychnine caused a relatively mild enhancement (14.33 ± 7.2% increase, N=9) of peak depolarization during the initial EPSP, but a significantly greater enhancement of excitation during the mixed-PSP component of the response(116.5 ± 18.73% increase; t(8)=8.54, P=0.0034; see Fig. 2D). This time course suggest a minor effect of strychnine on presynaptic
excitatory pathways and/or M-cell tonic excitability, but is consistent with a drug-induced disruption of feed-forward inhibition in the M-cell.

**GABAA receptors mediate peak inhibitory components of PPI**

We applied the same experimental approach as described above to test the effects of bicuculline (10 mM superfusion), a GABA_{A}R antagonist, on auditory prepulse inhibition (PPI) at the level of the M-cells. Figure 3A-C shows sample intracellular M-cell recordings at three ISIs before (black traces) and after (blue traces) bicuculline treatment. Similar to the effects we observed with strychnine, we found that the GABA_{A}R antagonist caused prominent changes in the amplitude and duration of sound evoked M-cell PSPs; these effects are analyzed in detail below. In contrast to the effects of strychnine, however, we found that treatment with bicuculline severely disrupted PPI. This was apparent in the overall reduction in PPI over the entire range of ISIs tested (see Fig. 3D), and in ISI-specific effects where the reduction of PPI was most pronounced. Figure 3A-C shows traces at 20, 50, and 75 ms ISIs where the disruption of PPI was most prominent, i.e., both prepulse and pulse stimuli evoked essentially identical PSPs in drug conditions. In figure 3D, the quantification of PPI is plotted across the range of ISIs tested. Our analysis of these data (GLMM) identified significant main effects of bicuculline treatment (F(1,50.96)=89.3722, P<0.0001, N=5) and the interaction of bicuculline x ISI (F(6,24)=5.519, P=0.001, n=5); that is, bicuculline causes both general and ISI-specific disruptions of PPI. Post-hoc analyses found the latter effect was attributable to ISI-specific reductions in PPI intensity at ISIs of 20 ms (P<0.0001), 50 ms (P<0.01), 75 ms (P<0.001), and 100 ms (P<0.01) relative to controls. At longer ISIs, the reductions in PPI in bicuculline conditions relative to controls were not statistically significant (P>0.05); however, this may reflect the generally weak effect of PPI at longer ISIs, generally. In sum, these results indicate that blockade of GABA_{A} receptors yields
general and ISI-specific disruptions of PPI, and the latter effects correspond to the ISIs where PPI effects are typically greatest, 20-100 ms.

**GABA$_A$ receptors mediate a tonic increase in sound-evoked excitation**

In these experiments, we tested the effects of the GABA$_A$R antagonist, bicuculline on sound-evoked depolarization independently of PPI (without prepulses) to determine how GABA$_A$Rs contribute to auditory processing. We again approached our analysis by examining the effect of the GABA$_A$R antagonist on the overall peak depolarization and duration of sound-evoked excitation. Figure 4A shows sound-evoked PSPs recorded in control (black trace) and bicuculline (blue trace) treatment conditions. We found that bicuculline significantly increased the mean overall peak of sound-evoked PSPs by 133.8 ± 10.3% (Fig 4B; t(4)=28.12, P<0.0001, N=5). Similarly, the duration of sound-evoked PSPs increased by 284.95 ± 65.64% in drug conditions (Fig 4C; t(4)=3.07, P= 0.037, N=5).

As in our analysis of GlyR-mediated components of sound-evoked excitation, we also measured the potentially differential effects of the GABA$_A$R antagonist on initial (EPSP) and subsequent components (PSP) of the sound-response (Figure 4A inset, light gray shading vs. dark grey shading). In contrast to strychnine, bicuculline produced large enhancement in both components of the response (EPSP: 90.95% increase; PSP: 164.32% increase); however, bicuculline's effect on the EPSP was not significantly different from the PSP (t(4)=1.116, P=0.327, N=5). The latter result is consistent with a drug induced increase in presynaptic excitation and/or by a decrease in inhibitory tone in the M-cell system which increases the neurons excitability (see below).
Strychnine and bicuculline disrupt tonic inhibition contributing to M-cell excitability

In prior experiments, we reported that GlyR and GABA_A antagonists enhance sound-evoked excitation in the M-cell (Fig. 2 and Fig. 4). Namely, strychnine treatment predominately enhanced later parts of the PSP, i.e., demonstrating a time-dependent effect, whereas bicuculline produced an enhancement of the entire PSP consistent with a tonic change in M-cell excitability. Accordingly, we next tested whether these antagonists mediate tonic excitability in M-cell. Since the M-cells’ soma-dendritic membrane is inexcitable, i.e., does not promote regenerative APs, changes in the magnitude of somatic APs provide a measure of corresponding changes in tonic membrane conductivity (excitability; reviewed in Korn & Faber, 2005; Curtin et al., 2013).

Figure 5A shows sample traces of APs elicited in control (black trace) and strychnine (red trace) treatment conditions. On average, treatment with strychnine increased the peak magnitude of APs by 15.07 ± 3.21% (Figure 5B; paired- t, t(6)=4.314, P=0.005) consistent with a decrease in inhibitory conductance. Importantly, resting membrane potential (RMP_control = -80.6 ± 0.82 mV; RMP_strychnine = -80.9 ± 0.86 mV) was not affected by treatment with strychnine (t(9)=0.4104, P=0.69), indicating the disruption of a shunting inhibition rather than a persistent hyperpolarization.

Similarly, bicuculline treatment increased the magnitude of APs by 16.23 ± 7.08% (Figure 5C, black vs blue traces; Figure 5D, t(4)=3.09, P=0.036, N=5), but had no significant effect on resting membrane potential (RMP_control = -78.6 ± 1.55 mV; RMP_bicuculline = -77.22 ± 1.35 mV), consistent with a shunting inhibitory process.

In sum, these results are consistent with the notion that tonic inhibitory processes regulate M-cell excitability, and these processes are mediated by GlyRs and GABA_ARs.
DISCUSSION

The aim of this study was to determine if glycine receptors (GlyRs) and/or GABA_\text{A} receptors (GABA_\text{A}Rs) mediate auditory prepulse inhibition in the decision-making neurons that initiate startle, the Mauthner-cells (M-cells). Our primary findings indicate that GABA_\text{A}Rs function as effector mechanisms mediating the onset and peak effect of PPI, corresponding to inter-stimulus-intervals (ISIs) ranging from 20-100 ms. GlyRs, in contrast, are primarily involved in the mediation of fast-onset feed-forward (sound-evoked) inhibitory processes that rapidly decay but overlap and contribute to the onset of PPI (20 ms). Independent of these distinct roles in sound-evoked inhibition, GABA_\text{A}Rs and GlyRs both act as mediators of tonic inhibitory processes that modulate M-cell excitability, causing corresponding shifts in the magnitude and duration of sound-evoked excitation. We discuss the evidence supporting these conclusions below, particularly in reference to past studies of tonic and phasic inhibitory processes within the M-cell auditory startle circuit, and comparable studies of PPI in mammalian (rodent) preparations.

GABA_\text{A} receptors mediate tonic excitability and sensorimotor gating

This study tested the effects of bicuculline, a selective GABA_\text{A}R antagonist, on auditory processing, sensorimotor gating (PPI), and post-synaptic excitability in the M-cells. Our results emphasize that GABA_\text{A}Rs mediate multiple inhibitory processes contributing to startle plasticity. Here we distinguish between a sound-evoked inhibitory process that mediates sensorimotor gating (PPI), and a tonic inhibitory process that modulates M-cell excitability. Importantly, although the latter tonic effect reflects a persistent process that is not evoked by auditory stimuli, the functional consequences of increasing post-synaptic excitability contributed to prominent changes in sound-evoked excitation.
We found that inhibition elicited by auditory prepulses was unambiguously abolished or reduced after treatment with bicuculline. Specifically, we found that PPI was reduced at all ISIs, but the effect of the GABA<sub>A</sub>R antagonist was most prominent at ISIs from 20 - 100 ms, corresponding to the time-course of peak inhibition, i.e., where PPI is strongest. We interpret the failure of PPI after pharmacological blockade as direct, in vivo evidence that GABA<sub>A</sub>Rs function as critical effector mechanisms mediating PPI at the level of the M-cells. These effects contrast sharply with the effect of the GlyR antagonist on PPI, which disrupted PPI only at a single ISI, confirming that bicuculline acted selectively on GABA<sub>A</sub>Rs, rather than on a common mechanism (i.e., GlyRs), that are also expressed in the M-cells.

Independent of bicuculline's effects on PPI, in experiments testing the antagonist's effects on sound-evoked M-cell post-synaptic potentials (PSPs) without prepulses we found a significant increase in the peak excitation and duration of sound-evoked PSPs. In contrast to our findings in parallel experiments with GlyR antagonists, the effects of bicuculline on sound-evoked excitation were not time-dependent relative to stimulus-onset. That is, sound-evoked excitation was equally enhanced in the earliest components of the response (EPSP), which is purely excitatory, and in the latter components of the response (PSP), which also include inhibitory inputs. The lack of time-dependency in bicuculline's effects on the sound-response is consistent with the disruption of a tonic (i.e., not stimulus-evoked) inhibitory process. In experiments testing the effects of bicuculline on antidromically-evoked action-potentials, we confirmed that these effects correspond with increased M-cell excitability, consistent with a post-synaptic action of the drug.

The GABAergic inhibition elicited with auditory prepulses thus matches the parameters expected of PPI; that is, inhibition evoked by weak sensory stimuli (prepulses) that attenuates
subsequent sensory inputs (and the initiation of startle), without affecting the initial response to the prepulse. GABAergic tonic inhibition driven by inhibitory synaptic noise (ISN) was first characterized by Hatta et al. (2001) in the M-cell system, and was shown to modulate M-cell excitability (measured in AP spike heights, as in the present study). Importantly, while our results confirm these effects, our findings additionally characterize the functional significance of tonic GABAergic inhibition in contexts of auditory processing, wherein it modulates sound-evoked excitation, and PPI, which operates independently of tonic processes. These findings are consistent with the conceptualization of tonic inhibitory processes as a sort of post-synaptic gain control that modulates startle sensitivity.

Glycine receptors mediate tonic excitability and feed-forward inhibition

In a parallel series of experiments we tested the effects of strychnine, a GlyR antagonist, on prepulse inhibition (PPI), auditory processing, and M-cell membrane properties. In contrast to the effects of bicuculline, strychnine had no general effect on PPI, but did cause an ISI-specific reduction in PPI effects at the shortest inter-stimulus interval (ISI) tested, 20 ms. PPI was fully recovered and comparable to control conditions within 50 ms and for all longer ISIs. Further, even at the 20 ms ISI, the GlyR antagonist never fully abolished PPI effects, suggesting that the GlyR-dependent component of PPI acts in concert with another inhibitory process at this short latency from prepulse onset. Indeed, in experiments with the GABA_{A}R antagonists we confirmed the onset of prepulse-evoked GABAergic inhibition at this ISI. These findings indicate a sound-evoked glycinergic process that contributes to the onset of PPI but rapidly decays and does not otherwise contribute to PPI effects. Importantly, though strychnine had little effect on PPI, the GlyR antagonist caused prominent changes in M-cell excitability that were superficially similar to the effects of bicuculline. The lack of effect of strychnine on PPI suggests that the disruption
of PPI in bicuculline experiments cannot be attributed to increased excitability in the startle circuit (e.g., as in Curtin et al., 2013).

As with the GABA\textsubscript{A}R antagonist, strychnine treatment significantly increased the peak magnitude and duration of sound-evoked excitation independently of PPI; importantly, some of these effects were time-dependent relative to the onset of the sound stimulus. That is, although strychnine increased the peak magnitude of sound-evoked excitation in the earliest phase of auditory processing (EPSP), prior to the onset of feed-forward inhibition, the enhancement of excitation during the mixed-PSP phase that includes inhibition was dramatically greater. The differential enhancement of the EPSP relative to the PSP reflects the disruption of distinct processes; namely, a tonic inhibition that persistently modulates excitability, and a sound-activated phasic inhibition that significantly influences the magnitude and time-course of the sound response, i.e., the temporal fidelity of auditory processing. In additional experiments we showed that the magnitude of antidromically-evoked APs increased after strychnine treatment, indicating a general increase in post-synaptic excitability consistent with the enhancement of the EPSP. Tonic glycinergic inhibition of the M-cell has been characterized in previous studies (Korn & Faber, 1990; Hatta & Korn, 1999); our results emphasize that tonic inhibition is critical to auditory processing. Below, we link the time-dependent effects of the GlyR antagonist on sound-evoked PSPs and PPI to the parameters of an associated feed-forward inhibitory circuit that is well-characterized in previous studies.

Our results show that feed-forward inhibition and PPI are overlapping phenomena; however, they are functionally distinct and operate over different timescales. Namely, feed-forward inhibition, like PPI, is recruited by weak auditory stimuli; unlike PPI, feed-forward inhibition attenuates sound-evoked excitation, generally, rather than selectively inhibiting
responses to subsequent stimuli. Experiments testing auditory responses without prepulses showed that the onset of sound-evoked glycinergic inhibition occurs during sound-evoked excitation; further, in PPI experiments, we showed that sound-evoked glycinergic inhibition persists to 20 ms but is fully decayed within 50 ms. Thus, put plainly, sound-evoked glycinergic inhibition is recruited too early and decays too rapidly to mediate PPI, but this time-course is entirely consistent with the well-characterized primary (disynaptic) feed-forward inhibitory pathway (Faber & Korn, 1982, 1988, 1989; Medan & Preuss, 2011). Indeed, previous reports have also reported that strychnine blocks auditory feed-forward inhibition (Diamond, 1973; Faber & Korn, 1988; Weiss et al., 2009), but the present study is the first to demonstrate that glycinergic feed-forward processes contribute to the summation of inhibition during PPI. In experiments with strychnine, we showed that PPI was reduced but not abolished, indicating an additional inhibitory component; we subsequently identified this as a GABA\textsubscript{A}R-dependent process.

**Mediators, modulators, and model systems**

These results highlight some striking similarities with advances in rodent model systems. Yeomans et al. (2010) showed that bicuculline disrupts the peak inhibitory components of behavioral PPI in rodents, and that PnC neurons (the sensorimotor interface equivalents of the M-cell in the mammalian startle circuit) are inhibited by GABA in an *ex vivo* brain-slice preparation. Moreover, the time-course of PPI mediated by GABA\textsubscript{A}Rs reported in rodents is similar to the ISI-specific effects we report here in the fish startle system.

Our findings are somewhat in contrast with studies of glycinergic inhibition in rodent preparations, however, Koch and Friauf (1995) showed that local and systemic applications of strychnine had no effect on phasic inhibitory processes including short-term habituation of startle
and PPI. Geis & Schmid (2011) used in vitro patch-clamp recordings to demonstrate that glycine directly inhibits PnC neurons in a rat brain slice preparation; however, they found no evidence that GlyRs were involved in phasic inhibitory processes, including feed-forward inhibition and short-term synaptic depression.

In contrast, our experiments identified GlyR-dependent phasic inhibitory processes that attenuate sound-evoked excitation in multiple contexts (Figs. 1, 2). These contrasting findings may reflect underlying differences in goldfish and rodents, or in experimental preparations or stimulus protocols. Whereas the present study measured in vivo synaptic processes in mature, awake goldfish, in vitro slice preparations used to record from PnC neurons were derived from embryonic rat brains (Yeomans et al., 2010; Geis & Schmid, 2011). Given the profound structural and functional shifts attributed to GlyRs and GABA_{A}Rs during development functional differences between mature and embryonic circuits may be expected (Erlich et al., 1999; Nabekura et al., 2004).

In sum, this study characterized in vivo synaptic signaling mechanisms that directly mediate the balance of excitation and inhibition at the sensorimotor interface of the startle circuit. Prior studies in the M-cell and other model systems have examined the role of neuromodulators, particularly monoaminergic transmitters (Medan & Preuss, 2011; Curtin et al., 2013), involved in PPI. Our results emphasize that in vivo electrophysiological methods can be applied to dissect overlapping inhibitory processes and effector mechanisms to directly test predictions drawn from advances in other model systems. Thus the M-cell presents an appropriate tool for dissecting the functional roles of synaptic processes as well as the effector mechanisms mediating their effects.
Acknowledgements

This work was supported by National Science Foundation grants IOS 0946637 and IOS 11471172 and by a grant from the Professional Staff Congress City University of New York (CUNY) Research Award Program, with additional support from Hunter College and the Graduate Center, CUNY. The authors additionally thank Dr. V. Medan, A. Curtin, and members of the Preuss laboratory for discussion and constructive feedback that contributed to the improvement of the manuscript.
Figure legends

Figure 3.1. Glycinergic inhibition contributes to the earliest components of PPI.

A-C. Sample intracellular recordings from the Mauthner-cell (M-cell) soma in response to paired (prepulse/pulse) sound pips at inter-stimulus intervals (ISIs) of 20 ms (A1, A2), 50 ms (B1,B2), and 75 ms (C1,C2) in control (black) and after application of the GlyR antagonist strychnine (red). Bottom traces show sound stimuli (200 Hz single-cycle "pips" at 80 dB re:20 µPa). Dashed lines and brackets indicate how PPI was quantified by comparing peak depolarization between the two evoked post-synaptic potentials (PSPs). D. Plots of the mean % PPI effect (± SEM, N = 9) across the full range of ISIs tested in control (black line) and strychnine (red line) conditions; asterisks indicate an ISI-specific reduction in PPI at the 20 ms ISI (see text).

Figure 3.2. Glycinergic inhibition mediates auditory processing in the M-cell.

A. Sample intracellular recordings from the Mauthner-cell (M-cell) soma in response to an individual sound pip before (black trace) and after (red trace) treatment with strychnine. The inset shows the initial part of the evoked response at an expanded time scale. Light and dark shaded areas distinguish the initial (EPSP; 0-5 ms) and later components (mixed PSP; > 5 ms) of the response, respectively. Bottom traces show sound stimulus (200 Hz single-cycle "pip" at 80 dB re: 20 µPa). B. Plots of mean (± SEM, N= 9) overall peak amplitude of sound-evoked depolarization in control (black bar) and strychnine (red bar) conditions. C. Plots of mean (± SEM, N = 9) PSP duration (Tau) before (black) and after (red) treatment with strychnine. D. Plots of the mean (± SEM, N= 9) relative change in sound-evoked depolarization for the initial (EPSP) and later parts (mixed PSP) of the sound response after treatment with strychnine.
Figure 3.3. GABA\textsubscript{A}Rs mediate peak inhibitory components of PPI.

**A-C.** Sample intracellular recordings from the Mauthner-cell (M-cell) soma in response to paired (prepulse/pulse) sound pips at inter-stimulus intervals (ISIs) of 20 ms (A\textsubscript{1}, A\textsubscript{2}), 50 ms (B\textsubscript{1}, B\textsubscript{2}), and 75 ms (C\textsubscript{1}, C\textsubscript{2}) in control (black) and after application of the GABA\textsubscript{A}R antagonist bicuculline (blue). Bottom traces show sound stimuli (200 Hz single-cycle "pips" at 80 dB re: 20 µPa). Dashed lines and brackets indicate how PPI was quantified by comparing peak depolarization between the two evoked post-synaptic potentials (PSPs). **D.** Plots of the mean % PPI effect (± SEM, N = 5) across the full range of ISIs tested in control (black line) and bicuculline (blue line) conditions; asterisks indicate an ISI-specific reduction in PPI at ISIs of 20, 50, 75, and 100 ms (see text).

Figure 3.4. GABA\textsubscript{A}Rs mediate auditory processing.

**A.** Sample intracellular recordings from the Mauthner-cell (M-cell) soma in response to an individual sound pip before (black trace) and after (blue trace) treatment with the GABA\textsubscript{A}R antagonist bicuculline. The inset shows the initial part of the evoked response at an expanded time scale. Light and dark shaded areas distinguish the initial (EPSP; 0-5 ms) and later components (mixed PSP; > 5 ms) of the response, respectively. Bottom traces show sound stimulus (200 Hz single-cycle "pip" at 80 dB re: 20 µPa). **B.** Plots of mean (± SEM, N= 5) overall peak amplitude of sound-evoked depolarization in control (black bar) and bicuculline (blue bar) conditions. **C.** Plots of mean (± SEM, N = 5) PSP duration (Tau) before (black) and after (blue) treatment with bicuculline. **D.** Plots of the mean (± SEM, N= 5) relative change in sound-evoked depolarization for the initial (EPSP) and later parts (mixed PSP) of the sound response after treatment with strychnine.
Figure 5. Strychnine and bicuculline increase Mauthner-cell excitability

A. Sample recordings showing antidromically-evoked Mauthner-cell (M-cell) action-potentials (APs) in control conditions (black trace) and after treatment with strychnine (red trace, superimposed). B. Plots of mean (± SEM) AP magnitude in control (black bar) and strychnine (red bars) treatment conditions. C. Sample recordings of antidromically-evoked M-cell APs, in control (black trace) and bicuculline (blue trace, superimposed) treatment conditions. D. Plots of mean (± SEM) AP magnitude in control (black bar) and bicuculline (blue bar) conditions.
FIGURE 3.1. GLYCINERGIC INHIBITION CONTRIBUTES TO THE EARLIEST COMPONENTS OF PPI.
FIGURE 3.2. GLYCINERGIC INHIBITION MEDIATES AUDITORY PROCESSING IN THE M-CELL.
FIGURE 3.3. GABA$_A$RS MEDIATE PEAK INHIBITORY COMPONENTS OF PPI.
FIGURE 3.4. GABA\textsubscript{A}RS MEDIATE AUDITORY PROCESSING
FIGURE 3.5. STRYCHNINE AND BICUCULLINE INCREASE MAUTHNER-CELL EXCITABILITY

A. 

B. 

C. 

D. 

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CHAPTER 4

4.1. GENERAL SUMMARY

The basic goals of this thesis were to identify novel neural mechanisms and/or functional dynamics involved in the selection of decision-making systems. Experimental approaches applied in vivo electrophysiology and pharmacology for studies of a sensorimotor gating process, prepulse inhibition (PPI), that inhibits startle to select "higher" decision-making systems. Intracellular recordings were used to measure sound-evoked excitation and inhibition of the Mauthner-cells (M-cells), the decision-making neurons of the goldfish startle circuit, and selective antagonists for serotonin (5-HT), GABA, and Glycine receptors were tested on those measures to identify neuromodulatory and neurotransmitter mechanisms contributing to auditory processing and PPI. This discussion offers a brief summary of key findings from those studies in the common functional contexts of startle excitability and sensorimotor gating (PPI), and emphasizes the likely shared effector mechanisms allowing those receptor systems to regulate different functional effects.

4.2. SELECTIVITY AND VALIDITY

The validity of conclusions drawn from these experiments rests on the basis that the selective antagonists used to study targeted receptors acted selectively on those receptors, i.e. without affecting other receptor systems. Three lines of evidence support this premise. First, the chemical properties and reactivity of the ligands used relative to their associated receptor targets. Second, the reported usage and effects of these antagonists described in the available literature. And, lastly, the functional effects observed following treatment with each antagonist, which were
consistent with selective effects. These lines of evidence are discussed below in the contexts of specific experiments and target receptor systems.

Experiments reported in Chapter 2 examined the functionality of the 5-HT$_{5A}$ receptor system by application of the 5-HT$_{5A}$ antagonists. **SB-699551**, the only antagonist used in all aspects of those experiments, was the only commercially-available selective antagonist available that preferentially blocks activation of the 5-HT$_{5A}$ receptor (Corbett et al., 2005). This ligand is deemed a "selective" antagonist, meaning it preferentially binds with and blocks activation of this receptor over others, because of its 50-fold affinity for the 5-HT$_{5A}$ receptor over other 5-HT receptors (Corbett et al., 2005). Given the binding affinities established by Corbett et al. (2005), the next-most likely binding site for this ligand would be the 5-HT transporter (5-HTT). Mintz & Korn (1991), however, previously showed that antagonizing the 5-HTT caused dissimilar effects than those reported here; that is, an increased excitability in the M-cell, rather than the reduced excitability caused by **SB-699551**. Further, we additionally showed that an alternative selective 5-HT$_{5A}$ antagonist, **A-843277**, obtained from the experimental work of Gannon et al. (2009), evoked nearly identical effects to those observed following treatment with **SB-699551**. It is additionally relevant that Whittaker et al. (2011) showed that only the 5-HT$_{5A}$ and 5-HT$_{6}$ receptors are expressed in the M-cells, and, consistent with post-synaptic effects expected of a selective antagonist for 5-HT$_{5A}$, we demonstrated these ligands reduced post-synaptic excitability at the level of M-cells. Lastly, the potential confound of dosage-driven non-selective effects were an important component of our experimental design, and for those reasons in these experiments we applied these drugs in dosage-concentrations far lower than were previously reported in the literature. Gannon et al. (2009) and Kessai et al. (2012), for example, applied these antagonists, with apparently selective effects, at concentrations more than 60X greater than were used in
these experiments. Thus, in sum, the most parsimonious interpretation of these lines of evidence is that the antagonists used in these studies likely acted selectively on the 5-HT$_{5A}$ receptor, as intended.

The antagonists used in the experiments reported in Chapter 3, strychnine and bicuculline, are well-documented selective antagonists used to discriminate glycinergic and GABAergic receptor functions, respectively (Triller et al., 1998; Seitanidou et al., 1988; Sur et al., 1995; Tapia et al., 1998). Further, the dosages applied in these studies were chosen to be within the mid-range of dosage-concentrations previously used with these drugs in the M-cell system by Diamond (1973), Faber & Korn (1991), and Hatta et al. (2001), which previously showed selective effects. Further, the functional consequences of drug application showed clearly distinct effects (discussed below). Again, these lines of evidence support the underlying assumptions of these experiments; that is, that the ligands used acted selectively on the targeted receptor systems.

4.3. TONIC INHIBITION: GAIN CONTROL FOR STARTLE EXCITABILITY

The experimental approaches reported here tested selective antagonists for GABA$_{A}$, glycine, and 5-HT$_{5A}$ receptors in the Mauthner-cell (M-cell) startle circuit. The effects of each antagonist on phasic, i.e., sound-evoked, inhibitory processes were temporally or functionally distinct (discussed in subsequent section). Those trends are important in consideration of the selectivity of pharmacological manipulations used in these studies. Had the antagonists used in these studies acted on similar mechanisms, i.e., non-selectively, then overlapping time-dependent effects would be apparent with different drugs. Nonetheless, despite clearly distinct effects, a noteworthy commonality emerged in that all of the receptors studied were involved in a form of
tonic inhibition of startle. The functional roles of GABA and glycine were opposite that of the 5-HT$_{5A}$ receptor, however, in that the serotonergic processes mediated via the 5-HT$_{5A}$ receptor elicited tonic inhibition when blocked, while the GABA$_A$R and GlyR antagonists blocked tonic inhibition.

Treatment with 5-HT$_{5A}$ antagonists reduced startle excitability, indicating that in typical contexts 5-HT would increase excitability via this receptor. Consistent with this notion, Mintz & Korn (1991) showed that M-cell excitability is increased by 5-HT. Though that study did not identify the associated 5-HT receptor, only the 5-HT5 and 5-HT6 receptors are expressed in the M-cell (Whittaker et al., 2011), and these are thought to have opposing functions (Barnes & Sharp, 1999). Importantly, and in contrast to effects described in other experiments, the 5-HT$_{5A}$ antagonists' effects on M-cell excitability caused no corresponding changes in sound-evoked inhibition. The consistency of auditory processing was apparent in the virtually identical time-course of sound-evoked post-synaptic potentials (PSPs) across drug treatment conditions, and, on longer time-scales, in the consistency of prepulse-pulse relationships in PPI trials. These results are particularly interesting in light of the findings of Mintz & Korn (1991) and Whittaker et al. (2011), who each independently report evidence of serotonergic modulation of the adjacent commissural feed-forward inhibitory network, which generates a fast-onset, rapidly decaying inhibition elicited by auditory afferences (Faber & Korn, 1978; Faber & Korn, 1998; Korn & Faber, 2005). These finding suggest against the expression of the 5-HT$_{5A}$ receptor in associated inhibitory networks (commissural inhibitory network, and/or putative PPI circuit) because if it were expressed then the antagonist would cause time-dependent effects in sound-evoked inhibition. As, for example, the time-dependent effects of the GlyR antagonist, strychnine, on sound-evoked PSPs (without prepulses), or the GABA$_A$R antagonists' effects on PPI.
The functional, rather than mechanistic, role of the 5-HT$_{5A}$ receptor is less clear, but the properties characterized in this study are consistent with a mechanism for selective potentiation, i.e., gain control, of startle excitability (as per Lashley, 1951). The M-cells receive multiple specialized types of 5-HT projections (Gotow et al., 1992) and these serotonergic inputs provide a pathway for descending control of startle excitability via activation of the 5-HT$_{5A}$ receptor.

The potential functional relevance of tonic glycinergic and GABAergic processes is less clear, though tonic inhibitory processes are well-characterized in the M-cells, and are thought to drive excitability (Hatta & Korn, 1999; Hatta et al., 2001). The studies reported here are the first to directly demonstrate the effects of persistent changes in tonic inhibition to auditory processing. In functional terms, the effects of GlyR and GABA$_A$R antagonists were unambiguous and opposite to those of the 5-HT$_{5A}$ antagonist. That is, both strychnine, the glycine receptor (GlyR) antagonist, and bicuculline, the GABA$_A$R antagonist, caused significant increases in sound-evoked PSPs. Further, both also caused increases in the magnitude of antidromically-evoked APs. The typical actions of glycine and GABA, then, are inhibitory, as expected. The M-cell, with its characteristic passively-inexcitable membrane, expresses no voltage-activated channels or similar mechanisms for propagating excitation. The increased magnitude of APs observed after treating with either drug, therefore, provides an unambiguous indicator of increased input resistance, i.e., excitability.

These results indicate that GABA$_A$Rs and GlyRs mediate tonic inhibition, as expected and previously reported (Hatta & Korn, 1999; Hatta et al., 2001). Importantly, and in contrast to the effects observed with 5-HT$_{5A}$ antagonists, both inhibitory neurotransmitters are also critical to sound-evoked inhibitory processes. This is likely true on multiple levels: first, as a consequence of greatly enhanced excitability, and secondly because the drugs block critical
inhibitory processes active during auditory processing and prepulse inhibition (PPI). Thus while one might speculate that these receptors could provide a gain control mechanism similar to the proposed function of 5-HT$_{5A}$, their broad, dense expression profiles in the hindbrain (Sur et al., 1999; Triller et al., 1985) and associated functional roles in sound-evoked inhibition are incompatible with highly selective control, as these processes would act to cancel each other (i.e., the gain control mechanism should not occupy the receptor sites necessary for auditory processing).

4.4. SELECTING AGAINST STARTLE: PREPULSE INHIBITION EXPERIMENTS

The relevance of the 5-HT$_{5A}$ experiments to PPI relates to the relationships between startle excitability and PPI predicted by the widely-cited theoretical perspective of Graham (1975). In this view, PPI is described as a distinct, exclusively "upstream" inhibitory process, since-identified in rodent model systems as originating from an anatomically-distinct midbrain circuit that projects to the hindbrain startle network (Koch & Schnitzler, 1997; Koch, 1999; Fendt & Yeomans, 2001; Yeomans et al., 2002; Yeomans et al., 2006). Accordingly, the excitability of the startle circuit, and the magnitude and time-course of PPI, are expected to vary independently. And, indeed, the electrophysiology experiments reported in Curtin et al. (2013) offer direct, in vivo evidence that PPI at the level of the M-cells was not affected by experimental/pharmacological manipulations of startle excitability, although startle excitability was, indicating discrete processes consistent with the predictions of Graham (1975).

The behavioral experiments reported in Curtin et al. (2013), however, indicated that the same treatments (with 5-HT$_{5A}$ antagonists, as described above) cause significant increases in PPI. This apparently contradictory result appears to oppose the physiological results described in
that study. The simplest explanation suggested to resolve this contradiction is that the 5-HT$_{5A}$ antagonist causes consistent effects in the startle network, i.e. a general suppression, whether startle excitation is measured as a behavioral rate (behavior) or as M-cell sound-evoked post-synaptic potentials (PSPs). We speculate that the effect of the antagonist does not manifest sufficiently to see effects on startle in the free-swimming conditions of the behavioral pharmacology experiments, but the inhibition elicited by prepulses is nonetheless superimposed on a latent antagonist-evoked suppression of startle, and the summation of these processes (typical PPI + drug-evoked tonic inhibition) yields an apparent enhancement of startle inhibition in trials with prepulse stimuli, yielding the apparent enhancement of PPI. In the context of behavioral decision-making, for this particular species and behavior, this is arguably a more relevant context for studying PPI than the physiological preparation. Considered again from the perspective of Graham (1975), though, these results identify a context in which startle excitability and the magnitude of PPI are not independent, as predicted, but rather the magnitude of the latter (PPI) depends entirely on the excitability of the former (startle). Similar inter-dependences were suggested by past authors (e.g., Blumenthal, 1997; Schicatano, 1999; Sandner & Canal, 2007), but were never previously connected across cellular, synaptic, and behavioral contexts.

Experiments with strychnine, the glycine receptor (GlyR) antagonist, nonetheless give evidence that the mechanisms generating PPI are independent of the circuit that initiates startle. Specifically, the increased excitability of the M-cell following treatment with strychnine enhanced sound-evoked post-synaptic potentials (PSPs). If the PPI circuit similarly expressed GlyRs, it would be similarly more excitable, and this would be detectable in its inhibitory outputs. Nonetheless, even in this context of increased startle excitability - the inverse of the
depressed-excitability scenario caused by 5-HT$_{5A}$ antagonists - the magnitude and time-course of PPI remained robustly intact and independently unaffected by strychnine treatment. The sole exception to this was at the earliest inter-stimulus interval (ISI) tested, 20 ms, where PPI was significantly reduced. Those results are discussed below.

Taken in sum with the tonic glycinergic inhibitory processes described, the functional significance of GlyRs in the startle network is unambiguously apparent in the context of auditory processing and decision-making. This evidence directly addresses discrepancies in the literature that include conflicting findings in various investigations of glycinergic inhibition of startle; e.g., Koch & Friauf (1995) and Geis & Schmid (2011) found no evidence of tonic glycinergic inhibitory processes acting on startle, but Kehn & Davis (1984) did. Hatta & Korn (1999) and Hatta et al. (2001) found that M-cell GlyRs mediate tonic inhibitory processes driven by inhibitory synaptic noise, and these determine excitability of the M-cells (Hatta & Korn, 1999; Hatta et al., 2001). The disruption of tonic inhibition in the present study emphasized, for the first time, that tonic glycinergic processes profoundly impact information-processing and decision-making parameters in the M-cell circuits. Thus, GlyRs are the effector mechanisms for multiple inhibitory mechanisms in the startle circuit, and are thus critical to establishing the parameters of information-processing and decision-making.

The disproportionate, time-dependent (relative to stimulus onset) increases in sound-evoked PSPs (without prepulses) following strychnine treatment are expected effects associated with blockade of inputs from the well-characterized feed-forward inhibitory process originating in the commissural inhibitory network (reviewed in Korn & Faber, 2005). Accordingly, the interpretation suggested is that strychnine blocked the fast onset (~5+ ms) and abrupt decay of a glycinergic inhibitory mechanism, likely the feed-forward process described above, and this
process, unexpectedly, contributes to the early onset of PPI at the 20 ms ISI, then rapidly decays prior to the 50 ms ISI, reflected in the rapid recovery of PPI to control levels. A consistent finding across experimental studies of PPI is that discrete temporal components of PPI, e.g. the inhibition present < 100 from prepulse onset, are mediated by distinct neurotransmitter systems (Koch, 1999; Braff, 2010; Yeomans et al., 2010). The results reported here, however, offer the first preliminary evidence that multiple inhibitory circuits are recruited to contribute discrete components to the PPI time-course. These data thus offer a novel perspective on PPI, while confirming some basic assumptions guiding theoretical formulations.

Last, experiments with bicuculline, the GABAA-R antagonist, identified GABAA-Rs as the effector mechanisms directly mediating M-cell PPI. These results confirm the findings of Yeomans et al. (2010), which tested GABA antagonists on PPI in neonatal ex vivo slice preparations comprising the primary startle circuit and associated midbrain pathways. This confirmation was critical to validating the rodent model system because it showed consistency in results between a neonatal ex vivo model and another vertebrate adult, in vivo. This was important because neonatal inhibitory systems are functionally and structurally immature, oftentimes yielding opposing properties (e.g., glycine can be excitatory in neonatal spines) to what is expected in adults (Tapia et al., 1994; Aguayo et al., 2004; Nabekura et al., 2004). Remarkably, given the differences in preparations, the time-course attributed to GABAA-Rs in the experiments reported here and in Yeomans (2010) are virtually identical.

The blockade of GABAA-Rs emphasized the mechanistic function of GABA in the context of PPI, but also offered an opportunity to examine startle in the absence of PPI, and thereby infer something of its theoretical function. As expected, without PPI the sound-evoked PSPs at shorter ISIs (20-75 ms) were integrated via temporal summation, such that the response
to subsequent stimuli was greater than lead stimuli, when usually the reverse is true. PPI is thus critical to computational and/or decision-making processes in the startle circuit, independently of its contributions to preserving higher information-processing functions. And GABA, explicitly, is a critical functional mediator of this process.

4.5. CONCLUSIONS

This thesis investigated novel neuronal mechanisms that enable the dynamic selection of decision-making systems. A novel serotonergic mechanism, the 5-HT<sub>5A</sub> receptor, was characterized in the context of modulating startle excitability. That is, relevant to the stated goals, the 5-HT<sub>5A</sub> receptor provides a mechanism for selectively potentiating the startle decision-making system over other circuits, as per Lashley (1951). Similarly, glycine receptors were shown to mediate tonic and phasic inhibitory processes that coordinate auditory processing in a central decision-making circuit. Lastly, the GABA<sub>A</sub>R, another well-characterized inhibitory mechanism, was here identified as the effector mechanism that mediates a sensorimotor gating process, prepulse inhibition (PPI), that selects "higher" decision-making processes by preventing the disruptive activation of startle. In sum, these and related processes are critical to the coordination of a decision-making hierarchy driven by the organizational principles of competition, interference, selective potentiation, and the ultimate arbitrating criterion of winner-takes-all.


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